



Uniwersytet
Wrocławski



**Università
degli Studi
di Ferrara**

University of Wrocław, Faculty of Chemistry

University of Ferrara, Dipartimento di Scienze Chimiche, Farmaceutiche ed Agrarie

Zincophore-based metal transport

MSc Kinga Garstka

Research group of the Biologically Active Metallopeptides

Supervisors: prof. Magdalena Rowińska-Żyrek

prof. Maurizio Remelli

Wrocław 2024

To my supervisor

prof. Magdalena Rowińska-Żyrek

Saying "thank you" is not enough for me to express how grateful I am for your support over the last four years. Everything I have achieved to date is because of you because you believed in me at the beginning of my chemistry career.

Thank you for the time you spent with me and everything you taught me, for your valuable advice, tips and for your invaluable help.

I am grateful for your support and all your words of encouragement, but also for all the opportunities you gave me.

Thank you, Magda, for making it such a pleasure to be at work each day.

This wouldn't have been possible without your support.

To my supervisor

prof. Maurizio Remelli

Thank you for giving me the opportunity to be a part of your research group.
I am grateful for your support and guidance. Moreover, Thank you for your help with the
Italian side of my PhD.

Thank you to my PhD mentor
prof. Henryk Kozłowski
for your time, your help, and your kind words. I'm incredibly grateful for all your support.

Thank you to my PhD mentor

dr Denise Belotti

for teaching me that everything and all the work you put in on my Italian side of PhD.

I couldn't have done it without you.

Thank you to Silvia Leveraro
for your great ideas and the time, we spent together.

Thank to my research group of Biologically Active Metallopeptides, you are amazing.
This team has set a new standard of excellence with their passion, skill, and dedication, and I
had the pleasure to work with you. Thank you for all your help, exchange of experiences
kindness and understanding.

Thank to research group of Biological Inorganic Chemistry
for your kind cooperation, all your assistance and your considerations.

Thank you to my fiancé (at the moment)

Kamil Litwin

I can't thank you enough for all of your support, but thank you for your unwavering faith in me, understanding and help. Thank you for the time you spent helping me become better at what I love to do.

Thank you to my wonderful parents

It is thanks to you that I am here, and all my dreams could come true. Thank you for your support, understanding, patience and faith in me, whose resources seem to be inexhaustible.

Thank you to my whole family
Thank you all for always being there for me.

This work has been performed in the framework of the NCN Project:

MAESTRO, 2017/26/A/ST5/00363

„Metalofory- zrozumienie (i wizualizacja) transportu metali w patogenach, by pomóc gospodarzowi oszukać najeźdźców”



NATIONAL SCIENCE CENTRE
POLAND

This work has been a contribution of the COST Action CA18202, NECTAR – Network for Equilibria and Chemical Thermodynamics Advanced Research, supported by COST (European Cooperation in Science and Technology).



This work has been performed in the frame of
International Joint Doctoral Study Program in Chemistry
Between the University of Wrocław and the University of Ferrara



Uniwersytet
Wrocławski



**Università
degli Studi
di Ferrara**

TABLE OF CONTENT:

I.	SYMBOLS AND ABBREVIATIONS	15
II.	ABSTRACT	17
A.	ABSTRACT IN POLISH	19
III.	CHAPTER I: INTRODUCTION	20
A.	Transition metal ion transport.....	23
1.	Human Zn(II) homeostasis.....	24
2.	Fungal Zn(II) homeostasis.....	34
3.	Bacterial Zn(II) homeostasis	42
B.	Analyzed microorganisms.....	55
1.	Zn(II) transport in <i>Aspergillus fumigatus</i>	55
2.	Zn(II) transport in <i>Paracoccus denitrificans</i>	61
3.	Zn(II) transport in <i>Streptococcus mutans</i>	66
C.	Coordination properties and biological significance of Zn(II).....	69
1.	Biological significance of Zn(II)	70
D.	Coordination properties and biological significance of Ni(II)	72
1.	Biological significance of Ni(II)	73
IV.	CHAPTER II: MAIN AIMS OF THE WORK	77
V.	CHAPTER III: EXPERIMENTAL SECTION	78
A.	Ligand synthesis.....	78
B.	Reagents and experimental procedures.....	79
1.	ESI-MS spectrometry.....	79
2.	Potentiometry	80
3.	UV-Vis, CD and NMR spectroscopy.....	82
VI.	CHAPTER IV: RESULTS AND DISCUSSION	83
A.	Thermodynamic and structural properties of Zn(II) and Ni(II) complexes with unstructured fragments of the Aspf2 zincophore	83
1.	Stoichiometry of Zn(II) and Ni(II) complexes with Aspf2 fragments	84
2.	Zn(II) and Ni(II) coordination modes in Aspf2.....	88
3.	Secondary structure analysis	105
4.	Comparison of the stability of Zn(II) and Ni(II) complexes.....	107
5.	Conclusions	113
B.	Thermodynamic and structural properties of Zn(II) and Ni(II) complexes with unstructured fragments of ZrfC transporter.....	114
1.	Stoichiometry of Zn(II) and Ni(II) complexes with ZrfC fragments.....	115
2.	Methods of Zn(II) and Ni(II) coordination.....	119

3.	Secondary structure analysis	129
4.	Comparison of the stability of Zn(II) and Ni(II) complexes.....	132
5.	Conclusions	138
C.	Thermodynamic and structural properties of Zn(II) and Ni(II) complexes with Ac-Ac-ACHA-NH ₂ and Ac-AHCA-NH ₂ ligands	139
1.	Zn(II) and Ni(II) coordination modes.....	140
2.	Comparison of the stability of Zn(II) and Ni(II) complexes.....	148
3.	Conclusions	156
D.	Thermodynamic and structural properties of Zn(II) and Ni(II) complexes with unstructured fragments of ZnuA and AztC transporters	156
1.	Stoichiometry of Zn(II) and Ni(II) complexes with ZnuA and AztC fragments	157
2.	Zn(II) and Ni(II) coordination modes.....	162
3.	Secondary structure analysis	179
4.	Comparison of the stability of Zn(II) and Ni(II) complexes.....	183
5.	Conclusions	187
E.	Thermodynamic and structural properties of Zn(II) and Ni(II) complexes with unstructured fragments of AdcA transporter	190
1.	Methods of Zn(II) and Ni(II) coordination.....	191
2.	Secondary structure analysis	199
3.	Comparison of the stability of Zn(II) and Ni(II) complexes.....	203
4.	Conclusions	208
VII.	CHAPTER V: FINAL DISCUSSION	209
VIII.	CHAPTER VI: CONCLUSIONS AND PERSPECTIVES	217
IX.	LITERATURE	221
X.	LIST OF TABLES.....	261
XI.	LIST OF FIGURES.....	262
XII.	LIST OF PUBLICATIONS	273
XIII.	SCIENTIFIC ACTIVITY.....	274

I. SYMBOLS AND ABBREVIATIONS

AMR – Antimicrobial Resistance

MDR – Multidrug Resistance

AMP – Antimicrobial Peptides
CDF – Cation Diffusion Facilitator
RND – Resistance-nodulation-division pump
MarR – Multiple antibiotic resistance repressor
ZIP – ZRT, IRT-like protein
CDF – Cation diffusion facilitator transporter
MFP – Periplasmic membrane fusion protein
TMD – Transmembrane domain
NBD – Nucleotide-binding domain
GTP – Guanosine triphosphate
CTP – Cytidine triphosphate
UTP – Uridine triphosphate
ITP – Inosine triphosphate
Zur – Zinc uptake regulator
Fur – Ferric uptake regulator
CTD – C-terminal domain
OMP – Outer membrane protein
IMP – Inner membrane protein
MFP – Membrane fusion protein
MDR – Multidrug resistance
NK – Natural Killer Cells
NAPDH – Nicotinamide adenine dinucleotide phosphate
IL-6 – Interleukin 6
EMT – Epithelial-Mesenchymal Transition
MTF1 – Metal regulatory transcription factor 1
LPS – Lipopolysaccharide
MTs – Metallothioneins
ROS – Reactive Oxygen Species
DNA – Deoxyribonucleic acid

Pra1 – pH-regulated Antigen 1

ZFD – Zinc Finger Domain

ZRE – Zn(II) Responsive Element

AD – Activation Domain

TPEN – N^1,N^1,N^2,N^2 -Tetrakis[(pyridin-2-yl)methyl]ethane-1,2-diamine

ARD – Acireductone dioxygenase

GloI – Glyoxalase I

NiSOD – Ni(II)-superoxide dismutase

LarA – Lactate racemase

ACS – Acetyl-coenzyme A synthase/decarbonylase

MCR – Methyl-coenzyme M reductase

II. ABSTRACT

Rapidly spreading antimicrobial resistance is a serious problem and a global threat to the modern society. Treatment of bacterial and fungal infections with drugs that have been effective so far turns out to be impossible in many cases. The seriousness of the situation is further intensified by the fact that the pace of the introduction of new antibiotics has decreased dramatically, and one of the greatest problems associated with finding new effective and selective antifungal drugs is the fact that fungi have common metabolic pathways and share basic cellular mechanisms with the cells of human hosts. Therefore, to design a highly specific antifungal drug, it is necessary to understand the difference in human and fungal metabolism. One of the main differences is based on the mechanism of Zn(II) uptake, which is one of the key aspects of their virulence and survival. Maintaining the correct concentration of Zn(II) is necessary for the proper functioning of all cells, where they perform a variety of important catalytic and structural functions. For proper development, it is important to maintain its concentration at an appropriate level, and in order to maintain the proper homeostasis of metal ions, organisms have developed a number of different mechanisms. The relationship between metal ion homeostasis and nutritional immunity seems to be a promising starting point in the search for new, effective and specific antibacterial and antifungal drugs. In response to infections occurring in the host cell, defense strategies are used such as lowering the concentration of free metal, which allows limiting its access to pathogen cells. By competing with the host organism for metal ions necessary for survival, bacteria and fungi have developed mechanisms that enable them to absorb them from the host's environment. These include, among others, the production of proteins with a high affinity for metal ions, which bind them and then deliver the pathogen to the body.

The main goal of this doctoral dissertation was to understand the bioinorganic chemistry of Zn(II) and Ni(II) complexes with selected unstructured fragments of zincophores and Zn(II) transporters present in the organisms of fungi and bacteria. In my work, I focused on fragments of five different proteins involved in Zn(II) transport: (i) zincophore Aspf2 from *Aspergillus fumigatus*, (ii) ZrfC transporter from *Aspergillus fumigatus*, (iii) ZnuA from *Paracoccus denitrificans*, (iv) AztC from *Paracoccus denitrificans* and (v) AdcA from *Streptococcus mutans*. All tested ligands met three criteria: (i) they are located in unstructured fragments of proteins (as predicted by Phyre2, a program for predicting protein structure), (ii) they have amino acid residues that are able to bind the analyzed metal ions, such as histidine imidazoles or cysteine thiol groups; and (iii) they are located in evolutionarily conserved places, which means that they are also present in other proteins performing similar functions. Bearing in mind that Ni(II) is necessary for the proper functioning and development of pathogens and may compete for binding sites with Zn(II), I also decided to determine the preferences of the analyzed fragments towards this metal ion. In my work, in order to have a better understanding of the preferred metal sites, I also focused on the characterization of the thermodynamic properties of two model sequences: Ac-ACHA-NH₂ and Ac-AHCA-NH₂. To carry out the physicochemical and thermodynamic characterization of the studied unstructured protein fragments involved in Zn(II) transport and their complexes, I used a number of complementary experimental methods: mass spectrometry, potentiometric titration, UV-Vis spectroscopy, CD spectroscopy and NMR spectroscopy. I carried out parts of the work under the supervision of prof. Maurizio Remelli at the University of Ferrara.

For all the studied systems, I determined the most probable coordination sites for Zn(II) and Ni(II) ions. I characterized the donor atoms involved in their coordination depending on the pH value of the tested solution and deciphered the geometry of the resulting Ni(II) complexes. Among the analyzed fragments, I determined the ligands that have the highest affinity for the analyzed metal ions, and the comparison of the thermodynamic stability of all unstructured fragments of the analyzed proteins allowed me to determine the most effective coordination modes. For Zn(II) these are the (i) {2N_{im}, 2S⁻} and (ii) {4N_{im}} coordination modes, the second one with a polymorphic motif. While for Ni(II), the most effective binding modes turned out to be: (i) the {6N_{im}} and (ii) the {2N_{im}, 2S⁻} type of coordination. Additionally, basing on the precisely calculated stability constants, I showed that the transport of Zn(II) from the zincophore Aspf2 to the ZrfC transporter present in *Aspergillus fumigatus* is possible. This result is particularly interesting because it makes a huge contribution to understanding the bioinorganic chemistry of Zn(II) transport in pathogens.

In the future, my findings may serve as a starting point for designing new, highly selective antibacterial and antifungal drugs based on STAMP technology. The indicated protein fragments with the highest affinity for Zn(II) are excellent examples of targeting domains that, connected with a drug substance by a flexible linker, ensure selective accumulation of the drug molecule near the pathogen.

Szybko rozprzestrzeniająca się oporność przeciwdrobnoustrojowa stanowi poważny problem i globalne zagrożenie współczesnego świata. Leczenie zakażeń bakteryjnych oraz grzybiczych, z wykorzystaniem skutecznych do tej pory leków, w wielu przypadkach okazuje się niemożliwe. Powagę sytuacji dodatkowo potęguje fakt, że tempo wprowadzania nowych antybiotyków drastycznie zmalało a jednym z największych problemów związanych ze znalezieniem nowych skutecznych i selektywnych leków przeciwgrzybiczych jest fakt, że grzyby posiadają wspólne szlaki metaboliczne i dzielą podstawowe mechanizmy komórkowe z komórkami ludzkich gospodarzy. W związku z tym, aby zaprojektować wysoce specyficzny lek przeciwgrzybiczy, konieczne jest zrozumienie różnicy w metabolizmie człowieka i grzybów. Jedną z podstawowych różnic opiera się na mechanizmie pobierania Zn(II), który jest jednym z kluczowych aspektów ich zjadliwości i przeżycia. Utrzymanie prawidłowego stężenia Zn(II) jest niezbędne do prawidłowego funkcjonowania wszystkich komórek, gdzie pełni one szereg istotnych funkcji, jak funkcje katalityczne czy strukturalne. Do prawidłowego rozwoju istotne jest utrzymanie jego stężenia na odpowiednim poziomie, a w celu utrzymania prawidłowej homeostazy jonów metali organizmy wykształciły szereg różnych mechanizmów. Związek pomiędzy homeostazą jonów metali, a odpornością odżywczą wydaje się więc obiecującym punktem wyjścia w poszukiwaniu nowych, skutecznych oraz specyficznych leków przeciwbakteryjnych oraz przeciwgrzybiczych. W odpowiedzi na infekcje występujące w komórce gospodarza stosowane są takie strategie obronne jak obniżenie stężenie wolnego metalu, co umożliwia ograniczenie jego dostępu dla komórek patogenu. Współzawodnicząc z organizmem gospodarza o niezbędne do przeżycia jony metali, bakterie oraz grzyby wykształciły mechanizmy umożliwiające im ich pobieranie z środowiska gospodarza. Można do nich zaliczyć między innymi wytwarzanie białek o wysokim powinowactwie do jonów metali, które wiążą go, a następnie dostarczają do organizmu patogenu.

Głównym celem badań przeprowadzonych w ramach niniejszej rozprawy doktorskiej było zrozumienie chemii bionieorganicznej kompleksów Zn(II) oraz Ni(II) z wybranymi nieustrukturyzowanymi fragmentami cynkoforów oraz transporterów Zn(II) obecnych w organizmach grzybów oraz bakterii. W badaniach skupiłam się na fragmentach pięciu różnych białek zaangażowanych w transport Zn(II): (i) cynkofor AspF2 z *Aspergillus fumigatus*, (ii) transporter ZrxC z *Aspergillus fumigatus*, (iii) ZnuA z *Paracoccus denitrificans*, (iv) AztC z *Paracoccus denitrificans* oraz (v) AdcA z *Streptococcus mutans*. Wszystkie poddane badaniom ligandy spełniały trzy kryteria: (i) znajdują się w nieustrukturyzowanych fragmentach białek, co zostało przewidziane przez Phyre2, program służący do przewidywania struktury białek, (ii) posiadają reszty aminokwasowe, które są zdolne do wiązania analizowanych jonów metali, takich jak pierścień imidazolowy histydyny czy grupa tiolowa cysteiny, (iii) znajdują się w miejscach konserwatywnych ewolucyjnie, co oznacza, że są obecne również w innych białkach, pełniących podobne funkcje. Mając na uwadze, że Ni(II) jest niezbędny do prawidłowego funkcjonowania i rozwoju patogenów i może konkurować o miejsca wiązania z Zn(II), postanowiłam również określić preferencje analizowanych fragmentów względem tego jonu metalu. W swojej pracy skupiłam się również na charakterystyce właściwości termodynamicznych dwóch sekwencji modelowych: Ac-ACHA-NH₂ oraz Ac-AHCA-NH₂. Do

przeprowadzenia charakterystyki fizykochemicznej i termodynamicznej badanych nieustrukturyzowanych fragmentów białek zaangażowanych w transport Zn(II) oraz ich kompleksów wykorzystałam szereg komplementarnych metod eksperymentalnych: spektrometrię mas, miareczkowanie potencjometryczne, spektroskopię UV-Vis, spektroskopię CD oraz spektroskopię NMR. Część przeprowadzonych badań realizowałam pod opieką prof. Maurizio Remelli na Uniwersytecie w Ferrarze.

Dla wszystkich badanych układów wyznaczyłam najbardziej prawdopodobne miejsca koordynacji jonów Zn(II) oraz Ni(II). Scharakteryzowałam atomy donorowe zaangażowane w ich koordynację w zależności od wartości pH badanego roztworu i określiłam geometrię powstających kompleksów Ni(II). Spośród analizowanych fragmentów wyznaczyłam ligandy, które charakteryzują się największym powinowactwem w stosunku do analizowanych jonów metali, a porównanie trwałości termodynamicznej wszystkich nieustrukturyzowanych fragmentów analizowanych białek pokazało mi wyznaczyć najbardziej skuteczne sposoby koordynacji. Dla Zn(II) są to (i) koordynacja typu $\{2N_{im}, 2S^-\}$ oraz (ii) koordynacja typu $\{4N_{im}\}$ z motywem polimorficznym. Podczas, gdy dla Ni(II) najsukuteczniejszymi sposobami koordynacji okazały się: (i) koordynacja typu $\{6N_{im}\}$ oraz (ii) koordynacja typu $\{2N_{im}, 2S^-\}$. Dodatkowo wykazałam, że transport Zn(II) z cynkoferu AspF2 do transportera ZrfC obecnych u *Aspergillus fumigatus* jest możliwy. Wynik ten jest szczególnie interesujący, ponieważ wnosi ogromny wkład w poznanie chemii bionieorganicznej transportu Zn(II) u patogenów.

W przyszłości te badania mogą posłużyć jako punkt wyjścia przy projektowaniu nowych, wysoce selektywnych leków przeciwbakteryjnych oraz przeciwgrzybiczych bazujących na technologii STAMP. Wskazane fragmenty białek, posiadające największe powinowactwo to Zn(II) stanowią doskonałe przykłady domen kierujących, które połączone elastycznym łącznikiem z substancją leczniczą zapewniają selektywną akumulację cząsteczki leku w pobliżu patogenu.

III. CHAPTER I: INTRODUCTION

Bacteria were first identified in 1670 by Antoni van Leeuwenhoek [1]. At that time, they were not suspected of being pathogenic, and only in the 19th century did Joseph Lisner put forward the theory that diseases were caused exactly by bacteria [2]. In 1928, Alexander Fleming discovered the first antibiotic, penicillin [3]. It was introduced into use 12 years later, when it was isolated by Howard Walter Florey and Ernest Boris Chain [4]. This was a revolution in the fight against infections caused by bacteria [5] and marked the first milestones in the history of antibiotic research (Figure 1). In the late 1930s, Selman Waksman defined an antibiotic as 'a compound made by a microbe to destroy other microbes' [6] for this class of natural growth inhibitors [7]. Subsequent discoveries gained rapid pace. Bacteria began to be isolated, cultivated and identified as potential disease agents or producers of bioactive metabolites [8]. At the same time, the first synthetic antibiotics were developed, and new classes of antibiotics were discovered. Thanks to this, in the middle of the 20th century, many diseases that had reached epidemic proportions at that time (such as cholera, syphilis, plague, tuberculosis or typhoid fever) could be combated using newly discovered antibiotics. However,

it quickly turned out that bacteria are able to develop, acquire and spread numerous resistance mechanisms [9]. Antibiotic resistance is a phenomenon characterized by the fact that some strains of bacteria, after causing disease, are not inhibited or killed by the concentration of antibiotics achieved in the tissues of the human body [10]. Consequently, exposure to antibiotics is the principal risk factor in the emergence and selection of antibiotic-resistant bacteria. These bacteria may exhibit innately resistant to antibiotics as well as acquired resistance, and both types of resistance are probably equally important in the context of the treatment. The acquisition of resistance genes is mediated by transferable extrachromosomal genetic elements, which are called plasmids or special genetic elements known as transposons and integrons [6]. The introduction of subsequent antibiotics, sooner or later, turned out to be ineffective, and new resistance genes were discovered. Although antibiotics have saved the lives of countless people, bacterial resistance to the antibiotics used is a dynamically growing clinical problem. The 'golden era' of antibiotics, which lasted from the early 1930s to the late 1960s, gave rise to many new medicinal preparations [11] but it ended because scientists were unable to maintain the pace of discovering new antibiotics against the emergence of resistant pathogens. Now antimicrobial resistance (AMR) is a serious global threat, raising increasing concerns not only about human and animal health, but also about the state of the environment. The seriousness of the situation is intensified by the emergence and spread of highly drug-resistant bacteria (MDR) [12] and 'superbugs' (viruses, bacteria, fungi, and parasites, that have developed high levels of resistance and a high morbidity and mortality rate [13]). Moreover, these bacteria continue to evolve and then become increasingly resistant to available antibiotics [14]. But it's not just bacteria that pose a serious medical problem. The incidence of drug-resistant fungal infections has increased significantly and is not limited to immunocompromised patients.

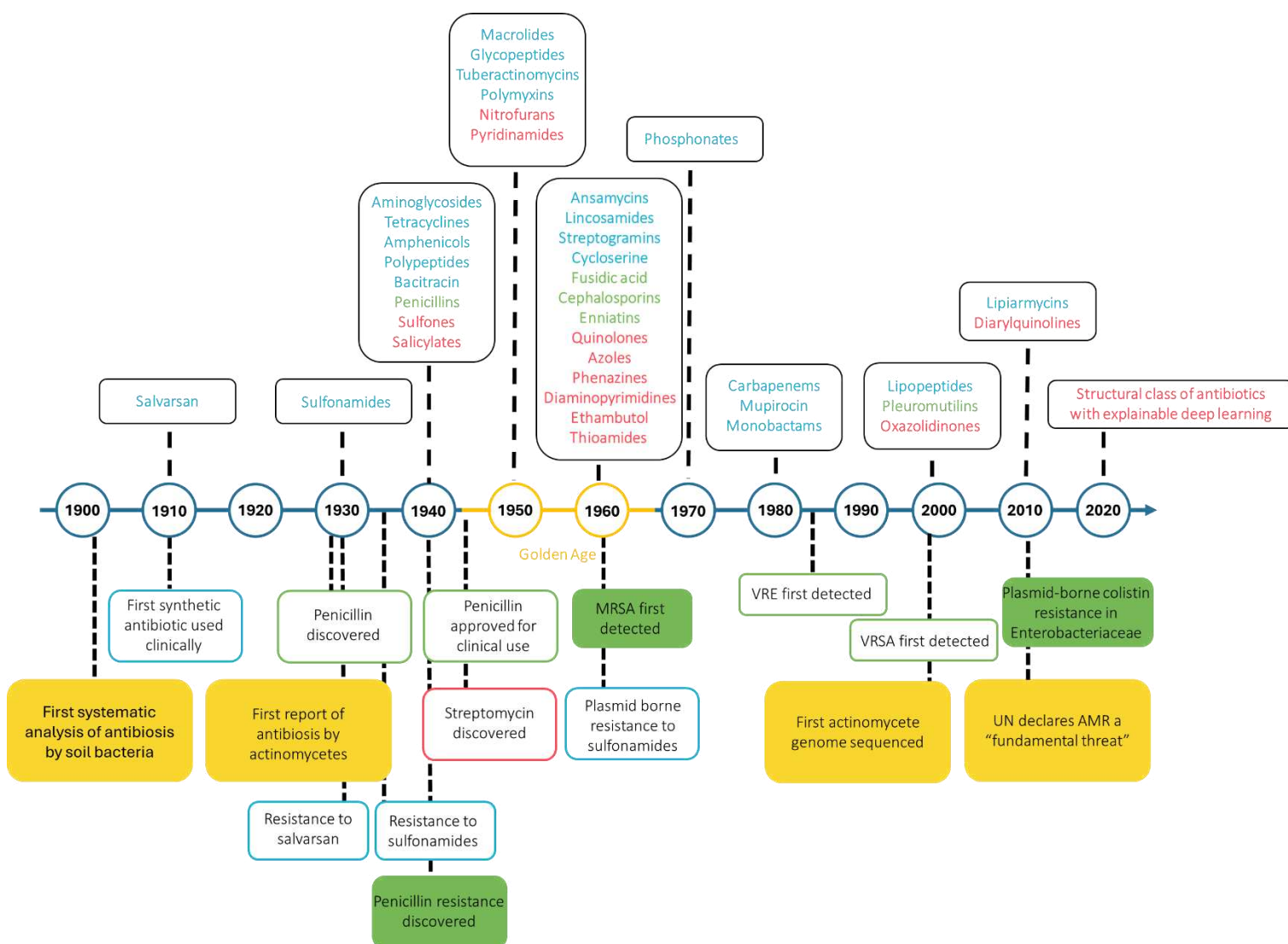


Figure 1 The timeline shows the introduction of antibiotics into treatment and the most important dates (at the bottom of the timeline). The antibiotics are colored according to their source: blue- bacteria; green- fungi; red- synthetic antibiotics

Probable causes of antimicrobial resistance include, among others, excessive use of antibiotics in farm, domestic and aquatic animals (widespread use of antimicrobials in farm animals such as cattle, chickens, and pigs to treat bacterial, viral, fungal, and parasitic infections, as well as the use of antibiotics as growth stimulants), but also in humans. Antibiotics were sold without a prescription and prescribed without taking swabs. Additionally, overcrowding, lapses in hygiene or poor infection control practices in hospitals, poor sanitary conditions, and the release of unmetabolized antibiotics and their residues have contributed to the spread of MDR bacterial infections in society [15]. The developing resistance is controlled by several physiological and biological mechanisms, the details of which are not yet fully understood. The analysis of available bacterial genomes allowed for the identification of over 20 000 potential resistance genes [16]. Consequently, microorganisms passed the selection process to develop mechanisms that allow them to avoid the lethal effects of antimicrobial substances. These include inactivation of the antibiotic, changes in the target molecules, repression of uptake systems, inhibition of drug penetration and distribution, activation of efflux pumps, or

production of enzymes that degrade antimicrobials [6]. This results in the fact that some bacterial infections are now almost untreatable with used antibiotics [17].

This phenomenon, combined with increasing difficulties in designing and introducing new antibiotics into treatment, poses a real threat and considerable medical problems. Thus, it is of importance that we introduce new treatment mechanisms and strategies to reduce the rate of appearance and spread of resistant pathogens.

A. Transition metal ion transport

One of the biggest obstacles in finding new effective and pathogen-specific therapies that do not cause serious side effects in patients, is the fact that bacteria and fungi share important metabolic pathways with humans. Especially in the case of fungi, which are also eukaryotes. To develop highly specific antifungal and antibacterial drugs, it is important to understand and target differences in human and pathogen metabolism. Although such pathogen-selective targets are rare, there is at least one significant difference between bacterial, fungal and mammalian cells- the transport of transition metal ions.

The transition metal ions are essential for proper functioning of all living organisms, both mammals and pathogens. Therefore, vertebrate organisms have developed mechanisms that limit the bioavailability of metal ions to pathogens in the so-called 'nutritional immunity' process [18]. It is a primary defense line put in place by vertebrate organisms to limit the efficiency of pathogens and describes the phenomenon of competition between a host and a pathogen for important resources (in this case, metal ions), during which both the host and the pathogen make enormous efforts to control their availability. It seems that both hosts and pathogens (bacteria and fungi) are aware of the importance of Zn(II) acquisition and both developed systems to maintain its concentration at the appropriate level. Bacteria and fungi have acquired highly effective Zn(II) sequestering modes (presented in point A.2 and A.3 of the Introduction, respectively), while hosts have developed mechanisms, which restrict its bioavailability for pathogens [19]. In response, the bacteria and fungi have developed efficient transport systems that are based on chelating molecules called metallophores. They are secondary metabolites secreted by bacteria into the host environment that bind metal ions with high affinity and selectivity and then deliver them back to the microorganism via specific membrane receptors and transport proteins [20], but they can also be used to protect yourself from toxic excess metal ions in your environment. The best known and most studied metallophores are siderophores (directed to catch Fe(II)), but analogous chelators are also secreted to capture other metal ions, such as Zn(II) [21]. Zincophores, named in analogy to the Fe(II) scavenging siderophores, are usually defined as small molecules involved in Zn(II) uptake in bacteria [22], [23], but this term has also been employed for proteins, that are able to chelate Zn(II) in fungi [24], [25].

1. Human Zn(II) homeostasis

The zinc gene regulation is much more sophisticated in mammalian cells than the one observed in fungi and bacteria (presented in point A2 and A3 of the Introduction, respectively). Human body Zn(II) homeostasis is predominantly regulated by its intestinal absorption [26] and changes in Zn(II) absorption and excretion are the primary mechanisms for maintaining Zn(II) homeostasis, because it is highly necessary for various cellular functions like catalytic and structural functions [27]. Moreover, Zn(II) is responsible for a factors in the human immune system [28] and for this reason, its deficiency causes among others depresses responses to T-cell-dependent and T-cell-independent antigens or reduces the amount of splenocytes [29] and every disruption of Zn(II) homeostasis may led to numerous illnesses like for example growth retardation hypogonadism, development of metabolic syndrome, impaired immune function or neuronal dysfunctions [30], [31], [32], [33]. Moreover, Zn(II) deficiency also causes decreased of serum thymulin activity, decreased NK and T-cytolytic cells activity and lowered IL-2 and IFN- γ mRNAs levels [34]. Zn(II) is also crucial component of Toll-like receptors in secondary immune cells, macrophages [35]. Thus Zn(II) affects the production and signaling pathways of cytokines and transcription factors causing the whole immune system to be compromised [36]. Due to its properties, Zn(II) also plays structural functions in numerous enzymes and transcription functions, in which it constitutes the active centers [37] and ensures their structural integrity [38]. In many cases, Zn(II) is a key factor in the information transfer between cells and within them. It can play a role as a neurotransmitter in the synaptic vesicles and/or an intracellular signaling molecule [30]. In addition to structural and catalytic functions, it was noticed that at least several dozen human genes encode cellular proteins involved in Zn(II) control [27]. They include membrane transporters [39], zinc sensors [40] and a family of at least 10 functional human metallothionein proteins [41]. In eukaryotic cells, there are considerable differences in Zn(II) concentrations. Zn(II) may reach millimolar concentrations in so-called zincosomes or lysosomes, but the affinity of Zn(II) for cytosolic Zn(II)-binding proteins is picomolar [27]. In turn, its excess is toxic and can disturb the Cu(II) homeostasis, thereby triggering cell death [42] [43].

10% of the human proteome is known to bind Zn(II) [44], [45], [46], and the total amount of Zn(II) dispersed in the human body is between 2 and 4 grams [47]. Thus, Zn(II) is the most abundant intracellular element and is found in all body tissues. Approximately 90% of the whole body's Zn(II) is stored in skeletal muscles and bones [48], [49], 5% in the liver and skin tissues and the remaining 2-3% in other tissues [50]. The remaining amount is in the liver, skin and in other tissues [51]. The schematic Zn(II) distribution in the human body is presented on Figure 2.

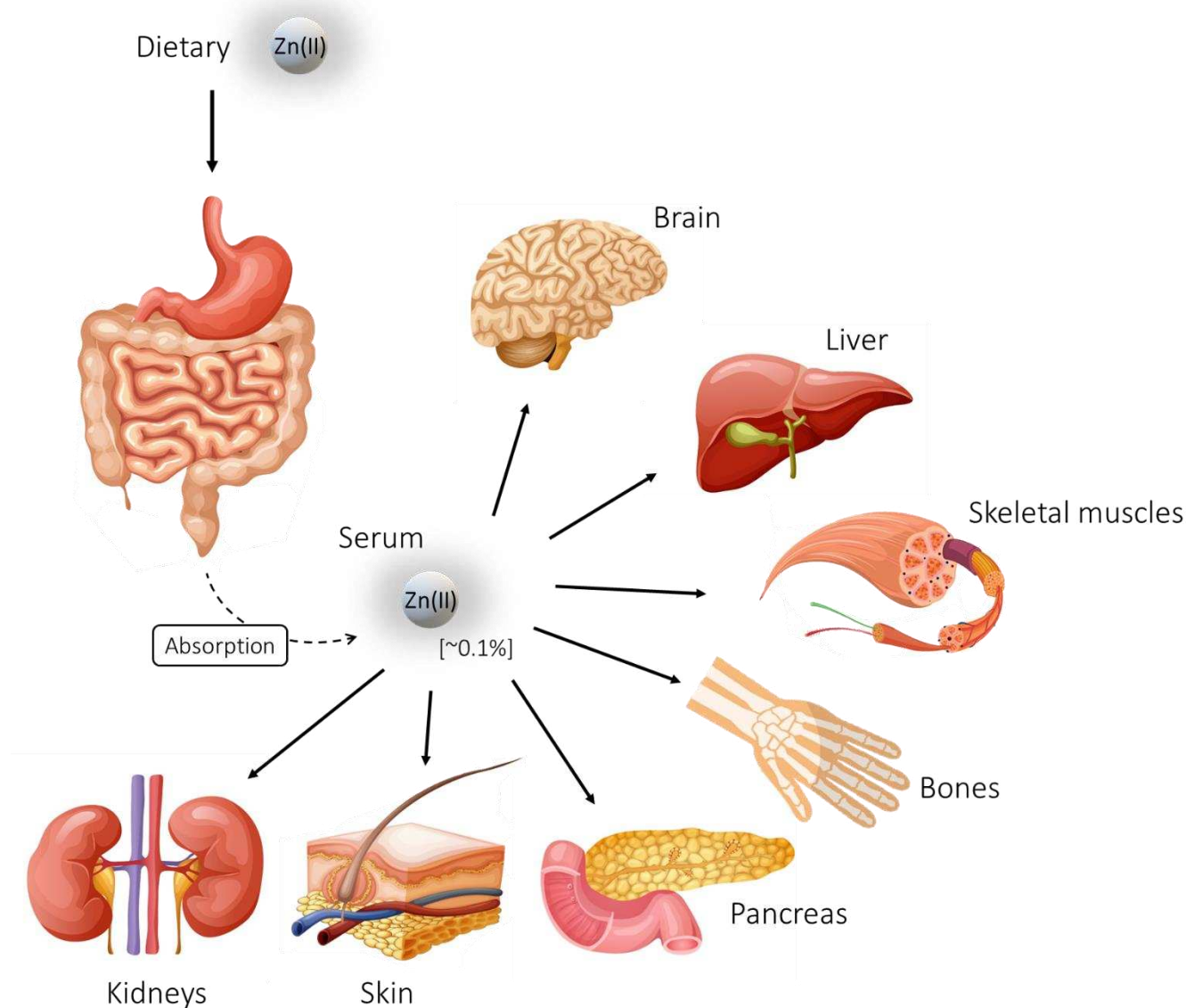


Figure 2 Schematic model of Zn(II) distribution in the human body. Dietary Zn(II) is absorbed in the small intestine (duodenum and jejunum) and then distributed to peripheral tissues. Most of the serum Zn(II) is stored in skeletal muscles, bones, liver and skin. The remaining amount of Zn(II) is distributed to other tissues, like the brain, kidney or pancreas

In cells, Zn(II) is distributed in the cytoplasm (50%), nucleus (30-40%) and membranes of plasma and organelles (10%) [52], [53]. However, Zn(II) is bound with a countless number of proteins, like albumin (80%), α -macroglobulin (20%) [50], [54] or transferrin [55], and

sequestered into organelles and vesicles. Thus the 'free' Zn(II) concentration is very low [56], [57], [58], [59] and the Zn(II) serum accounts for approximately 0.1% of the total Zn(II) in the human body [60]. Moreover, it should be taken into account that Zn(II) uptake is diminished by the presence of other divalent metal cations like for example Fe(II), Ni(II), Cu(II) or Cd(II) [61]. The homeostasis of Zn(II) is regulated both at the systemic and cellular levels [60] and the gastrointestinal tract is the major site for regulation of systemic Zn(II) homeostasis [49]. These mechanisms can maintain zinc homeostasis by decreasing the loss of endogenous zinc, mainly through feces and urine [62], but also by increasing the efficiency of absorption in cases where its intake is low or when its needs are increased due to factors, such as growth, pregnancy, or lactation [63]. Zn(II) absorption depends both on the dietary intake and on its physiological demands and intestinal availability from the diet [64]. In order to maintain cellular Zn(II) homeostasis, it must be delivered to and removed from the cells and organelles. These mechanisms of Zn(II) homeostasis are illustrated in Figure 3. Metallothioneins are mainly responsible for these processes, as are Zn(II) transporters, which are distributed on the cells and organelles' membranes [65]. Zn(II) transporters regulate the cytosolic Zn(II) concentrations and their levels in the cellular compartments, while metallothioneins can regulate their concentrations under normal physiological conditions. Moreover, they are responsible for the Zn(II) storage and its intracellular release [60].



Figure 3 Zn(II) is absorbed throughout the whole small intestine, as shown in Figure 2. The losses of Zn(II) include fecal Zn(II) excretions and excretions with urine, sweat, loss of hairs and nails, exfoliation of the skin or menstrual flow, and semen for adults

a) Membrane transport

Zn(II) homeostasis and transport in humans are regulated by two main families of proteins: ZIP transporters (shown on Figure 4) and ZnT family proteins (shown on Figure 5) [66]. ZIPs and ZnTs have antagonistic functions in mediating Zn(II) homeostasis. ZIP proteins belong to the SLC39A family and constitute importers. They export Zn(II) from the cytosol to the extracellular space or intracellular organelles, while ZnT proteins belong to the solute carrier 30A (SLC30A) family [60] and are exporters, responsible for its transport from extracellular space or intracellular organelles into the cytosol [67] (such as endosomes, Golgi network or synaptic vesicles [60]). However, both ZIP and ZnT are built of 6-8 transmembrane domains (TMDs) and possess largely unstructured loop domains, which stick outside the membrane together with the *N*- and *C*-termini [36]. In the binding of different divalent metal ions are crucial: (i) loops between the second and third transmembrane domains, which are responsible for metal selectivity, (ii) the extracellular *N*-terminus [68], (iii) loops between third and fourth transmembrane domains, which contains His-rich domains and most probably also are

responsible for metal binding [69], [70]. A total number of Zn(II) transporters is 24 and includes 10 Zn(II) transporters – ZnTs, and 14 Zrt-like proteins, ZIP [60], but in the intestinal Zn(II) absorption is mainly mediated by the ZIP4 transporter, which imports Zn(II) from the lumen into enterocytes [71], [72] and ZnT1, which is a basolateral membrane protein exporting Zn(II) on the basolateral side of enterocytes into the portal blood [73].

(1) ZIP transporters

In humans, ZIP transporters belong to the solute carrier 39 (SLC39) family, which consists of 14 members [74]. Most of the ZIP transporters are localized to the plasma membrane, on the cell-surface [75], [76] and their substrate specificity is not strict and they are also involved in the transport of Fe(II), Mg(II), Cu(II) or Cd(II) [48]. The predicted membrane topology of ZIP transporter is shown in Figure 4. The ZIP1 transporter functions as an importer of Zn(II) at the plasma membrane [77], [78] and Zn(II) taken up by this transporter contributes to the activation of microglia by inducing ATP release and autocrine/paracrine activation of P2X₇ receptors [79]. Moreover, most probable ZIP1 expression is necessary for normal prostate metabolism [48]. Next transporter, ZIP2 imports Zn(II) from the extracellular space [80] and its function is necessary for the differentiation of keratinocytes and for the turnover of the epidermis [81]. ZIP3 transporter imports Zn(II) from the extracellular space and is localized in the plasma membrane or intercellular compartments [82]. Its expression increases during differentiation to a secretory phenotype in mammary epithelial cells in response to prolactin [83], and its role is to re-uptake Zn(II) from the lumen of the lung alveoli [84]. Both, ZIP1, ZIP2 and ZIP3 expression is often regulated in a synchronized manner [85] and the protein expression of these transporters decreases in prostate carcinomas [86]. The ZIP 4 is an essential transporter in dietary Zn(II) absorption in mammals [87], which is accumulated at the apical surface of enterocytes [88]. Mutations in the ZIP-4 are responsible for acrodermatitis enteropathica (Table 1), caused by inherited Zn(II) deficiency [71], [72]. Moreover, ZIP4 transport Zn(II) from the lumen into the enterocytes [89] and plays a key role in intestinal integrity [87] and its expression is strictly regulated by Zn(II) concentrations (the low Zn(II) levels upregulate the expression level of ZIP-4, while the high levels downregulate its expression [89], [90], [91], [92]) and by the translational, transcriptional and post-transcriptional mechanism [76], [93], [94], [92]. Its abnormal expression is associated with the pathogenesis and progression of cancer [95], [96], [97]. Another transporter, ZIP5, functions as a Zn(II) importer [48] and antagonizes ZnT1 and ZIP4 functions by importing Zn(II) from the blood circulation into the enterocytes [98]. Thus, its expression is not increased but decreased in Zn(II)-deficiency environments [48] and is also expressed during the whole process of eye development, above all in the retina and sclera [99]. On the other hand, ZIP6 is localized to the plasma membrane [100], [101] and may be a growth factor-induced signaling molecule through functions of Zn(II) influx from the extracellular space [48]. Its elevated expression is involved in the epithelial-mesenchymal transition (EMT) of breast cancer cells [102], in prostate cancer cells [103] or in pancreatic carcinoma [104] and is associated with estrogen receptor α status [105], [106]. Moreover, it is positively correlated with lymph node environment [107]. ZIP6 often plays a role in conjunction and in immune functions

[48]. It contributes to dendritic cell maturation by negatively regulating LPS-induced cell surface MHC class II expression [108] or to tuning the TCR activation threshold in T-cells through increasing Zn(II) influx [109]. Another member of the LIV-1 subfamily is ZIP7, which is localized to the early secretory pathway including the endoplasmic reticulum and Golgi apparatus [110], [111]. Its Zn(II) transport activity is regulated by phosphorylation by casein kinase 2 protein kinase and the regulated release of Zn(II) from intracellular stores promotes the activation of tyrosine kinases, involved in the regulation of cell proliferation and migration [112]. Moreover, its expression level inversely correlates with phosphorylated glycogen synthase kinase 3 levels and cellular Zn(II) concentration [113]. Next transporter, ZIP8 is localized to the plasma membrane, to the apical surface in polarized cells [114], [115] and to the lysosome [116]. In both cases, its activity increases the cytosolic Zn(II) status and the ZIP8 mRNA expression is induced significantly by the stimulus of the Bacille Calmette-Guerin in monocytes [117]. This transporter is involved in the negative-feedback loop, which directly regulates the innate immune function [114] and its higher expression is found in the cartilage of patients with osteoarthritis, which is characterized by higher levels of serum Zn(II) [118]. It leads to an increase in Zn(II) influx into the cells, which activates Metal regulatory transcription factor 1, MTF1, thereby enhancing the expression of matrix-degrading enzymes and causing cartilage breakdown. And most probably, it causes the pathogenesis of osteoarthritis [119]. Moreover, ZIP8 can transport another metal ions, such as Cd(II), Mg(II) and Fe(II) [103], [120], [121]. Among ZIP transporters, only ZIP9 belongs to the ZIP1 Subfamily [122], [123], which is localized to the Golgi apparatus and the cell surface [124], [125]. The first plays a crucial role in B-cell receptor signaling through the Zn(II) translocation from the Golgi lumen [126]. While the other one may function as a membrane androgen receptor and contributes to testosterone-induced prostate and breast cancer apoptosis [125], [127] and its increased expression is reported in malignant breast and prostate biopsies [125]. Another member of the LIV-1 Subfamily, ZIP10, is a cell surface Zn(II) importer [128]. It is also, like ZIP9, indispensable for B-cell development in early and mature stages [129], [130], because it plays an important role for anti-apoptotic signaling by inhibiting caspase activity in the early B-cell survival [130] and is essential for proper humoral immune responses following BCR activation in mature B-cell functions [129]. Moreover, ZIP10 is also involved in invasive behavior of cancer cells [131] and its transcription is regulated by cytokine signaling pathway [130]. These transcription is upregulated in Zn(II)-limiting cells [132] and attenuated in Zn(II) excess [48]. One of the last ZIP transporters is ZIP11, which is the sole member assigned to the gupA Subfamily [80], [123] and is localized to the nucleus or Golgi apparatus [133], [134]. The next ZIP transporter, ZIP12, which most probably has the highest affinity to Zn(II) among all ZIP members [135]. It is localized to the plasma membrane and imports extracellular Zn(II) into the cytosol [135] and has critical functions in neuronal differentiation, like for example neurite extension and tubulin polymerization [135]. Next transporter, ZIP13, mobilizes Zn(II) from the lumen of Golgi apparatus and cytoplasmic vesicles, where is localized [30], [136], [137]. It plays pivotal roles in the endoplasmic reticulum homeostatic maintenance [137] and in cellular signaling [30]. Moreover, its gene is responsible for spondylodysplastic Ehlers-Danlos syndrome [30], [138]. The last one, ZIP14, has two

alternative isoforms: ZIP14A and ZIP14B [48]. Both are localized to the plasma membrane and are responsible for Zn(II) import [94], [121], [129]. ZIP14 has the same localization and function as ZIP5 [98]. Moreover, both show exclusive apical surface localization in polarized cells [121] and its expression is upregulated significantly by cytokines and lipopolysaccharide, LPS [139]. But it is also induced by the unfolded protein response stimulated by Zn(II) deficiency [140] and is important for regulation of systemic growth due to the fact that it modulates G protein-coupled receptor [141]. Moreover, similar to ZIP8, this transporter can also mobilize other divalent cations such as Fe(II), Mg(II) or Cd(II) [120], [142].

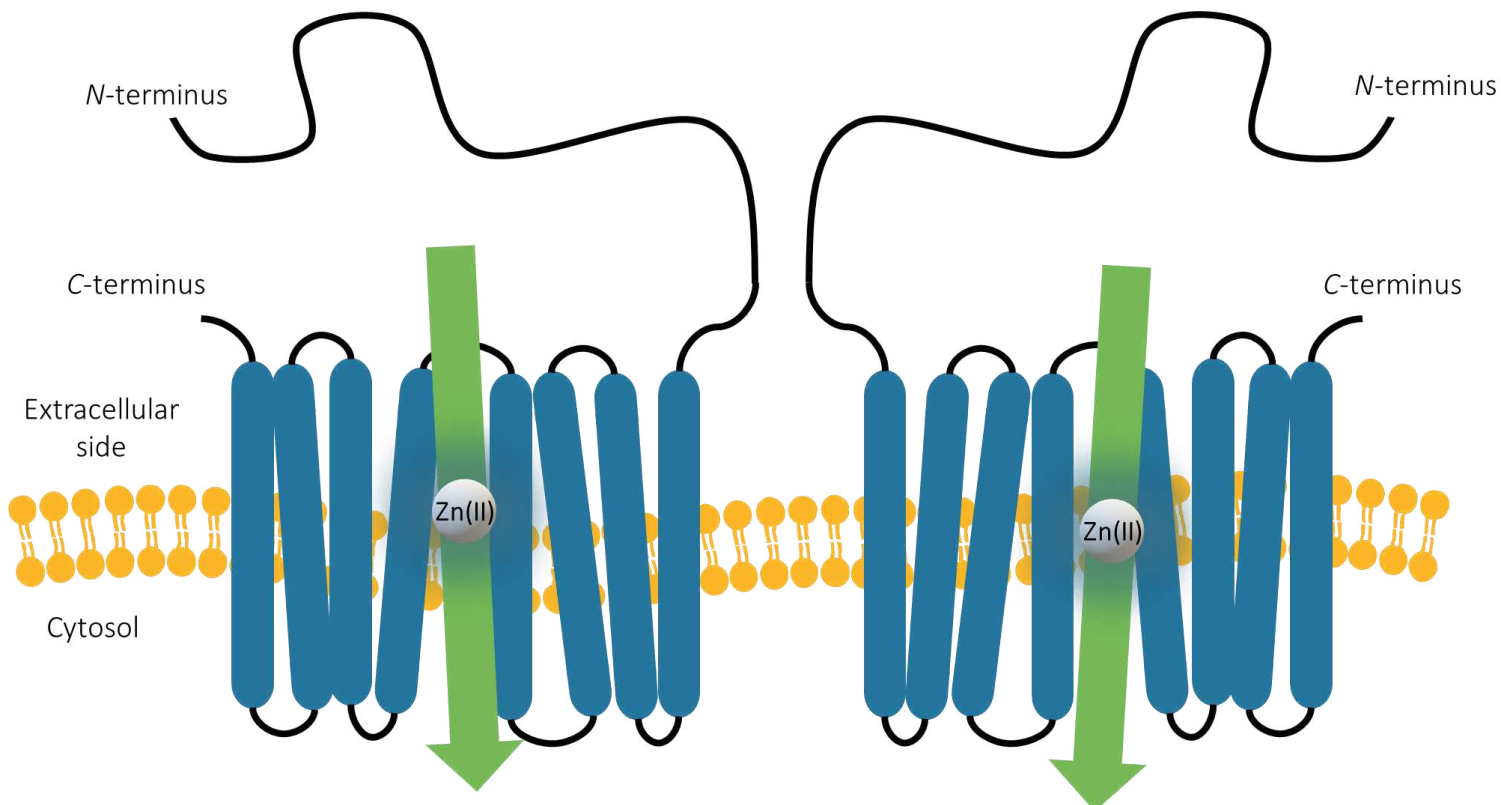


Figure 4 The predicted topologies of ZIP transporters. They form homodimers with eight transmembrane helices

(2) ZnT transporters

ZnT transporters form an important class of Zn(II) efflux proteins in mammals. There are nine ZnT transporters, designated ZnT1-ZnT8 and ZnT10. ZnT transporters operate on the principle of Zn(II)/H⁺ exchange [143], [144]. The schematic topology of ZnT transporters, proposed based on the X-ray structure of their *Escherichia coli* homolog, is presented in Figure 5. The ZnT1 is the only one ZnT transporter that is localized mainly to the plasma membrane and functions by exporting cytosolic Zn(II) into the extracellular space [145]. In the intestine, it is localized on the basolateral surface of enterocytes and transport Zn(II) on the basolateral side of enterocytes into the portal blood [146]. This efflux transporter is expressed in the whole body [92]. Its function is to protect cells from excess Zn(II) influx under pathological conditions [147]. ZnT1 interacts with various intracellular proteins and thus contributes to its roles in cellular signaling [48]. ZnT2 confers cells with Zn(II) resistance by transporting surplus cytosolic Zn(II) into the lumen or vesicular compartments (including endosomes, vacuoles in the intestine and

lysosomes) [60], [148], [149]. It is indispensable for Zn(II) mobilization to breast milk [48] and is expressed in intracellular structures in enterocytes [60]. ZnT3 is a critical Zn(II) transporter into synaptic vesicles of a subset of glutamatergic neurons in the hippocampus and neocortex [150]. In addition, ZnT3 expression is also detected in pancreatic β cells, where it is involved in the regulation of the production of insulin [45]. Most probably, ZnT4 may be involved in a Zn(II) absorption process in adults [48]. It is found in the endosomes, lysosomes, cytoplasmic vesicles, Golgi apparatus and trans-Golgi network [151], [152], [153]. ZnT5 is localized for example in COPII-coated vesicles and the Golgi apparatus [30], [154] and uniquely forms heterodimer complexes with the ZnT6 [155], [156], [157]. Its role is to contribute to the homeostatic maintenance of the secretory pathway functions by supplying Zn(II) into the lumen during Zn(II) deficiency [140], [158], [159] and implicate in the regulation of cellular signaling [160]. Next transporter, ZnT6 is localized to the Golgi apparatus and trans-Golgi network [151] and lacks Zn(II) transport activity. This is caused by the substitution of two His residues of the intramembranous Zn(II)-binding site in the fourth TMD with leucine and phenylalanine [156], [161]. Furthermore, as mentioned, ZnT6 constitutes a modulator subunit of Zn(II) transport activity of ZnT5-ZnT6 heterodimers [156]. Another transporter, ZnT7 is localized in the Golgi apparatus [162] and it is the homolog of the ZnT5 transporter, but does not interact with ZnT6 [156], [161]. It is involved in the activation of alkaline phosphatases [163], [164], and cellular signaling [165] and contributes to homeostatic control in the early secretory pathway [159]. Moreover, the intracellular Zn(II) concentrations do not affected on the action of ZnT6 and ZnT7, since their mRNA expression level is not affected by the high or low dietary Zn(II) level [98]. Both, ZnT6 and ZnT7 transporters are detected in the cytoplasm of absorptive epithelia cells [98]. Penultimate, ZnT8 was identified as a pancreatic β -cell-specific Zn(II) transporter and enhances glucose-stimulated insulin secretion in a high glucose challenge [135], [166]. Moreover, it plays an indispensable role in supplying Zn(II) to insulin granules in β -cells to form insulin-Zn(II) crystals [48]. Last ZnT transporter, ZnT10 is localized to early/recycling endosomes or the Golgi apparatus [167], [168]. Expression of ZnT10 is downregulated by IL-6 [169] and most probably is under the control of ZTRE [170].

Also, the ZnT9 transporter has been classified as a member of the ZnT family. However, ZnT9 is thought to have no Zn(II) transport functions, because it lacks the necessary His residue in the intramembranous Zn(II)-binding site [48]. Moreover, it is localized in the cytoplasm [171].

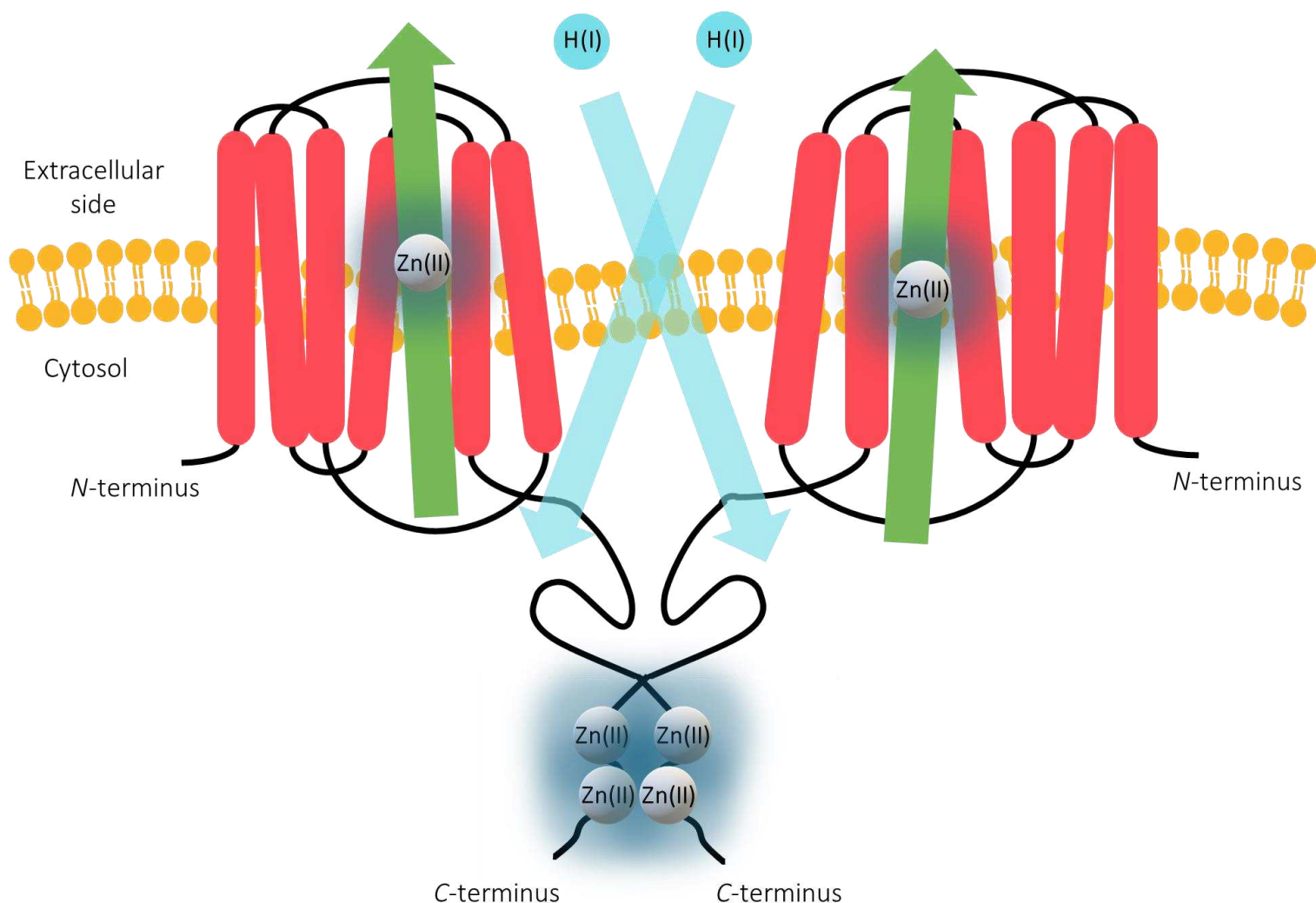


Figure 5 The predicted topologies of ZnT transporters. They form dimers with six transmembrane helices

One of the differences between eukaryotic and prokaryotic transporters consists of ZnT-3 and Znt-8, the representatives of the eukaryotes CDF transporters [69], while in prokaryotic cells are present CzcB, YjiP, ZitB and CzcD homologues [172].

Both ZNT and ZIP transporters play different but key roles in the functions of immune cells. They control Zn(II) signaling and their incorrect expression results in abnormal immune functions [35], [52], [173], [174]. Due to Zn(II) functional role in the human body, it must be kept in a constant condition of homeostasis. Even a small change can cause significant physiological or pathological consequences, causing inherited diseases. A few examples are presented in Table 1. These transporters are also involved in 'nutritional immunity' [175], [176].

Table 1 Mutations of Zn(II) transporters and the diseases they cause

Mutated Gene	Disease	Clinical Features
ZIP4/SLC39A4	Acrodermatitis enteropathica	eczematous dermatitis on the perioral, perianal, and acral areas, diarrhea, alopecia and growth

		retardation [71], [177], [178]
ZIP13/SLC39A13	Spondylocheiro dysplastic Ehlers-Danlos syndrome	growth retardation, skeletal and connective tissue abnormalities, tapering finger, articular hypermobility and protruding eyes with bluish sclera [138]
ZnT2/SLC30A2	Transient neonatal Zn(II) deficiency	erosive dermatitis around the mouth, genital region, neck, and fingers; diarrhea and alopecia [179], [148]
ZnT10/SLC30A10	Syndrome of hepatic cirrhosis, dystonia, polycythemia, hypermanganesemia	dysarthria, fine tremor, hypertonia, bradykinesia, and spastic paraparesis [180]

b) Zinc absorption, regulation, storage, release, buffering and signaling

Zn(II) concentrations can fluctuate under various conditions and Zn(II) may be released intracellularly from the metallothioneins, MTs or secreted from specialized organelles [27]. One of the most important Zn(II) storage and buffering proteins are metallothioneins [181], which, in addition to Zn(II) transporters, also play a key role in its absorption [60]. Approximately 5-15% of the cytosolic Zn(II) pool is bound by metallothioneins [48], including eleven functional human MT-isoforms, encoded by genes MT1-MT4 [182], [183]. They are small proteins, that regulate the level of 'free' Zn(II), bind it and passing it to its transporters [184], [185]. They are composed of 30% of Cys residues (critical to its structure and function are 20-21 Cys residues in the sequence [186], [48]), and that's why they have high affinity towards Zn(II) [37], [68], [187]. The sulfur-containing proteins have an important role in the cellular mobility of Zn(II), because they provide sufficiently strong interactions with Zn(II) and can undergo rapid exchange of ligands [27]. These donor atoms bind up to seven Zn(II) in two clusters [27] and their high Zn(II) binding capacity allows them to keep the cytoplasmic Zn(II) concentration in the picomolar range [188], which constitutes a minute fraction of the few hundred micromolar concentrations of total cellular Zn(II) [27]. They can incorporate up to 7 atoms of Zn(II) and other divalent metals with different affinities [48]. The Zn(II)-binding domain is divided into two clusters and the first cluster has three Zn(II) and contains of nine Cys residues, Zn₃S₉, while the other has four Zn(II) and contains 11 Cys residues, Zn₄S₁₁ [189]. The first cluster constitute more stable α -domain and the second is more reactive β -domain [48]. Formally, the thiolate ligands are shared between all Zn(II) and form ligand bridges [27]. In humans, affinity of metallothioneins is vary and is different for all classes of binding sites, even though the tetrahedral geometry and the set of donor atoms are the same [27]. One Zn(II) is bound rather

weakly, two are bound with intermediate affinity, while last four are bound with high affinity [27]. Metallothioneins are activated by the metal-responsive transcription factor-1 [190] and can be produced everywhere, such as MT-1 and MT-2 [191], or occur in specific places, such as MT-3, which is biosynthesized mainly in the nervous system [192], or MT-4, which is expressed in the skin and gastrointestinal system. The structure of the metallothioneins depends on the availability of Zn(II) and the redox poise in the cellular environment [27]. In the case of oxidative stress, the oxidation of their-SH motives, their buffering capacity is lowered, and the more zinc is bound the less reactive metallothioneins become toward thiol-oxidizing or modifying agents [27]. It is due to their sulfur donors, which coordinate Zn(II). They are redox reactive and their oxidation leads to the release of Zn(II), which contributes to intracellular Zn(II) signaling [48]. The expression of MT is a great influence on the level of Zn(II) in enterocytes [98]. In Zn(II)-deficiency, their expression decreases while it increases when the level of Zn(II) is increased [184], [193]. Furthermore, MTs upregulation appears to affect Zn(II) transport kinetics and decreases luminal Zn(II) absorption [194], [195], which in response to elevated intestinal MT, leads to decreased serum and body Zn(II) concentration [195], [196].

In the serum, Zn(II) is bound mainly to albumin, α 2-macroglobulin and transferrin [197] and the cellular Zn(II) homeostasis is regulated by gene-expression mechanisms, in which Zn(II) plays a significant role [198]. The concentration of available Zn(II) is strictly correlated with the expression of transporters responsible both for the influx and efflux of Zn(II) across the membranes and for cellular Zn(II)-binding proteins [36]. The availability of cytosolic Zn(II) is quite low and in the host is quite limited. Only 0.1% of total Zn(II) in human body is located in the plasma and most of this 0.1% is bound to plasma proteins, which further limits Zn(II) availability. Moreover, metallothionein-bound Zn(II) accumulates in the liver, causing a decrease in the plasma Zn(II) levels [199]. Low Zn(II) concentration reduces the chemotaxis of polymorphonuclear cells, while its high levels enhance the same process [200]. An inappropriate concentration of Zn(II) within the cells leads to the inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) actions, which are extremely important in superoxide anion production [201], maturation of dendritic cells by a drastic change in Zn(II) transporter expression and reduction of the amount of B cells and altering antibody production [202].

c) Local Zn(II) limitation

In addition to changes on a systemic scale, the hosts have evolved excellent strategies for local Zn(II) reduction, like local chelators, which could be the antimicrobial peptides from the S100 family [203]. These include: psoriasin (S100A7) [204], calprotectin (S100A8/S100A9 complex) [205], or S100A9 [206]. They manipulate the microbial access to Zn(II). After pathogen invasion, the neutrophils in abscesses release extracellular traps to restrict extracellular Zn(II) via a Zn(II)-binding protein, calprotectin [207], a heterodimeric protein composed of S100A8 and S100A9 subunits [208], [209]. The amount of free Zn(II) is also reduced intracellularly, where infected macrophages pump out of the phagosome the Zn(II)-bound to the metallothioneins [210]. In other host niches, the availability of the free Zn(II) is also limited due

to systemic zincemia [211]. More information about local Zn(II) limitations in fungi and bacteria is described in point A.2 and A.3 of the Introduction, respectively.

2. Fungal Zn(II) homeostasis

Fungal pathogens represent an enormous burden on human health and the most prevalent of these pathogens include *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans*. All of them are opportunistic, causing disease primarily in individuals with perturbations in immune function [212]. For them, just as for humans (point A.1 of the Introduction) and bacteria (point A.3 of the Introduction), the transport of Zn(II) is important. It serves as a cofactor for transcription factors like zinc fingers [213] and several enzymes [214], like superoxide dismutase, ligase, oxidoreductase, alkaline phosphatase or peptidase [215], [216]. Members of these families are involved in the regulation of several biological processes, like amino acid and sugar metabolism, mitosis, meiosis, or nitrogen utilization [217]. Canonical sequences related to Zn(II) binding consist 5-6% of their proteome [33]. The majority of these proteins are located in the nucleus and cytosol, the rest is secreted from cells or is located within an intracellular compartment [218]. In Zn(II)-deficient conditions, like those in the cells during infection, fungi activate the expression of different systems to increase the uptake of Zn(II). Like other eukaryotes, to Zn(II) transition across cell membranes, fungi use two families of transporters. They include proteins from the ZIP and ZnT families. For this reason (both fungi and humans are eukaryotes), the Zn(II) metabolic pathways of pathogenic fungi intersect with human biopathies and they are much more closer than bacteria pathways. Moreover, Zn(II) transport in fungi is distinguished by a process analogous to the well-understood siderophore-mediated Fe(II) acquisition. This strategy relies on an extracellular zincophore that is able to sequester Zn(II) from the host tissue and deliver it near the pathogen. From the functional point

of view, the zincophore based Zn(II) transport system is analogous to the ABC transporters present in bacteria(Figure 6).

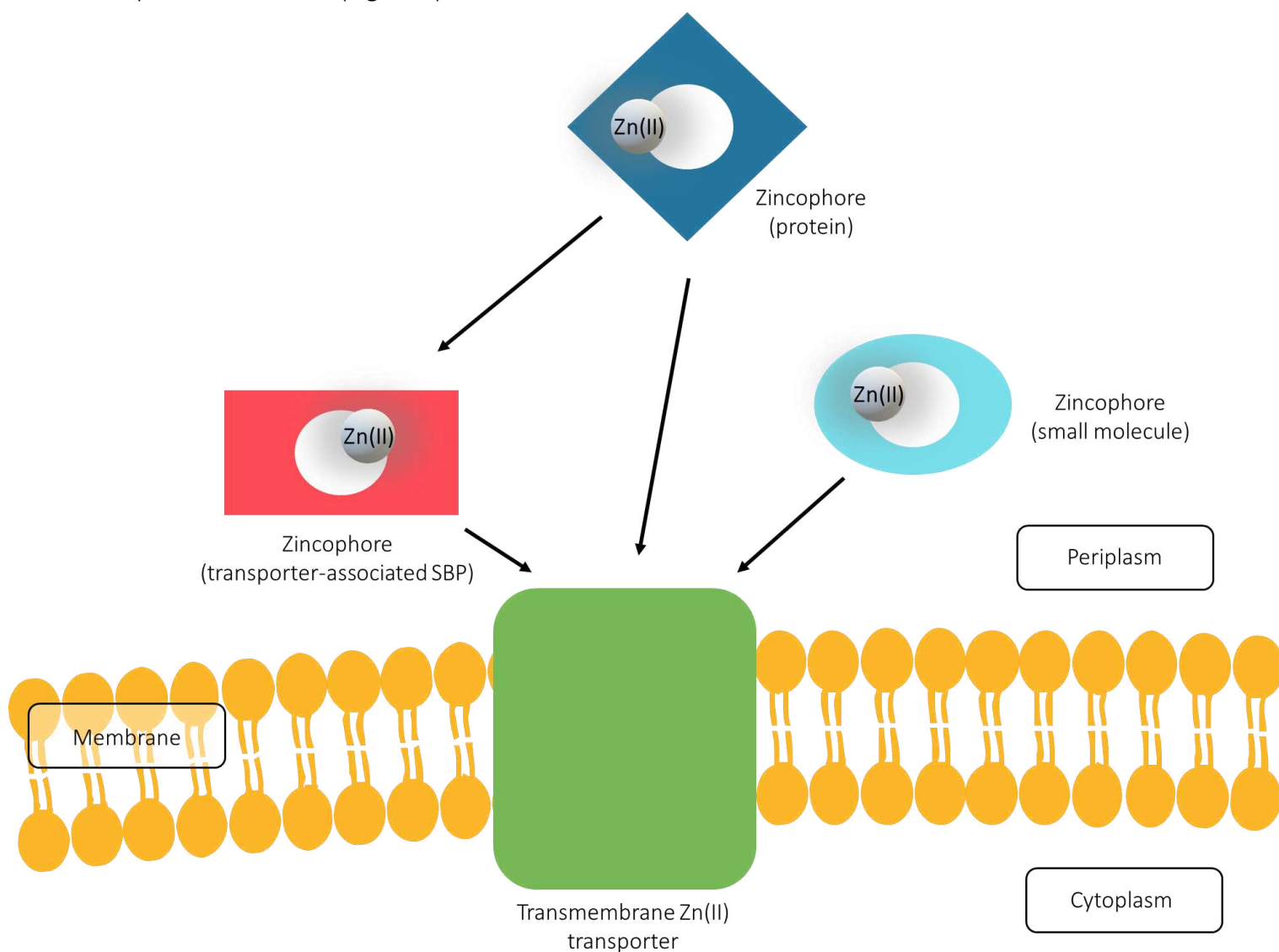


Figure 6 The schematic model of the defined zincophore systems

However, excess of cellular Zn(II) cause cell toxicity by binding to inappropriate sites in proteins [216], like the interaction with the mitochondrial aconitase in cellular respiration [219]. Zn(II), unlike Fe(II) or Cu(II), is redox inactive, and therefore does not generate reactive oxygen species [216]. Notwithstanding, Zn(II) may generate an imbalance in oxidative metabolism and, consequently, elevate ROS levels [220] and it can displace other redox-active metals from their cognate sites [218]. In fungi, too high exposure to Zn(II) can have effects ranging interference in ergosterol biosynthesis to reduced metallothioneins activity [221], [222], increased chitin deposition within the cell wall [223], [224], increased hyphal branching and apical swelling [224], increased endoplasmic reticulum stress and reduced cell viability [225], [139] or interruption of conidia and conidiophore development [223]. Unlike bacteria, fungi do not appear to actively Zn(II) efflux system [39] and vacuoles are the main organelles that storage and detoxification of unnecessary ions, such as Zn(II) [226]. Despite the importance of Zn(II) in the virulence of pathogenic fungi, its mechanisms are still largely unclear [216]. However, the

influence of the environmental conditions on the expression of Zn(II) transporters might slightly differ among fungi. The proposed model of Zn(II) homeostasis in fungi is presented onon Figure 7.

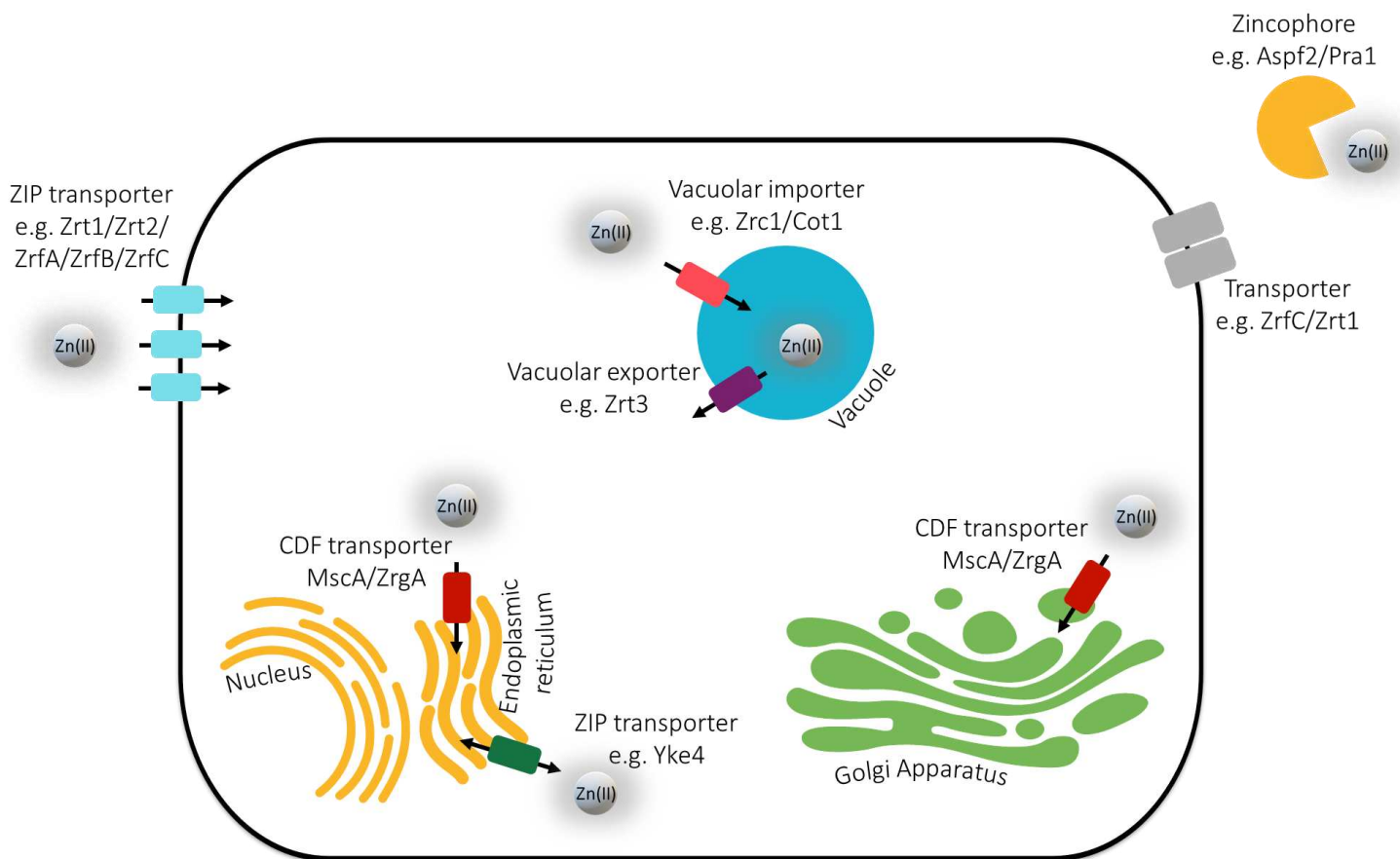


Figure 7 Proposed model of Zn(II) acquisition in fungi

a) Fungal Zn(II) uptake

Metals can exist in various forms such as oxides, salts, sulfates or nanoparticles, but fungi are able to utilize metal ions from these compounds. The dissociation leads to unbound ions, which are available for their uptake and transport [226]. Zn(II)-deficient conditions reduce fungal growth [227] and fungal cells must acquire Zn(II) from the host for proper development during their life cycle [215]. It is not trivial because mammalian hosts reduce the level of Zn(II) and other divalent metals [19]. The 'free' Zn(II) concentration in humans is approximately 150-fold lower than the minimum required concentration [207]. Moreover, when the host will detect fungal infection, the Zn(II) chelating calprotectin is released by neutrophils, monocytes/macrophages, keratinocytes and plasmacytoid dendritic cells [21] to reduce its levels [228]. In neutrophils, calprotectin constitutes approximately 40% of the cytoplasmic protein [229] and they contain high levels of calprotectin and decorate their neutrophil extracellular traps with this potent antimicrobial peptide [230]. Although calprotectin binds Zn(II), Mn(II), F(II) and Cu(II), its antimicrobial activity is associated with Zn(II) and Mn(II) sequestration [231]. In response, pathogenic fungi have developed efficient strategies to Zn(II)

uptake from host [215]. The central role in Zn(II) transport in fungi play ZIP (Zrt-, Irt-like protein) family [39].

(1) ZIP and ZnT

Members of the ZIP family are implicated in Zn(II) uptake, and they transport it into the cytoplasm, either from outside the cell or from an intracellular compartment (like, the endoplasmic reticulum or vacuole) [122], [215]. These ZIP protein family contains Zrt1, Zrt2 and Zrt3 [232]. For example, Zn(II) importation in *Saccharomyces cerevisiae* is performed by high affinity Zrt1 transporter, low affinity Zrt2 transporter [210], [211] and ScYke4, which functions as a bidirectional Zn(II) transporter in the endoplasmic reticulum membrane [228]. It transport Zn(II) into the secretory pathway under at sufficiently high Zn(II) concentrations and under Zn(II)-deficiency conditions it takes out it from the reticulum [233]. *Aspergillus fumigatus* also possess some orthologues of ScMsc2, ScZrg17 and ScYke4: AfMscA, AfZgrA and AfZrfG, respectively [234]. The ScZrg17 orthologues have a conserved His-rich region between third and fourth domains [155]. Moreover, *Candida albicans* also presents Zrt1 and Zrt2 plasma membrane Zn(II) transporters [235]. ZIP proteins are predicted to have eight putative transmembrane regions. Its N- and C-termini are often located on the extracellular or luminal side of the membranes [236] and they consist of a His-rich region present between third and fourth transmembrane regions, which is necessary for Zn(II) selectivity [237]. Within cells, Zn(II) is transported to various compartments, including the endoplasmic reticulum, nucleus and vacuole, through the action of specific transporters unrelated to the ZIP family [39]. As mentioned earlier, Zrt1 and Zrt2 are high and low affinity plasma membrane transporters and both are upregulated in Zn(II)-deficiency conditions. Moreover, they are repressed when Zn(II) conditions are favorable [210], [211]. The level of ZRT1 transcription is highly upregulated in the cells grown in Zn(II)-deficiency conditions and is regulated exactly by its levels [210]. In the case of Zrt2, its role in Zn(II) uptake is marginal, and it contributes to Zn(II) accumulation [216]. It is worth mentioning that the Zn(II) uptake by Zrt 1 and Zrt 2 may also trigger a transmembrane gradient of another metal ion [210], [211]. Vacuolar Zn(II) levels are also modulated by Zrt3, which is also a member of the Zrt- and Irt-related protein family of Zn(II) transporters. In Zn(II)-limiting conditions, the Zrt3 releases the stored Zn(II) back into the cytosol from the vacuole [238]. The role of those transporters in the vacuole is critical for intracellular Zn(II) homeostasis and they are also present in other organelles, like Msc2 or Zrg17, which are the members of the cation diffusion facilitator localized in the endoplasmic reticulum [216] and compose a heteromeric complexes that import Zn(II) into their space for proper protein processing [158]. They form heterodimeric complex and transport Zn(II) into the endoplasmic reticulum, which is required for the proper functioning of the organelle [155]. In the environment with Zn(II)-excess, Zrc1 mediates Zn(II) transport from the cytosol into the vacuole to prevent their toxicity [239]. Exactly that gene, Zrc1, was the first isolated gene associated with Zn(II) transport and its homeostasis [240]. It encodes a protein with six transmembrane domains, which is localized in the vacuolar membrane [241]. Together with Zrc1, the Cot1 contributes to the detoxification of intracellular Zn(II) in the cytoplasm [216]. Moreover, Cot1 also confers tolerance to cobalt [242]. Cot1/Zrc1 is the ZnT type transporter.

(2) CDF

In fungi, the import of Zn(II) from the endoplasmic reticulum is performed by a heterodimeric transporter composed of two CDF proteins: ScMsc2 and ScZrg17 and the cell wall biogenesis is disturbed in *msc2* and *zrg17* mutants [158], explained by the reduction of chitin content and repression of glycosylation events [243]. In Zn(II) import are involved *zrfA*, *zrfB*, *zrfC*, UmZRT1/2, Zip1/2 [205], [244]. To transport Zn(II) fungi also use the CDF protein family, the ferrous transport protein and others [215], [230].

(3) Zincophores

Some fungal species possess additional smart strategies to sequester Zn(II) from the host cells and tissues, that help them overcome Zn(II) limitations in a process analogous to Fe(II) chelation, which is based on secreted siderophores [215]. The Fe(II)-loaded siderophores deliver Fe(II) back to the microorganism via specific membrane receptors and transport proteins [245]. An analogous process occurs for zincophores [230]. It assumes that the binding of zinc by zincophores (generally small molecules), which occurs during infection, allows it to bypass the strategy developed by immune cells to inhibit the growth of the pathogen [205]. It is based on Zn(II)-binding proteins, zincophores, that are able to scavenge Zn(II) from tissues invaded by the fungus. They could interact with the Zn(II) transporters [235] to deliver the metal to the cell [216]. *Candida albicans* secretes a pH-regulated antigen 1, Pra1, which binds Zn(II) in the extracellular environment and delivers it to the cell through the interaction with the Zrt1 transporter. Both, zincophore and transporter share the same promoter and are induced by Zn(II)-deficiency and by neutral-alkaline pH [235]. The Zrt1 transporter of *Candida albicans* has dual transporter and receptor functions [230]. These genes are generally clustered with Zrt1 orthologs in a highly syntenic manner [235], representing a conserved mechanism for Zn(II) acquisition during host-fungal interactions in other species, including Basidiomycota and Ascomycota phyla [228]. Pra1 and its orthologues possess the predicted Zn(II) binding HRXXH motif (where X may stand for any amino acid) [235]. The *Candida albicans* Pra1 zincophore and Zrt1 transporter collaborates with the secreted aspartyl protease, Sap6, which is involved in Zn(II) scavenging and acts upstream in the zincophore pathway delivering the substrate to Pra1 and Zrt1 [21]. The proposed mechanism of Zn(II) transport in *Candida albicans* based on peptide zincophores is presented on Figure 8.

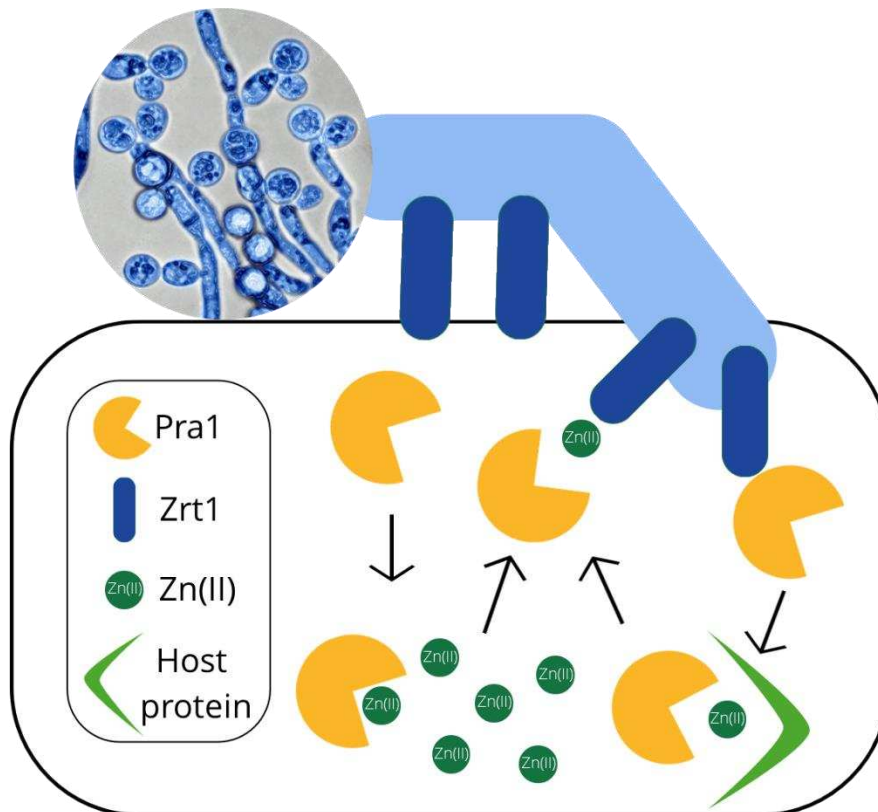


Figure 8 Mechanism of Zn(II) transport in *Candida albicans* based on peptide zincophores

In *Aspergillus fumigatus* the locus is represented by the Asp2 zincophore and ZrfC transporter [228]. The transport mechanism of Zn(II) in this fungus is more accurately presented in point B.1 of the Introduction. Zn(II) transport in *Aspergillus fumigatus* in the slightly alkaline environment of the host is mediated by ZrfC, the Zn(II) importer [207]. Therefore, ZrfC is a potential drug target and all compounds that impairs its function inhibit the growth of the fungi in the host [226]. Moreover, ZrfC has no homolog in humans [207], and consequently, all compounds targeting the N-termini of ZrfC would be effective [226] and free of side effects. The Blast similarity analysis was also made it possible to determine the similarity of the Asp2 zincophore to proteins found in *Fonsecaea pedrosoi*, *Cladophialophora carrionii*, *Cladophialophora bantiana* and *Exophiala dermatitidis*. They show 62%, 54%, 61%, and 59% identity, respectively [228]. However, many other fungal pathogens do not encode zincophores [235]. These other species must rely on alternative acquisition systems and it is possible, that some species rely solely on transporters for Zn(II) uptake or secrete small molecule Zn(II) chelators to sequester it [230]. Moreover, this Zn(II) uptake system is also analogous to bacterial Zn(II) ABC transporters, described in point A.3 of the Introduction. Both, fungi and bacteria encode an extracellular Zn(II)-binding protein (zincophores in fungi and ZnuA in bacteria species) and Zn(II) transporter (ZnuB in bacteria). Additionally, bacteria encode an ATP-binding protein (ZnuC), which provides the energy to its transport.

b) Fungal Zn(II) storage

Vacuole is the main space for Zn(II) storage in fungi, and its detoxification [246] (because fungi do not appear to have a plasma membrane Zn(II) efflux system [39]) and Zn(II) transport into the fungi vacuole is performed by the CDF transporters [228]. The detoxification process

depends for example on Zrc1 and Cot1 Zn(II) importers present on the vacuolar membrane [247]. Another Zn(II) transporter family, important for its homeostasis, is the cation diffusion facilitator family, which takes part in the transport of excess metals from the cytoplasm to intracellular organelles [248]. They include ScCot1 and its paralogue ScZrc1 [238]. Moreover, *Aspergillus fumigatus* presents a vacuolar Zn(II) importer AfZrcC, which is related to ScZrc1, and paralogues: AfZrcA and AfZrcB [234]. Members of the CDF family share some structural characteristics, like six predicted transmembrane domains and the loop region placed between the fourth and fifth domains, which usually contains a His-rich region [122]. The topology of vacuolar Zn(II) importers in fungi is presented on Figure 9.

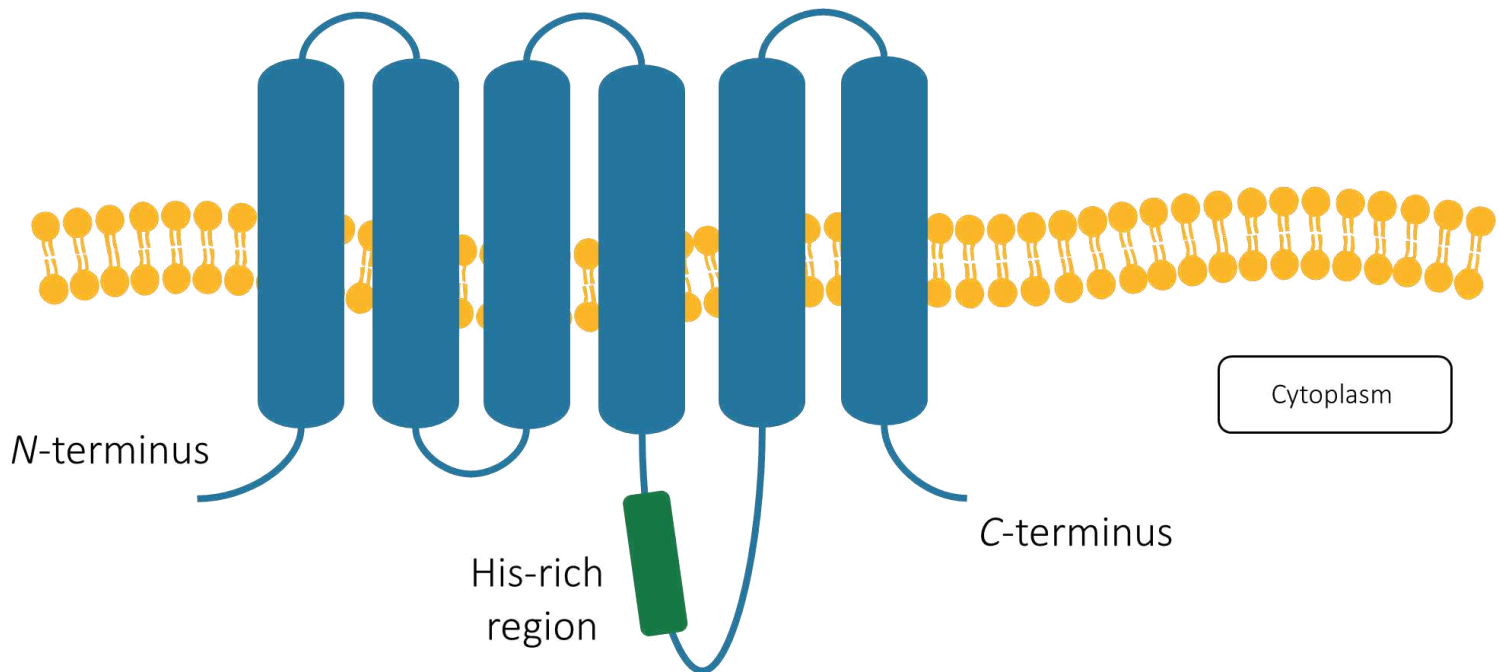


Figure 9 Topology of vacuolar Zn(II) importers

Moreover, zincosomes represent another potential intracellular Zn(II) storage. They are vesicles, which contain labile Zn(II) and are present both in mammalian and yeast cells [230]. Most probably, they serve to detoxify excess Zn(II) and mobilize it upon deprivation [39].

c) Regulation of fungal Zn(II) homeostasis

Zn(II) homeostasis in fungi is primarily regulated at the transcriptional level by transcription factors. These include, for example, Zap1, which is the major regulator of Zn(II) homeostasis in Zn(II)-deficient conditions [249]. It activates Zrt1 and Zrt2 in *Saccharomyces cerevisiae* [250], [251] and contains seven Cys₂His₂ zinc finger domains (ZFDs: ZF1 – ZF7) of which five (ZF3 – ZF7) is on the C-termini and is responsible for recognizing and binding to the specific Zn(II) responsive elements (ZREs) at promoter Zap1 [228]. In Zn(II)-limiting conditions, Zrt1 is expressed, N-glycosylated and localized in the plasma membrane. Zn(II) binds to the ZF1 and ZF2 at the activation domain AD2 or in the Cys/His region in AD1. It leads to inactivation of the activation domains and results in decreased expression of target genes. Under mildly Zn(II)-limiting conditions, Zn(II) binds to AD1 and AD2 and promotes their inactivation, suppressing even more the transcription of Zap1 targets. In the case of Zn(II) excess, Zrt1 is ubiquitinated by the Rsp5 ubiquitin-protein ligase and the Ubc4 and Ubc5 ubiquitin-conjugates enzymes. Then

comes to rapid internalization via endocytosis and degradation in the vacuole [249], [252] and DNA-binding domains are independently regulated by Zn(II) and the global Zn(II)-responsiveness of Zap1 is a combination of these different mechanisms [253]. Moreover, Zap1 binds to Zn(II) responsive elements on its own promoter and regulate its expression [211]. Consequently, the Zap1 level rapidly increase allowing an expression of several genes, such as *zrt1*, *zrt2* or *zrt3*, in Zn(II)-deficient conditions [254]. A Zap1 orthologues was also found also in *Cryptococcus gattii*. Similar to other fungi, it is necessary for the activation of the plasma membrane Zn(II) transporters: ZIP1 and ZIP2, in the presence of the Zn(II) chelator, TPEN. Its absence reduces *Cryptococcus gattii* survival in a mouse model of the infection [255], and this change in virulence may be partially explained by increased susceptibility to nitrogen-responsive species and ROS accumulation [215]. The ZIP family of *Cryptococcus gattii* includes also ZIP3 and ZIP4, which are homologs of *Saccharomyces cerevisiae* ZRT3 and YKE4, respectively [216]. Zap1 is indeed the major Zn(II) transporter in *Cryptococcus gattii* and also *Aspergillus fumigatus* possesses its homolog, the ZafA [256]. It activates the Zn(II) uptake by inducing expression of the high affinity ZIP family membrane transporters: ZrfA and ZrfB in Zn(II)-limiting media [228]. The ZafA protein has no homolog in humans, and due to the presence of Cys₂Hys₂-type Zn(II) fingers, its inhibition should be based on a specific blockade of its transcriptional activation domain or interference with its DNA binding domain [226]. In *Aspergillus fumigatus* the adaptation to Zn(II) availability is also regulated by the pH-responsible factor PacC. In acidic Zn(II)-deficiency conditions ZafA binds to Zn(II) responsive elements on promoters of *zrfA* and *zrfB* genes and activate its expression. ZafA binds also to the promoter of *zrfC*, whose expression is repressed. In contrast, in alkaline Zn(II)-deficiency conditions PacC binds to *zrfA* and *zrfB* promoters and represses their transcription. ZafA overcomes *zrfC* repression by PacC strongly indicating its expression. ZrfA and ZrfB are responsible for the Zn(II) uptake under acid conditions, while ZrfC functions as Zn(II) transporter in Zn(II)-deficiency, alkaline niches [234]. In proper function of *Candida albicans* is also important Csr1/Zap1, which is another homolog of Zap1] and constitutes a high copy number suppressor of ZRT2 in *Saccharomyces cerevisiae* [216]. It contributes not only to Zn(II) uptake and homeostasis but also to the morphological transition in this pathogen [257] and it has a negative regulatory role in the production of extracellular matrix for biofilm production [258]. Similar to *Candida albicans*, *Candida dubliniensis* also possesses the Zap1 homolog, named Csr1 [216] and is a transcriptional activator for Zrt1, Zrt2 and Zrt3. Although Zap1 is widely dispersed in fungal kingdom, *Schizosaccharomyces pombe* genome lacks a homolog of this protein and its *zrt1* gene is highly expressed in Zn(II)-limited cells, while *zym1* is responsible for Zn(II) storage [259]. Alternative regulator is Loz1, which contains two Cys₂His₂ zinc finger domains and an adjacent accessory domain at C-terminus [260] and acts as a gene repressor in the Zn(II) excess. There are three members of the cation diffusion proteins in *Schizosaccharomyces pombe* that are localized to the secretory pathway. They include Cis4 and Zrg17 (homologs of Msc2 and Zrg17), which transport Zn(II) into the cis-Golgi, and Zhf1 (homolog of Zrc1 and Cot1), which transports excess Zn(II) into the endoplasmic reticulum [261]. Besides, Zn(II) transporters and transcription factors in Zn(II) homeostasis are other types of proteins, such as metallothioneins or chaperones [226]. The essential for the maturation of mitochondrial MnSOD in *Saccharomyces cerevisiae* is Mtm1 – a manganese-trafficking factor [262], which is important for proper Zn(II) homeostasis [263].

3. Bacterial Zn(II) homeostasis

All organisms must regulate the cellular uptake, efflux and intracellular trafficking of essential elements, such as Zn(II). Thus, Zn(II) is also an essential and cardinal metal ion for the proper growth and survival of bacteria and the bacterial Zn(II) homeostasis plays critical roles in a variety of contexts [264]. In bacteria Zn(II) is primarily used as a metalloenzyme cofactor [265] and the percentage of Zn(II)-binding proteins in the bacterial proteome is about 5–6% [265]. Moreover, in a few bacterial pathogens, Zn(II) is also involved in the execution of pathogenesis ability [46]. So, it is extremely important, but its intracellular free concentration in bacteria is maintained within a narrow range [266], on average around 0.1 to 1 mM [56], [267]. However, Zn(II) can also be toxic. In excess, competes for non-cognate metal binding sites in metalloproteins and renders them dysfunctional [268]. However, its excess is toxic for the cells, when its concentration is too high, Zn(II) occupies the binding sites of other essential metal ions. For example, Zn(II) has a greater affinity for magnesium enzymes than magnesium itself [269].

Therefore, the concentration of intracellular Zn(II) must be strictly regulated and in response to this, bacteria have developed several mechanisms to control cellular Zn(II) level [270]. They include both high- and low-affinity uptake, but also efflux systems, which are necessary to precise maintenance of Zn(II) homeostasis [271], [272] and storage mechanisms, which enable safe-keeping of Zn(II) for later use [273]. However, in the export and uptake of Zn(II) in bacteria ABC transporters, P-type ATPases, cation diffusion facilitators (CDF) and protein families of resistance-nodulation-cell division (RND) are mainly involved [39], [271] and almost all metal acquisition genes are regulated by repressor-type regulators that are linked to related metals as corepressors [274]. Moreover, the most important Zn(II)-sensing regulators include members of the ferric uptake regulator family (Zur), MarR family (AdcR), MerR family (ZntR) and ArsR/SmtB family (CzrA) [275]. All this due to the fact that extremely efficient Zn(II) acquisition and increased Zn(II) tolerance contribute to the survival of bacteria in the host [264]. The major proteins involved in bacterial Zn(II) homeostasis are presented on Figure 10.

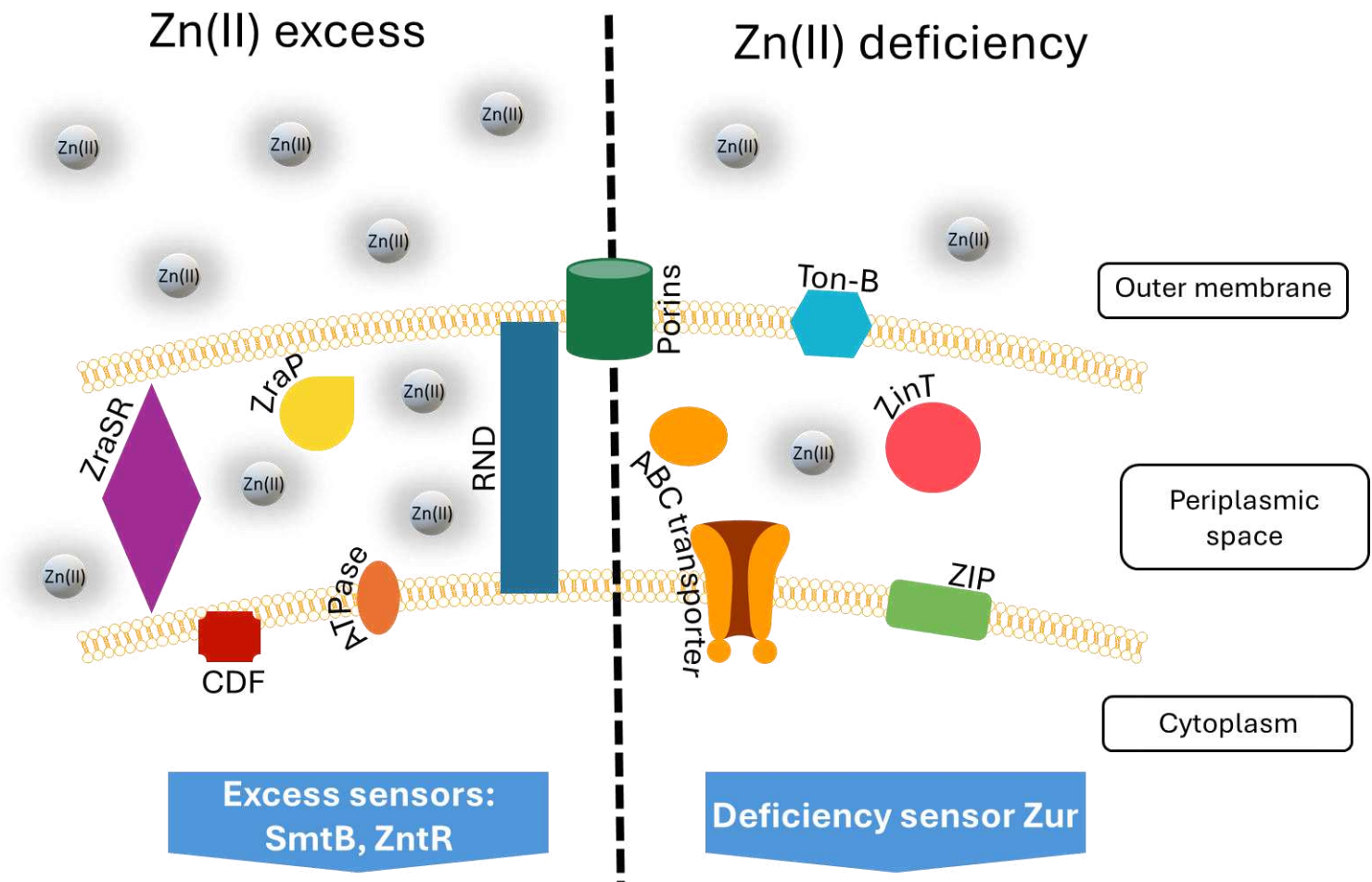


Figure 10 Main proteins involved in bacterial Zn(II) homeostasis. From left: transporters (ATPase, CDF and RND), periplasmic proteins (ZraP and ZraSR) and sensors (SmtB, ZntR) involved in Zn(II) homeostasis in the case of Zn(II) excess; from right: different molecules and systems involved in Zn(II) uptake in Zn(II) deficiency

a) Uptake systems

To maintain and control appropriate Zn(II) levels at extremely low Zn(II) concentration bacteria use high-affinity zinc uptake system [276], [277], which includes several binding protein-dependent ABC transporters [278]. The second major type of zinc import system is low-affinity zinc uptake, which most probably is involved in the Zn(II) supply at a high but nontoxic concentration of Zn(II) [279].

(1) ATP-binding cassette (ABC) transporters

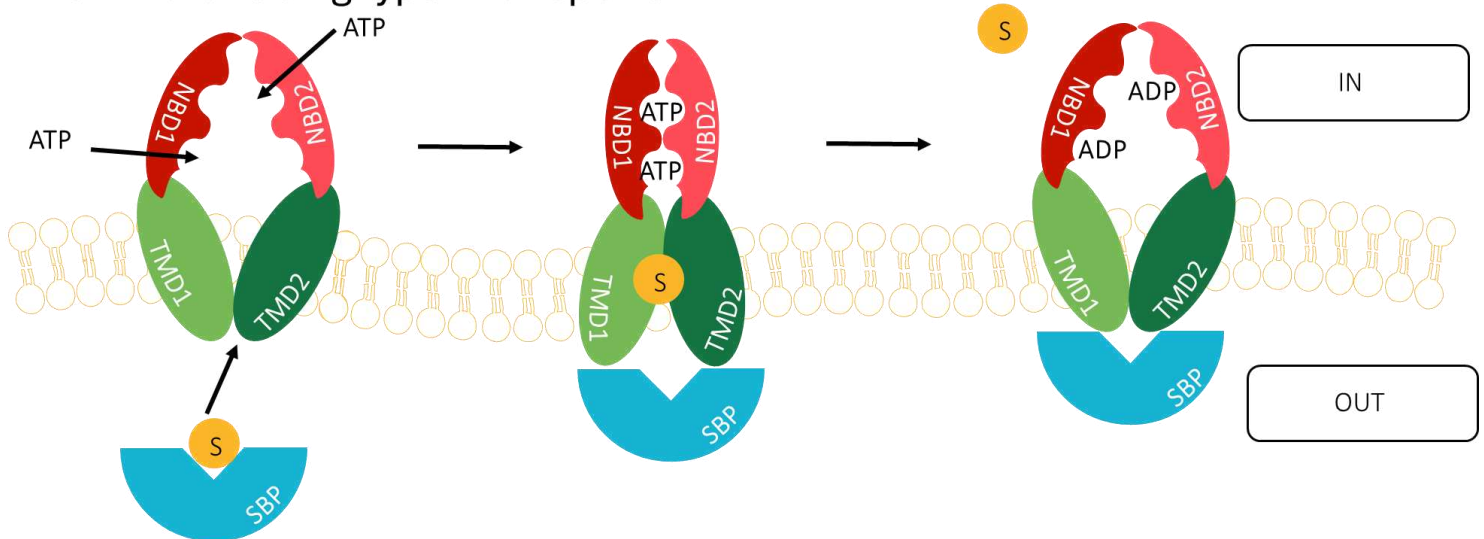
The superfamily of adenosine triphosphate ATP-binding cassette (ABC transporters), play a role in the ATP-powered import and export of different molecules across membranes against a concentration gradient, such as amino acids, lipids, virulence factors inorganic ions, peptides, proteins, saccharides, drugs, or metal ions, but they also play an intermediate role in the transport of many other physiological substrates [280], [281] and are involved in the regulation of several physiological processes, signal transduction, protein secretion, antigen presentation or drug and antibiotic resistance [281]. Moreover, they are necessary for the cell

viability and virulence [282] because they are involved in DNA repair and chromosome maintenance [283]. ABC transporters operate in a single direction, either import or export. Due to the details of their mechanics and architecture, two classes are distinguished among importers: I and II [283], [284]. Moreover, they are present in bacteria, archaea and eukaryote [285] and their number differs widely between species [286]. They have a characteristic architecture consisting minimally of four core domains [287]. The first two constitute two ATP-binding cassette domains that provide the energy to translocate substrates across cell membranes from ATP hydrolysis [288] or nucleotide-binding domains (NBD1 and NBD2), and the next two are transmembrane α -helical domains (TMD1 and TMD2), which form pores in the membranes [283]. In bacteria, these four domains may be a combination of single, identical pairs of subunits or a combination of fused NBDs and/or TMDs [284]. Furthermore, in addition to the four main domains, bacterial importers require an additional subunit that is responsible for capturing the transport solute substrate and delivering it to the binding site in the TMDs. In Gram-negative bacteria, the additional subunits are soluble proteins with a mass of 30–50 kDa, which are located in the periplasm, while in Gram-positive microorganisms, the additional subunits are lipoproteins anchored in the outer leaflet of the cell membrane [289]. That transporters use the hydrolysis of the ATP to pump molecules across the membrane, they are called ATPases, and their large superfamily includes the rotary motor F-, A- and V-ATPases, the P-type ATPases and the ABC transporters [290]. Many ABC transporters are major determinants of genetic human diseases. The most common diseases are cystic fibrosis, Stargardt disease, progressive familial intrahepatic cholestasis-3, adrenoleukodystrophy, anemia, Tangier disease, or Dubin-Johnson Syndrome [291].

The substrate specification of each transporter is determined by the amino acid sequence in the transmembrane domain [291], what results in little similarity between TMD of different ABC transporters. The transmembrane domains contain 6-11 membrane-spanning α -helices and in the contrast to the hydrophilic NBDs are highly conserved. NBDs consist of approximately 90-100 amino acids, and each is encoded by a single polypeptide [292]. Every nucleotide-binding domain contains two short sequence motifs in their primary structure: the α -helical Walker A and β -standard Walter B motif [285]. The Walker A-site constitutes of GXXGXGKS/T motive, where X may stand for any amino acid, while Walker B-site contains hhhhD motif, where h stands for hydrophobic residues [293]. Moreover, the Walker B-site is immediately preceded by a high conserved LSGGQQ/R/K/KQR motif sequence [294], [295] and can be useful in the identification of putative new members of the family [285]. Both motifs contain invariant amino acid residues (lysine residue in the Walker A motif, which is crucial for the binding of the β - and γ -phosphates and the aspartate in the site B, which plays a role in liganding the Mg(II) that accompanies the nucleotide) and are linked by an extended peptide fragment, which consists of approximately 100 residues. The basic catalytic cycle of ABC transporter consists of a series of steps, which include the binding of solute-binding proteins (in the case of importers) or the direct binding of a substrate (in the case of exporters) to the TMDs; dimerization of the NBDs; switching of the TMDs; ATP hydrolysis; and release of the transported substrate accompanied by dissociation of the NBD to prepare the transporter to the ground state for the next cycle [289]. Thus, two NBDs are engaged in a symmetric dimer with two ATP molecules in the dimer interference, where ATP is bound to NBD1 coordinated by Walker A

residues from NBD1 and residues from the signature sequence of NBD2. The situation is the same for the second ATP [289] and therefore the hydrolysis of two ATP molecules results in the transport of one substrate molecule [291]. The details of this transport, taking into account various types of importers, are illustrated in Figure 11. It is worth underline that amino acids changes in both in A and B motifs are generally not tolerated with respect to ATPase activity [296] and in cases where one of the four domains is absent, one of the remaining domains acts as a homodimer so as to maintain full complement [297].

The inward-facing type I transporter



The outward-facing type II importer

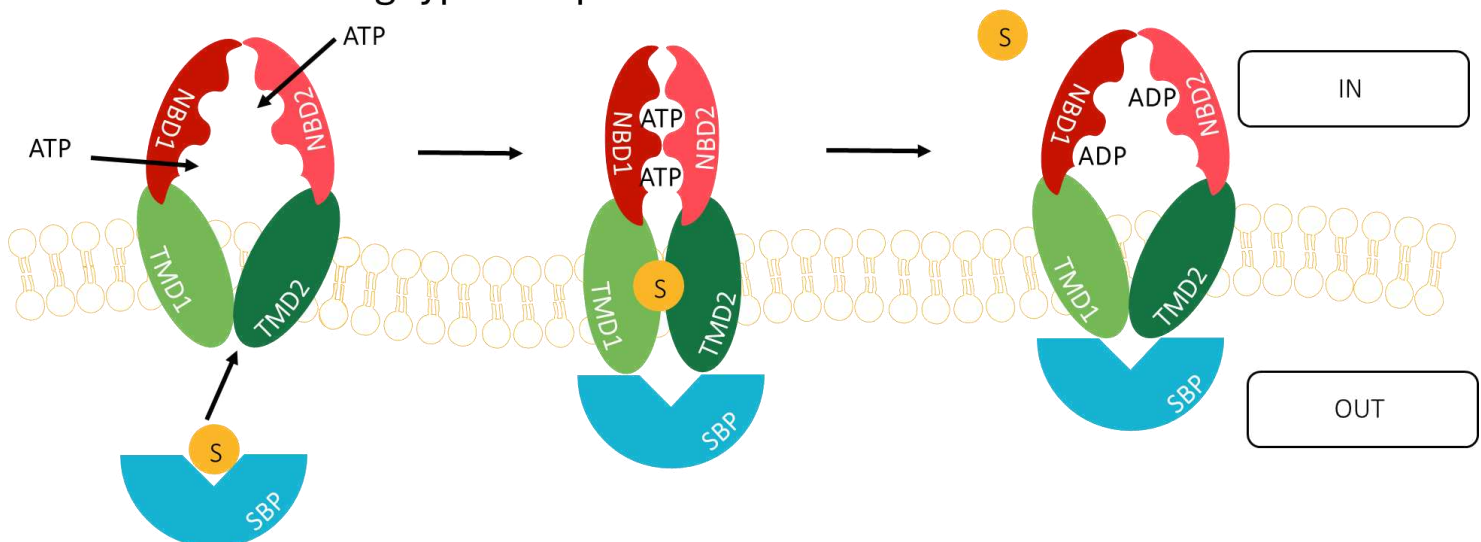


Figure 11 The inward-facing type I transporter binds to the substrate binding protein and two molecules of ATP. NBDs dimerize and result in an outward-facing conformation. The substrate leaves the binding protein and binds to the TMDs mid-membrane. In the last step, the ATP is hydrolyzed and the product is released, together with the dissociation of the NBD. The outward-facing type II importer binds to the substrate binding protein and two molecules of ATP. The dimerization of the NBDs results in an occluded conformation, with the substrate confined to a sealed cavity mid-membrane. The ATP hydrolysis and NBD dissociation allows the substrate to escape into the cytoplasm

Some ABC transporters appear to be a part of ion channels and are not directly involved in the transport of substrates. In these cases, the hydrolysis of ATP is associated with the movement of the protein itself or to other proteins to regulate the opening and closing of the ion channels [298]. The transport against a chemical gradient requires a source of free energy, which can be provided either by the potential energy of an existing chemical gradient or by a coupled enzymatic reaction [289]. The preferred substrate of ABC domains or purified transporters is the ATP, but other nucleotides, such as guanosine triphosphate (GTP) [299], [300], cytidine triphosphate (CTP) [299], uridine triphosphate (UTP) [299] or inosine triphosphate (ITP) [299] are also accepted although with much lower affinity and greatly reduced hydrolysis rate [285]. Furthermore, the ATPase activity of most ABC proteins and transporters has been found to be sensitive to ADP and non-hydrolysable nucleotide analogs, like AMP-PNP or ATP γ S [299].

One of the most widespread high-affinity zinc-uptake transporters in Gram-negative bacteria is the ZnuABC transporter, which is essential to growth under different environmental conditions characterized by low availability of Zn(II) [301]. Bacteria use it to transport Zn(II) from the periplasmic space to the cytosol, where it is accumulating [268], [272]. The ZnuABC transporter is composed of three proteins [302]: i) ZnuA – a periplasmic Zn(II)-binding protein, which contains a flexible region composed of a large number of histidine and/or glutamic acid residues (His-rich loop) and is responsible for Zn(II) delivery to the ZnuBC transporter [303]; ii) ZnuB – transmembrane protein which executes the transportation of Zn(II) across the membrane [303], [304] and iii) ZnuC – an ATPase-binding protein of the high-affinity Zn(II) ABC transporter, responsible for providing energy for the Zn(II) uptake [303], [304]. The conventional arrangement of ABC transporters genes includes the transmembrane permease gene and the gene encoding the ATPase adjacent to SBP, and all these three genes are co-transcribed [305]. In some Gram-negative bacteria, such as *Salmonella enterica*, *Escherichia coli* or *Bacillus subtilis*, there may be an additional element of the ABC transporter, ZinT [302]. It is a soluble Zn(II) trafficking protein, that facilitates the Zn(II) acquisition, as it is capable of deliver it to the soluble periplasmic ZnuA domain [19], [302]. Thus, ZinT and ZnuA are strictly correlated [306], [307] and form stable complexes when a metal is bound to them, but they do not interact each other in the absence of Zn(II) [306], [307]. During the complexation process, the His-rich loop of ZnuA is inserted into the ZinT cavity, forming the ZinT–ZnuA complex. Its role is to participate in zinc homeostasis in the periplasmic process of Zn(II) recruitment, and the role of the periplasmic chaperone that binds Zn(II) with a very high affinity and transfers it to other periplasmic zinc-binding proteins [308]. ZinT is active at an extremely low concentration of Zn(II) and does not occur in bacteria that lack the ZnuA protein.

The zinc uptake regulator, Zur, acts as a repressor of the uptake (*znuA*) and the export (*zitB*) genes for Zn(II), which is responsible for controlling the ZnuABC system and contributing to zinc homeostasis in most Gram-positive and Gram-negative bacteria [279], [307] such as proteobacteria, firmicutes, cyanobacteria or actinobacteria [272]. This regulator is an abundant protein, that is member of the ferric-uptake regulator family proteins (Fur) and regulates genes encoding proteins but also a noncoding RNAs [309]. Its level does not fluctuate within a wide range of changes in Zn(II) concentration [274] and it forms oligomers and multimers at Zn(II) concentrations from femtomolar to picomolar and further to micromolar [310]. Zur has a dual

role, both as a repressor and an activator [274] and its dimers can adopt at least two conformations: closed with high DNA-binding affinity and open with low DNA-binding affinity [264] and each Zur monomer can bind more than one Zn(II) [311], [312]. Under Zn(II) sufficiency, Zur exists in a Zn(II)-bound form, which is capable to DNA-binding and keeping the genes involved in Zn(II) uptake and mobilization in a repressed state [305]. However, the Zn(II)-bound Zur not only represses genes for Zn(II) acquisition, but also induces the *zitB* gene encoding a Zn(II) exporter in *Streptomyces coelicolor*. Besides Zur, the Fur family also includes Ferric Uptake Regulator (Fur) [313], Nickel Uptake Regulator (Nur) [314], Manganese Uptake Regulator (Mur) [315] and further members include sensor of cytoplasmic peroxide (PerR) and sensor of haem (Irr) [316], [317]. Figure 12 shows a schematic representing the mechanism of Zur, taking into account the Zn(II) concentrations.

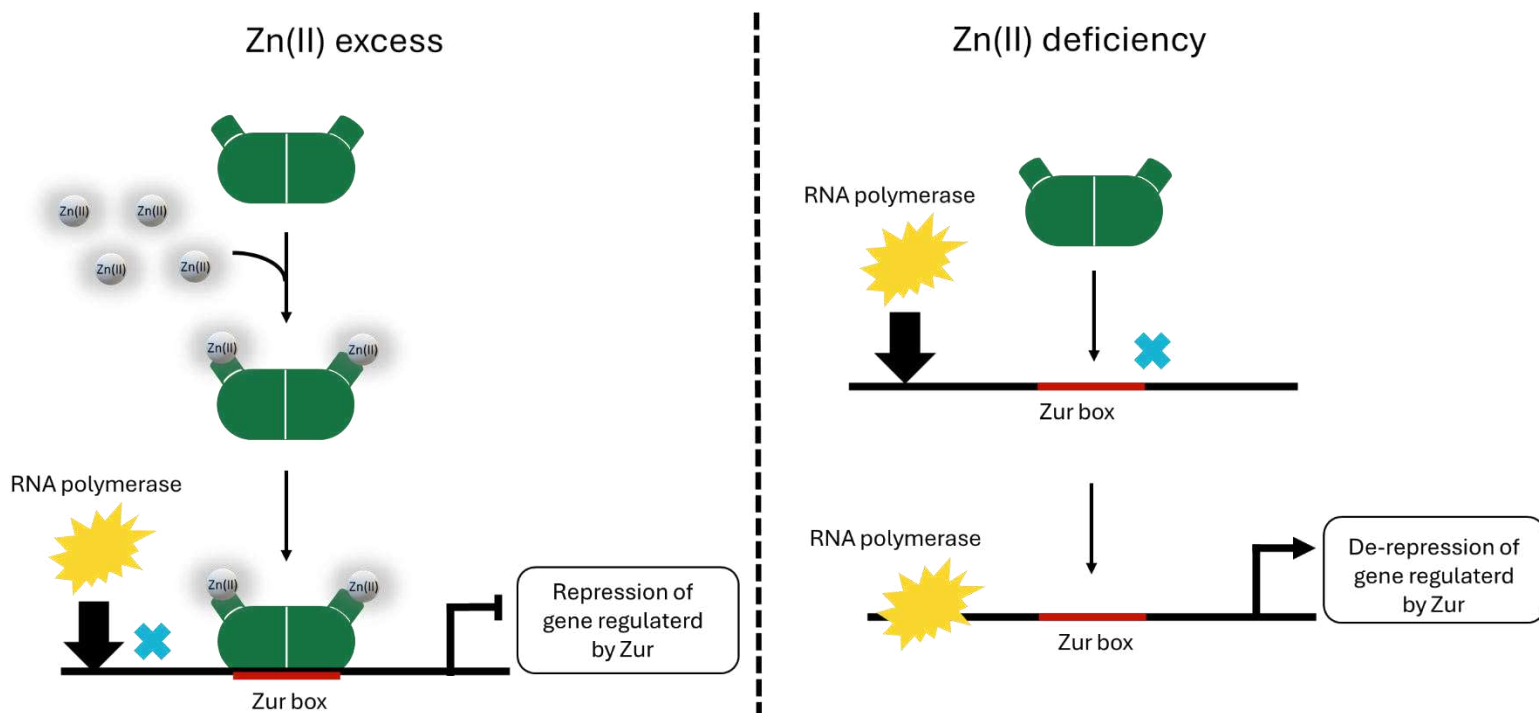


Figure 12 The schematic mechanism of Zur under Zn(II) excess and Zn(II) deficiency conditions

In most bacteria the Zn(II) limitations are controlled by the Zur, zinc uptake regulator, but in some Gram-negative bacteria zinc selective ABC transporter genes are also regulated by the Zn(II)-dependent adhesin competence repressor, AdcR. The transition metal-sensing bacterial repressors are transcriptional regulatory proteins, that allow organisms to control the intracellular availability of biologically required metal ions [309] and in streptococci this function is performed by the structurally dissimilar adhesin-competence regulator, AdcR [268], [318]. The AdcR is a member of the MarR (multiple antibiotic resistance repressor) family, which are known to expression of virulence factors, to regulate aromatic catabolism [319] and response to antibiotic and antimicrobial stress and oxidative stress [275]. Moreover, it is also responsible for the regulation of alternative ribosomal genes in some streptococci [320]. Consequently, its regulation action includes the negatively regulate the expression of genes involved in metal uptake, metal storage and metal-sparing responses [275], [321]. AdcR is a homodimeric DNA-binding protein, which possess two domains: (i) a DNA-binding domain, which has a helix-turn-helix motif and (ii) a dimerization domain [321]. The Zn(II) binding sites in the AdcR are located

in its interdomain, which changes after the metal occupancy and becomes capable of DNA binding [321]. When sufficient Zn(II) is available, the metalated form of the AdcR binds operator sequences in the AdcR-regulated promoters, what resulting in the repression of gene expression [275]. Thus, the AdcR mechanism involves both repression and activation [322].

Furthermore, some Gram-negative bacteria, like for example *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Yersinia pestis*, *Salmonella enterica* and *Proteus mirabilis* possess an additional high affinity Zn(II) uptake system, ZevAB [323]. It plays only a secondary role in Zn(II) transport, where the ZnuABC is the major system, but is particularly essential for the proper growth of bacteria in severely Zn(II)-deficient media host defense against pathogens during lung infections. This locus important for Zn(II) utilization is referred as 'zinc binding essential for virulence', *zev*, and consists of the operon composition of two genes: (i) the potential DsbA substrate *zevA* and (ii) the *zevB*, a gene encoding a putative membrane protein [323]. Though, ZevA constitutes periplasmic solute-binding protein and ZevB a membrane permease component of an ABC-type transporter, the *zevAB* locus does not encode a protein with the Walker A and Walker B motifs [282].

A Gram-positive bacterium, *Listeria monocytogenes*, has two zinc uptake systems, which are regulated by Zur: ZurAM and ZinABC. Both are related to the ABC-type Zn(II) permease system, and their presence may improve the bacterial response to Zn(II) deficiency conditions in various environments and at various stages of infection [324]. The presence of two related ABC-type Zn(II) transport systems provides the ability to react during different stages of infection and in a variety of environments and each system can make a distinct contribution under different loading conditions [325]. Moreover, mutants lacking both systems are unable to grown in the absence of Zn(II), but the lack of one of them do not influence on their survivability.

(2) ZIP transporters

In bacteria, there are two major Zn(II) uptake transporters, the ZnuABC transporter (described above) and the ZIP family (ZRT, IRT-like protein) [326]. While the ZnuABC system is used during zinc starvation, in less extreme environments where zinc is present in higher concentrations, the uptake of this metal is ensured by lower affinity transporters, just like the ZIP family [270], [327]. They are present in prokaryotes, eukaryotes but also archeocytes, where they are involved in the transport of divalent metal ions from the extracellular environment into the cytoplasm [328]. Many of eukaryotic proteins are also involved in the efflux metals from intracellular organells/vesicels into cytoplasm [329]. Biochemically, ZIP family members participate in the transport of Zn(II) [272], [330] but also Cd(II), Fe(II), Cu(II), Co(II) or Ni(II) [330], thus they play a role in uptake, homeostasis of essential trace elements but also absorption and distribution of toxic metals [331]. From the biological point of view, they exert diverse functions, which depend on their distribution in organs and tissue, intracellular localization, species but also biochemical properties [331]. Most of the ZIP family members have conserved sequence elements like simple domain architecture with a transmembrane domain preceded (or unstructured fragment) by an *N*-terminal soluble domain, followed by a short *C*-terminal sequence [331]. Most of them have eight transmembrane helices [332] (with both termini exposed to the extracellular space for plasma membrane [326]) and often their loops between transmembrane domains are quite short and a longer one is frequently found between third

and fourth domain [332]. This region often contains a potential metal-binding histidine-rich loop with the sequence (HX)_n, where X may stand for any amino acid and n is generally in the range of 3-5 [329]. The most conserved portion of the ZIP family proteins occurs in the fourth transmembrane domain, which contains a completely conserved histidine residue, and which forms an amphipathic helix. This histidine residue together with the adjacent (semi)polar residue may constitute a part of the intramembrane heavy metal binding site [333]. Topological model of ZIP proteins is presented on Figure 13. Regulation of their activity in response to Zn(II) availability occurs both at the transcriptional and posttranscriptional levels [326], [329]. One of the member of the ZIP family is a ZupT, a low-affinity Zn(II) transporter and serves as a key protein, which is involved in the pathogenicity [270].

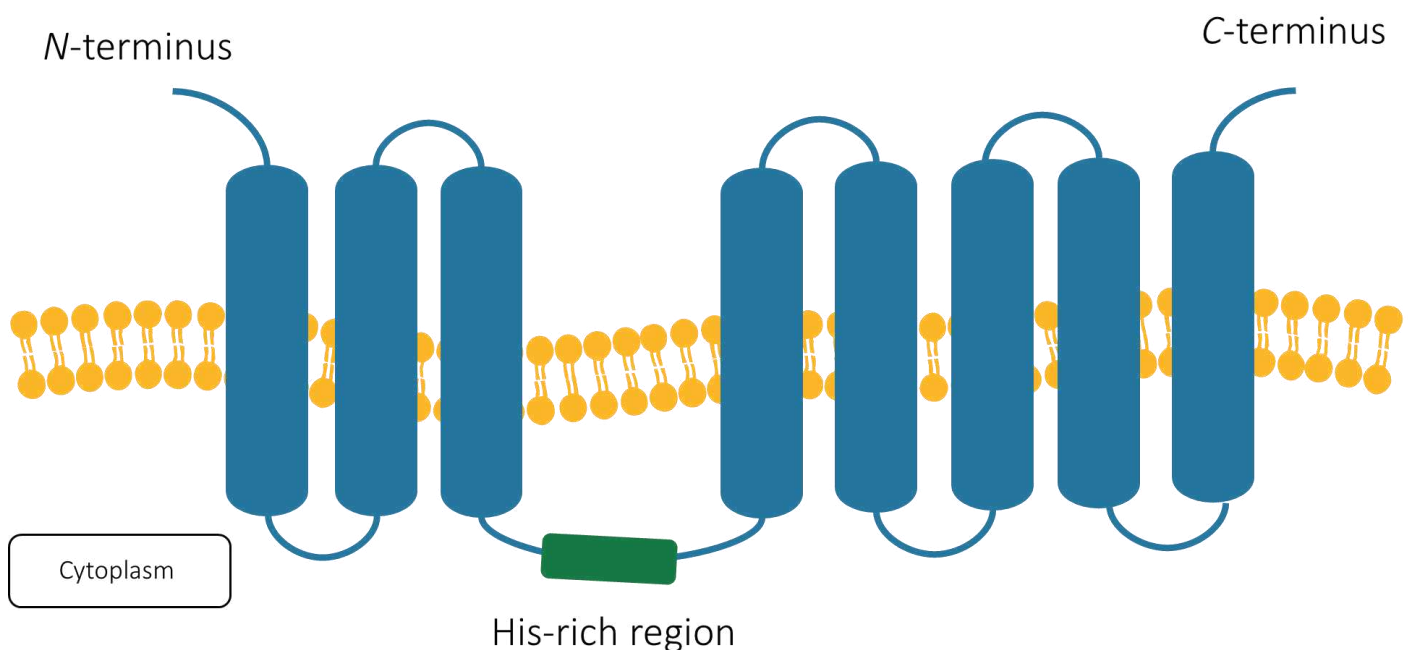


Figure 13 A schematic topological model of ZIP proteins for most bacterial ZIP proteins

b) Export systems

In view of the fact that a high concentration of Zn(II) may have a negative impact on the cell, bacteria have developed a number of transporters that enable them to export Zn(II). Among them, we can distinguish: the P-type ATPase transporters, cation diffusion facilitator transporters (CDF), resistance-nodulation division pumps (RND) and ZntB exporters, which expression is tightly controlled by appropriate transcriptional regulators [272]. Moreover, other transporters, like low-specificity iron transporters or inorganic phosphate transporters can also facilitate the translocation of Zn(II) [334].

(1) P-type ATPases

The P-type ATPases are a highly conserved, large family of integral membrane transporters, which generate and maintain crucial electro-chemical gradients across cellular membranes and utilize the energy from ATP hydrolysis to the translocation of cations, heavy metals, and lipids across cell membranes [335] or sequestering them into membrane-

compartmented organelles [336]. Therefore, that enzymes play a key role in metal homeostasis and their exports. The ATP-dependent pumps, P-type ATPases, are classified in six main classes (P1, P2, P3, P4, P5 and P6) [337] of which the P1B-type ATPases are involved in the transport of heavy metals (Cu(I), Cu(II), Zn(II), Co(II), Cd(II) and Pb(II)) across the biological membrane against a concentration gradient [338]. The typical structure of the P-type ATPases is generally identified by conserved sequence motifs in the cytoplasmic domains, which include three domains: a nucleotide-binding domain, a phosphorylation, and an actuator domain [214], [339] and one transmembrane domain [339] typically contains 10 transmembrane helices (M1-M10) [336]. The actuator domain is directly connected to M1, M2 and M3 helices [340]. To achieve active cation transport against an electrochemical gradient, the transporter must prevent the formation of an open passage across the entire membrane, which would lead to rapid reverse flow of ions and destroy any charge or concentration gradient. To overcome this problem, P-type ATPases operate according to an "alternating access" model [341]. Access pathways to both sides of the membrane are temporarily closed before opening to each side alternately, which blocks ions, and the actual translocation of ions occurs through extensive conformational changes driven by ATP hydrolysis in the ion pumping cycle [338]. The phosphorylation and dephosphorylation reactions are the two key events to drive the conformational cycle of the P-ATPase [336] and the ATPase alternates in two conformational states (enzyme 1 (E_1) and enzyme 2, (E_2) which are associated with autophosphorylation by ATP (high affinity for the cation) and with autodephosphorylation (lower affinity for these cations, they can bind counterions instead), respectively [335]). The P-type ATPases form a phosphorylated intermediate state during their ion transport cycle [342]. The catalytic phosphorylation of Asp residue in the DKTGT sequence (the ion bound E_1 state) [343], occurring alternates between phosphorylated and dephosphorylated states, creates the high-energy E_1P -ADP state, which promotes a subsequent conformational change to the functionally distinct lower-energy E_2P state (the phosphorylated form of enzyme 2) [335]. It promotes a subsequent conformational change to the functionally distinct lower-energy E_2P state and is associated with the opening of an extra cytoplasmic exit pathway from the ion-binding sites. The distortion of these sites lowers the affinity for the E_1 -bound cations and then induces re-occlusion and dephosphorylation of the transporter (E_2P to E_2) [335]. As a result, the counterions are released during dephosphorylation of E_2P is followed by the spontaneous conversion of E_2 to E_1 [337] and the P-type ATPase keeps the cycling along the path of: $E_1 \rightarrow E_1P \rightarrow E_2P \rightarrow E_2 \rightarrow E_1$ regardless of whether substrate binding has occurred [344]. The E_1 state is associated with Na(I) and the E_2 state with K(I) binding [340] and they have very different structures [345]. Due to the fact that ATP hydrolysis and ion binding and release occur at sites that are ~ 50 Å apart it is necessary to combine the release of mechanisms between the substrate-binding and phosphorylation sites [346]. Three linker regions that connect the actuator domain to the transmembrane helices M1, M2 and M3 are important for this energy coupling [347]. When phosphoryl transfer releases the tight combination between the nucleotide-binding and phosphorylation domains and when the actuator domain rotates into the resulting phosphoenzyme gap, this extensive rotation exerts attraction on the three linkers. This in turn leads to a change in the tilt but also a position of the attached transmembrane helices M1, M2 and M4 [340]. They separate from the remaining transmembrane helices (M5-M10) and open up the ion exit pathway [348] (because except for the P1 subfamily, they have an additional supporting domain of transmembrane segments M7-M10 [340]). Furthermore,

the phosphorylation domain moves up and down relative to the membrane and at the same time, these rearrangements distort the geometry of the ion binding site, leading to a drastic reduction in affinity for the bound ion and thus the release of it (the transported ions and ligands bind to the middle of the transmembrane domain [337], coordinated by negatively charged and polar residues, in the region between transmembrane α -helices M4, M5, M6 and M8) [335]. A schematic model of the action of P-type ATPases is shown on Figure 14.

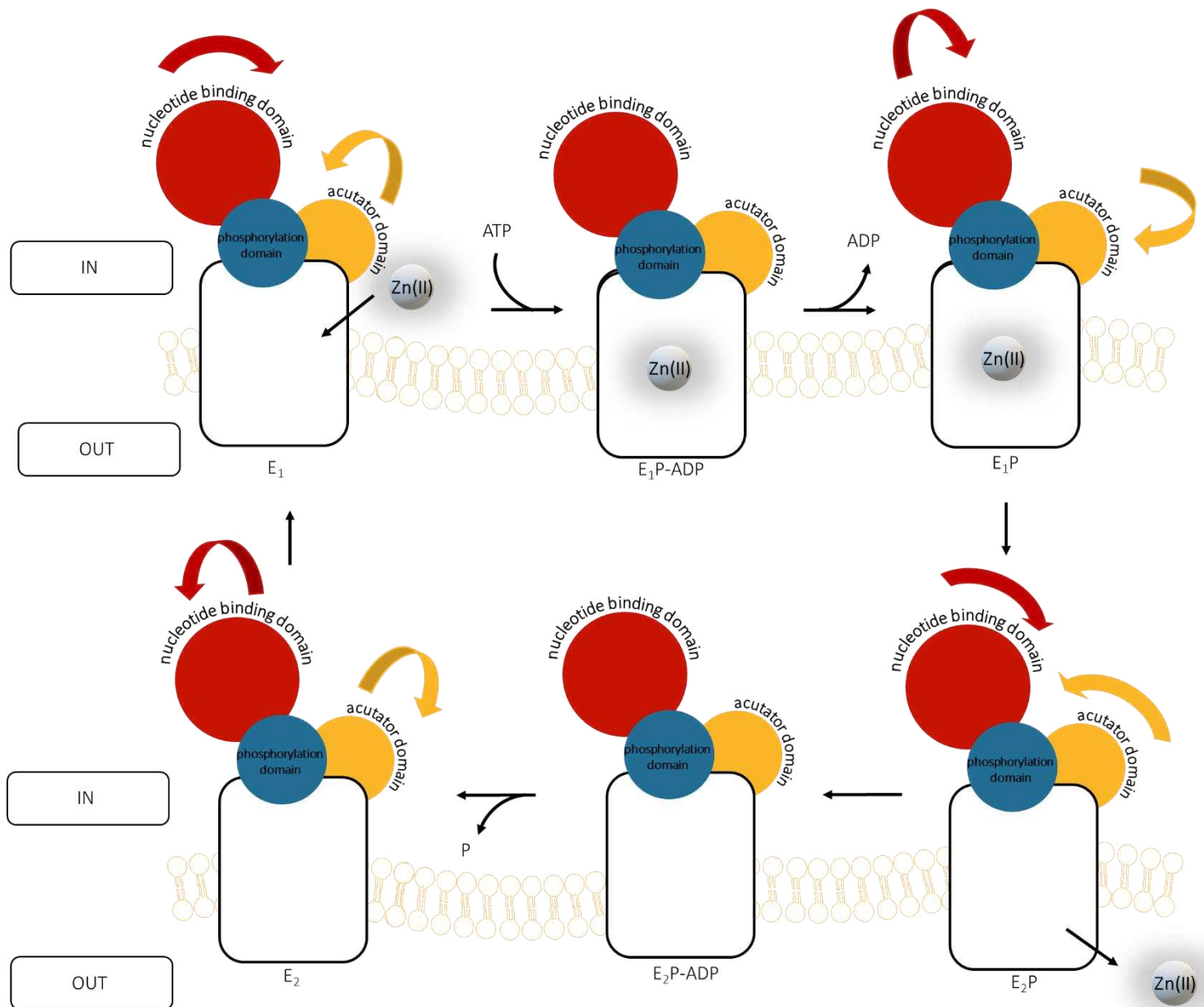


Figure 14 The Post-Albers (E_1 to E_2) reaction cycle of Zn(II)-transporting P-type ATPase. A high-affinity state (E_1), which is open to the intracellular space, where it binds Zn(II) and then enters an occluded state and phosphorylation. After that, (E_1P) triggers the release of the Zn(II) and goes into a low-affinity state (E_2P). In the next step, the inorganic phosphate (P_i) is released, which yields the fully dephosphorylated conformation (E_2). After the E_2 state, conformation is restored to the inward-facing conformation (E_1), which ends this reaction cycle and initiates a new one.

Another crucial domain for correct function of most P1-type ATPases is the *N*-terminal domain, which has a repeated 1-6 times, conserved metal-binding motif: GXXCXXC (X may stand for any amino acid) [349]. The ATP is bound in this conserved pocket and its phosphate moiety pointing towards the reactive aspartate residue in the phosphorylation domain [335]. On the other hand, the P-1B-2 ATPases (ZntA and CadA) enzymes contain the Cys-Pro-Cys motif in the center of the sixth transmembrane domain but also Lys in seventh transmembrane domain and Asp and Gly residues in the eight [350]. The activity of most P-type ATPases is tightly controlled by additional regulatory domains or protein subunits [338] and ATPs themselves maintain the asymmetric distribution of aminophospholipids in membranes by moving phosphatidylserine and/or phosphatidylethanolamine from one bilayer to the other. This asymmetry is crucial for membrane fusion events at the plasma membrane and in the trans-Golgi network [351].

(2) Cation-diffusion facilitator proteins

Cation diffusion facilitators (CDFs) are secondary active intermembrane transporters, which selectively transport divalent *d*-block metal ions (Zn(II), Fe(II), Co(II), Cd(II), Ni(II) and Mn(II) [352]) out of the cytosol and either outside the cell or to cellular compartments [353] and play a role in their homeostasis at the cellular level [354] and tolerance to trace elements [355]. There are three major groups of CDFs, according to metal specificity: Zn(II)-CDFs, Zn(II)/Fe(II)-CDFs and Mn(II)-CDFs [352] dividing them into 18 independent clades according to their metal specificity [356] (each CDF protein effectively bind and transport only specific metals [248], [356] or sole cation [248], and some can transport more than one metal [332]) in which eight are Zn(II)-transporting CDFs [248], making its transport polyphyletic [248]. Members of the CDF family exist as a homodimers [354] and every monomer consists of three domains: a transmembrane domain formed by six helices, through which cations are transported, an *N*-terminal domain and a long, regulatory cytoplasmic *C*-terminal domain that protrudes into the cytoplasm [248], [353] and usually use the proton driving force for transport (use the proton-motive force of M(II)/H(I) antiporters [354]). Thus, they use the energetic capital from the entropically favorable diffusion of one cation to facilitate the less favorable diffusion of another [354]. CDFs can exist as fully functional M(II) transporters in both forms: classical and truncated with lack a pronounce cytoplasmic domain [248]. As previously mentioned, the CDFs proteins usually have six transmembrane domains [357] and can be grouped into two independent subdomains (a bundle of four domains involving TM1, TM2, TM4 and TM5 and a bundle of two domains involving TM3 and TM6 [338]). Moreover, they have a conserved signature sequence between the first and second transmembrane domains. Moreover, their structural characteristic includes His-rich cytosolic regions at *N*- and *C*-termini and between another two transmembrane domains (IV and V) [181]. Due to the fact that CDFs can vary greatly in sequence and size, these rules are often broken [358]. Mostly CDFs proteins contain three highly conserved Zn(II)-binding sites, termed as A (located between TM2 and TM5 [359], [360]), B (located in the loop between TM2 and TM3 on the cytoplasmic membrane [359], [360]) and C (located at the CTD-CTD interface [332]). The CTDs are proposed to play a crucial role in the scissoring mode of action illustrated on Figure 15, but most likely, Zn(II) binding to the CTD occurs also through a rocking-bundle mechanisms and suggest the alternating access to the Zn(II) binding site [361]. Metal bonding rigidifies it, which might then restrict the movement of TM3 and TM6 and acts as a scaffold to aid conformational changes of other TMs (TM1, TM2,

TM4 and TM5) [332]. This periplasmic conformation facing Zn(II) facilitates its removal [248]. Moreover, the flexibility of the CTD might affect the regulation of Zn(II) transport [362]. CDF proteins may also function as importers of divalent metal ions when its concentration in the cytoplasm is too low [248].

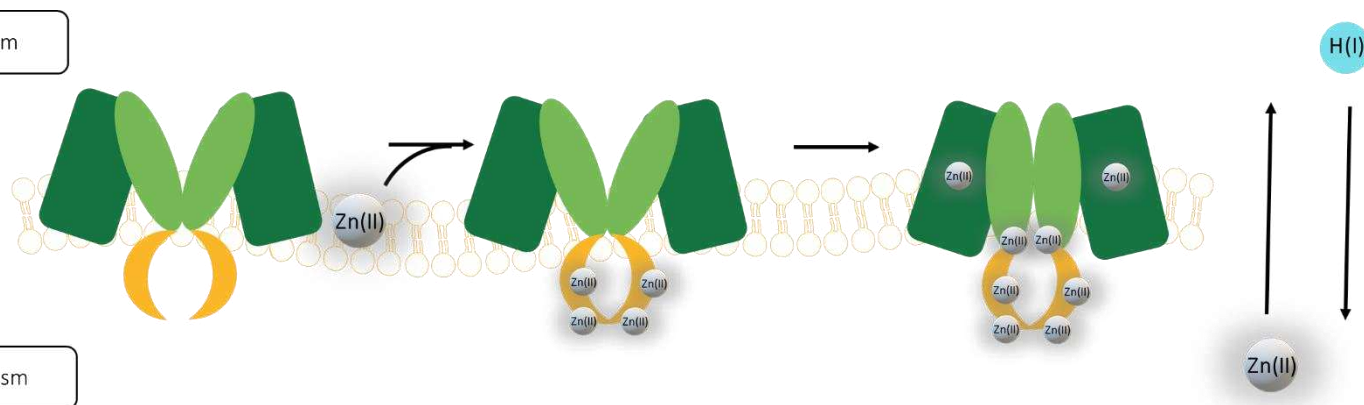


Figure 15 Proposed CDF mechanisms of action from *Escherichia coli*

(3) RND proteins, ZntB and ZraP

Efflux pumps of the RND (Resistance nodulation division) superfamily are ubiquitous in bacteria, archaea and eukaryotes [363] but are predominant in Gram-negative bacteria, where they are the major cause of multidrug resistance (MDR) [364]. Besides, they are also involved in the pathogenesis, virulence or stress response [365]. They use a proton as a motive force [365] and this superfamily includes a central RND-substrate antiporter (the inner membrane component), a periplasmic membrane fusion protein (MFP) and an outer membrane channel factor [366]. Efflux pumps are an envelope or cytoplasmic membrane proteins, which function as pumps that eliminate into the outside of cells antibiotics but also dyes, biocides, heavy metals, antimicrobials, fatty acids chemotherapeutic agents, and other chemicals [365], [367], [368] and the efflux of antibiotics is not their primary physiological function [368]. They are composed of multiple or single units [365] and work synergistically with the outer membrane barrier [365]. In Gram-negative bacteria, they usually constitute three portions' pumps, located in the inner and outer membrane and passing laterally through the membrane fusion protein. While in Gram-positive bacteria, they primarily constitute an efflux pump composed of a single polypeptide from the cytoplasmic membrane [369]. In general, they consist of outer membrane protein (OMP) [370], inner membrane protein (IMP) and membrane fusion protein (MFP) [371], that links the IMP and OMP [372]. The schematic model of the tripartite pump complex is presented on Figure 16. Thus, they function as a tripartite complex [373] and form a continuous channel across the Gram-negative cell ensuring that a substrate molecule, captured from the outer leaflet of the inner bilayer flows directly through the periplasm and outer membrane [368]. It allows Gram-negative bacteria to remove substrates into the external medium, from periplasm (or locations that are in rapid equilibrium with periplasm) [374]. Each of these components is essential for drug efflux, and the absence of even one component makes the entire complex totally nonfunctional [375]. RND transporters contain 12 α -helical segments (sometimes 13 or 14 [374]) and two periplasmic domains (large external loops) between TMS-

1 and TMS-2, but also TMS-7 and TMS-8 [376], [374]. The periplasmic portion is connected to the single tube formed by trimeric outer membrane factor, which is open to the extracellular space on the site. Its second end is connected to an opening on the top of an RND trimer [363]. The periplasmic fusion protein plays a stabilizing role between two trimers by creating a trimeric or hexameric ring around the RND trimer and outer membrane proteins [376]. Importantly, even in the absence of the one component, the membrane protein can use the proton-motive force to translocate the metal ions [377] and the RND transporters accept their substrates from the periplasm or in the case of hydrophobic substrates from the cytoplasmic membrane (not from the cytoplasm) [378]. Their expression is even regulated by an intricate balance between local regulators (repressors, which are encoded upstream of the operon) and global regulators [368]. Overexpression of RDN efflux pumps may result from interactions of selected molecules with the local repressor [379], mutations in the repressor encoding genes [380] or activation of expression by means of a global regulator [381]. The RND protein family also contains the CzcABC-like transporters, which are very efficient in the cell and protect it against toxic metal ion concentrations [367] and ZneB, a component of the ZneCAB heavy-metal efflux system, which is highly specific for Zn(II). Moreover, pathogenic γ -proteobacteria contains ZntB zinc efflux protein, which belongs to the CorA family of metal transporters [382] and in some bacteria was found the ZraP protein, which has the properties of metallochaperones [383].

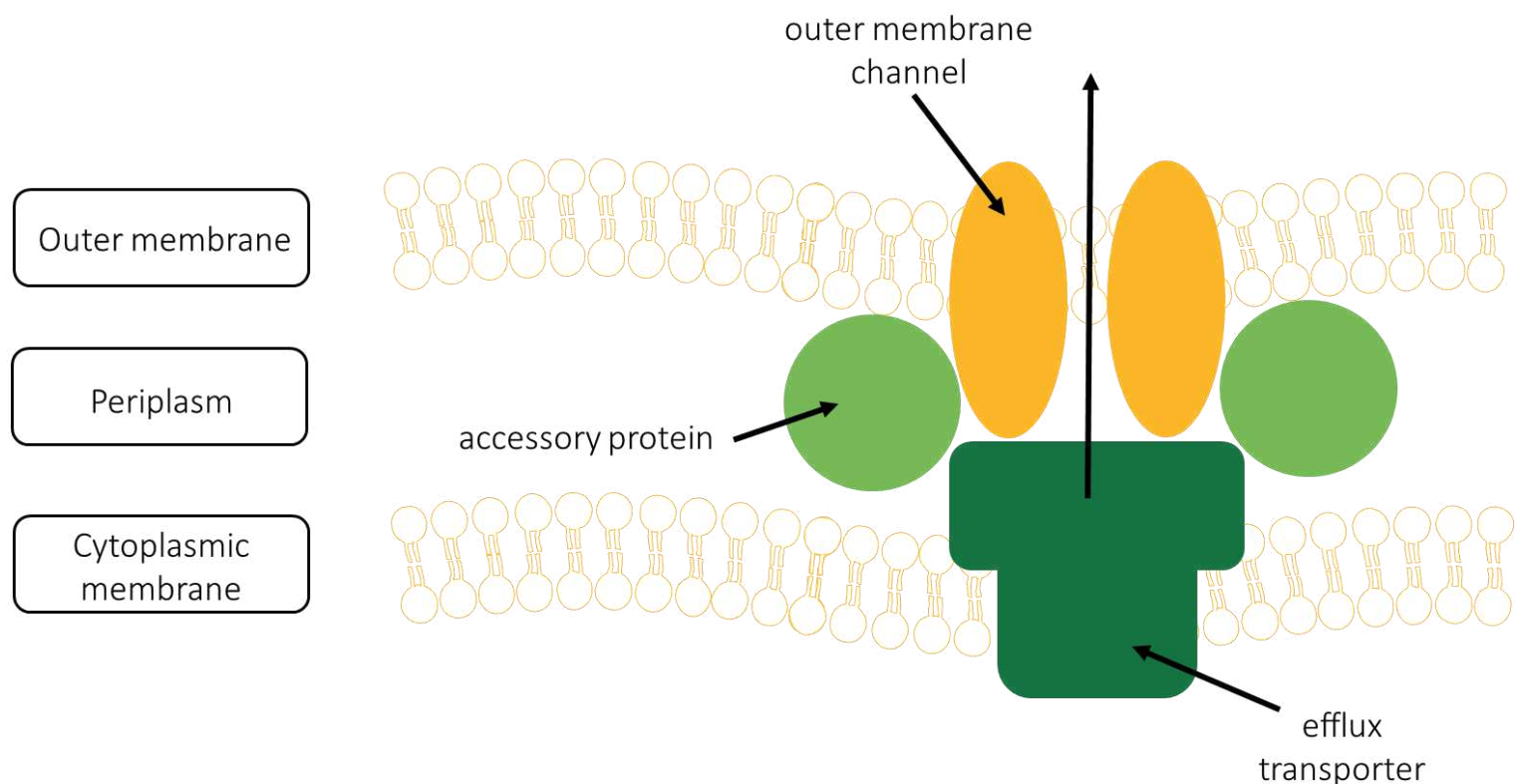
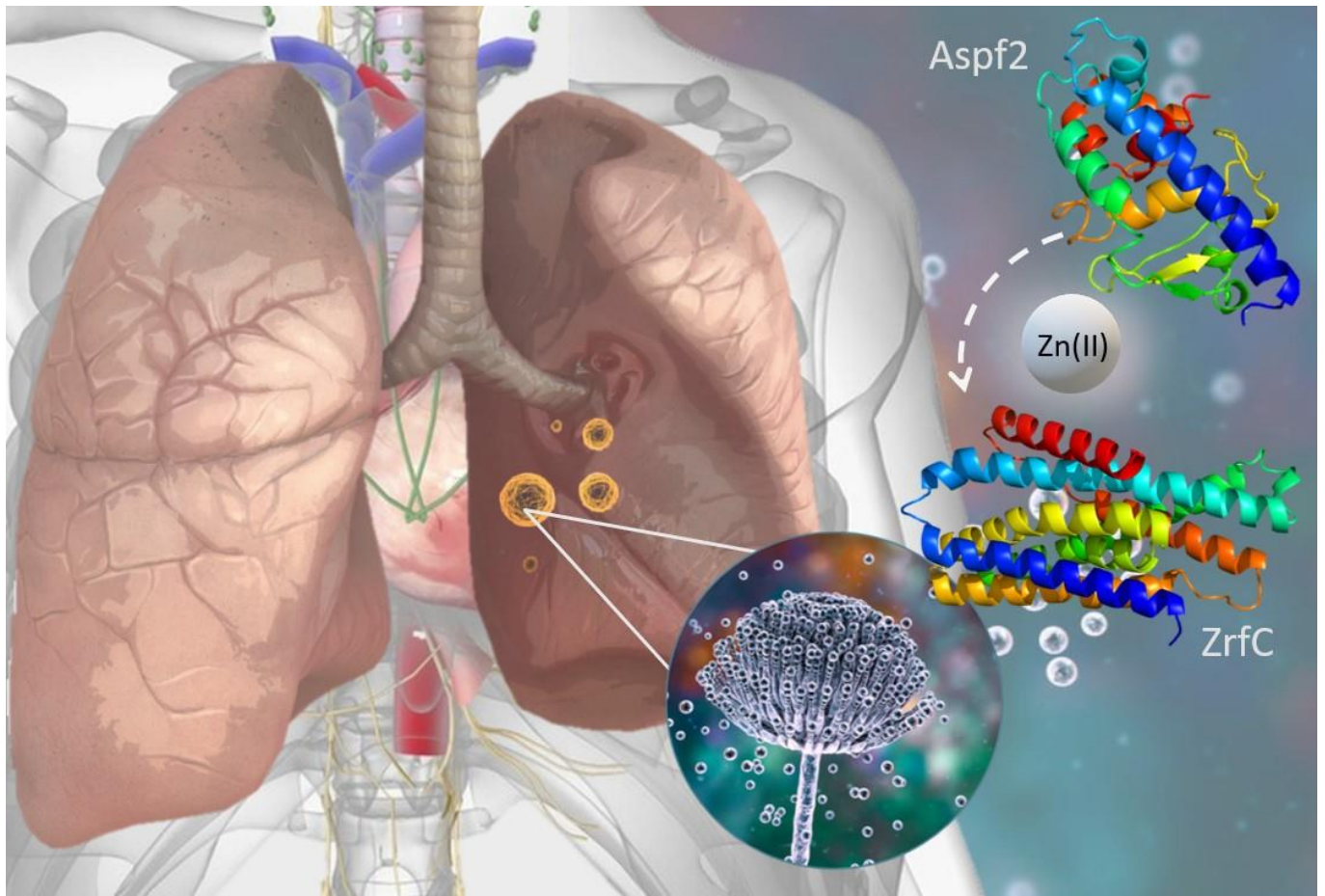


Figure 16 An early schematic view of the tripartite pump complex in Gram-negative bacteria

B. Analyzed microorganisms

1. Zn(II) transport in *Aspergillus fumigatus*



Scheme 1 Schematic model of zincophore-based transport mechanism in *Aspergillus fumigatus*

Aspergillus fumigatus is a well-adapted saprophytic and an opportunistic fungal pathogen, that produces many small airborne spores, proliferates in the lungs of immunocompromised patients and causes among others invasive pulmonary aspergillosis [212], [384], [385], [386], [387], [388], whose mortality rate may reach up to 90% [389]. Aspergillosis is one of the most common invasive fungal diseases of humans [212], but *Aspergillus* species are found in most domestic animals and birds as well as in wild animals [390]. Every day, people are in continuous contact with its spores, inhale several hundred of them during each day [386]. Despite this constant contact, most humans do not get sick as a result of their presence and only the immunocompromised people are at the risk. Aspergillosis can be considered as a spectrum of diseases, each associated with a spectrum of abnormal host immune responses [391]. These may include allergic asthma, hypersensitivity pneumonitis or allergic bronchopulmonary aspergillosis [392], [393]. In animals causes mainly respiratory infections [390], but in humans' treatment of aspergillosis is much more complicated among other to the azole resistance [394] that has been found in many countries in recent years [395], [396], [397].

Currently, there are three main antifungal drugs: polyenes, azoles and echinocandins. Azoles are the first-line antifungal drugs in clinical treatment [398]. The conducted research shows that approximately 30% of isolates show resistance, and its mechanism is not identifiable [399], although efflux pumps are known to play a role in its resistance [400]. Moreover, the toxicity and side effects of antifungal drugs make the development of new selective methods imperative to fight fungal pathogens. Zinc binding proteins and transcription factors constitute approximately 9% in *Aspergillus fumigatus* [215]. The concentration of free Zn(II) in plasma is about 10^{-4} μ M [401] and depends on the pH value of the environment and decreases with increasing pH due to its tend to form insoluble metallic complexes, like for example: metal oxides or phosphates [402], [403], [404], [405]. At physiological pH (pH 7.4) the concentration of the free Zn(II) in the host cells is very low and the most Zn(II) is bound to human's intracellular and extracellular zinc-binding proteins such as albumin and α_2 -macroglobulin [183], [406], [407], [408], making its concentration 150 time less than the minimum concentration proper for normal fungal growth [207]. For example, the average concentration of total Zn(II) in human lungs, which constitute an environment for the development of *Aspergillus fumigatus*, is around 12 μ g/g of lung tissue [209]. In addition, an increase in pH enhances the toxicity of Zn(II) to microorganisms [409]. Therefore, genes involved in the regulation of Zn(II) homeostasis are important for fungal virulence [226] and fungal must use of several different mechanisms to scavenger Zn(II) from the environment of the host [21] and to keep up the right Zn(II) levels [208].

The first characterized zinc transporters present in human fungal pathogens come from the *Aspergillus fumigatus* [410], which contains eight ZIP-type proteins (Zrt-like, Irt-like Protein): ZrfA, ZrfB, ZrfC, ZrfD, ZrfE, ZrfF, ZrfG and ZrfH [228], [234]. *Aspergillus fumigatus*, which is able to survive in a wide pH range and under different Zn(II)-limiting conditions, has developed different pathways to acquire Zn(II) [226]. Among the eight transporters encoded by *Aspergillus fumigatus*, three (zrfA, zrfB, zrfC) are involved in zinc uptake under acid or alkaline conditions; four (zrfA, zrfB, zrfC, zrfF) are ZafA targets and five (zrfD, zrfE, zrfF, zrfG, zrfH) are predicted to play a role in Zn(II) homeostasis [208]. At neutral and alkaline pH the zrfC gene is activates by the C₂H₂ zinc finger ZafA protein and a transcription factor responsive to the surrounding pH, PacC [411], [256]. *Aspergillus fumigatus* uses ZrfC to acquire Zn(II) in alkaline Zn(II)-limiting conditions [412], [208]. It is a 522 amino-acid protein with a predicted molecular mass of 54.3 kDa [411], which is necessary for Zn(II) uptake in lung tissue [207], [411] and presents four putative Zn(II)-binding motifs on its N-terminus [207], [413].

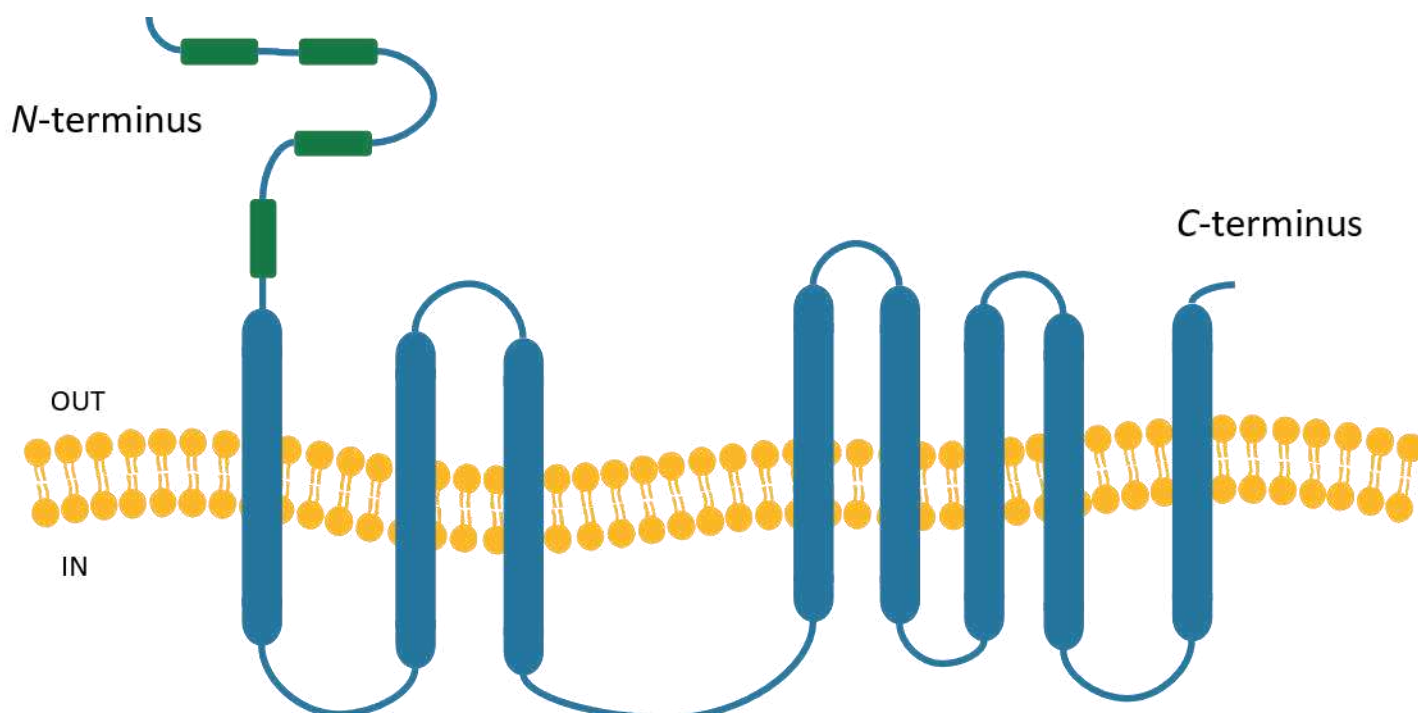


Figure 17 Topological model of ZrfC transporter insertion in the plasma membrane

The ZrfC protein contains nine supposed hydrophobic transmembrane domains (Figure 17) and most likely its *N*- and *C*-terminus are located on the outer surface of the membrane [411]. So, the remaining transmembrane domains cross the membrane eight times [414] and the *N*-terminus part of the ZrfC protein consists of approximately 200 residues toward the extracellular side and four motifs that are defined as the CHXHX₅CX₆E/D [411]. These repeats were labeled ZBM1 to ZBM4 (for Zn(II)-binding motifs) [411], because they are similar to zinc-binding motifs found in other proteins [415] and are highlighted in the box on Figure 2. It is also worth focusing on the CHFHAGVEHC fragment of ZBM4, which has remained unchanged throughout evolution in all ZrfC-like proteins that are collected in the GenBank database [411] and does not present in LZT subfamily in their transmembrane V domain [416]. For this reason, it is postulated to create a new subfamily of ZIP transporters that have the CHFHAGVEHC motif located at the *N*-terminus, close to transmembrane I domain [411]. Moreover, no orthologues of the ZrfC protein have been found neither in humans nor in nonpathogenic yeasts such as: *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* or *Kluyveromyces lactis* [417]. Most probably, the binding of Zn(II) to the ZBMs induces the proper folding of the *N*-terminus necessary for the proper interaction with the membrane-embedded part of ZrfC protein that is responsible for Zn(II) transport across the plasma membrane or to promote the formation of Zn(II) bridges between ZrfC molecules that lead to the formation of homo-oligomers, thus simulating its endocytosis [411]. Furthermore the endocytosis of ZrfC might be simulated by the Zn(II)-mediated interaction of the ZBM with the Aspf2 zincophore, which possess a CHTHXGX₂HC motif at its *C*-terminus [411].

It is assumed that the Aspf2 protein is important during Zn(II) starvation [418] and is located in the fungal periplasm, where could bind Zn(II), and forming a Zn(II)-Aspf2 complex, which could interact with the ZrfC. So this 'zincophore' system, named in analogy to the Fe(II) scavengers siderophores, includes ZrfC, a plasma membrane ZIP transporter that receives Zn(II)

and the Aspf2 zincophore, which *Aspergillus fumigatus* synthesizes a large amounts under Zn(II)-limited conditions when invading host cells [244], [419], [420], [230]. These two genes, aspf2 and zrfC are similarly regulated by pH and Zn(II) via PacC and ZafC, respectively [411] and the proposed scheme of Zn(II) transport in *Aspergillus fumigatus* is presented in Figure 18. Asp2 binds the host's Zn(II), both free and bound to other biomolecules [421].

The activity of Aspf2 extends beyond Zn(II) binding and it binds also factor H, factor H like protein or plasminogen [422], giving the multi-level immune interactions [421]. The ZrfC has a dual role in fungal virulence. It is needed to mediate Zn(II) uptake, but also to counteract the inhibitory effect of calprotectin in abscesses [413]. Moreover, most probably the arrangement of histidine residues, whose protonation state and chemical properties are pH dependent, is optimized for its function both at acidic and alkaline pH [410]. In this context, the ZrfC protein could be essential for obtaining Zn(II) from host environment and the targeting Zn(II) homeostasis of *Aspergillus fumigatus* holds promising expectations to find novel therapeutic strategies.

The activity of Aspf2 extends beyond Zn(II) binding and it binds also factor H, factor H like protein or plasminogen [422], giving the multi-level immune interactions [21]. The ZrfC has a dual role in fungal virulence. It is needed to mediate Zn(II) uptake, but also to counteract the inhibitory effect of calprotectin in abscesses [413]. Moreover, most probably the arrangement of histidine residues, whose protonation state and chemical properties are pH dependent, is optimized for its function both at acidic and alkaline pH [410]. In this context, the ZrfC protein could be essential for obtaining Zn(II) from host environment and the targeting Zn(II) homeostasis of *Aspergillus fumigatus* holds promising expectations to find novel therapeutic strategies.

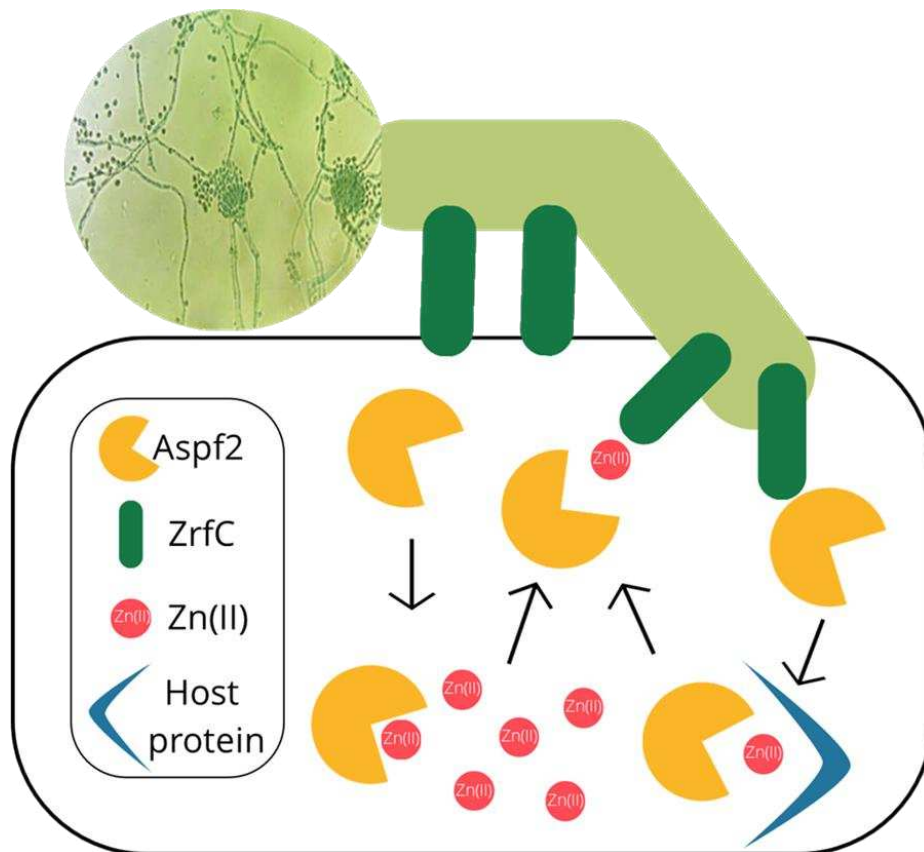
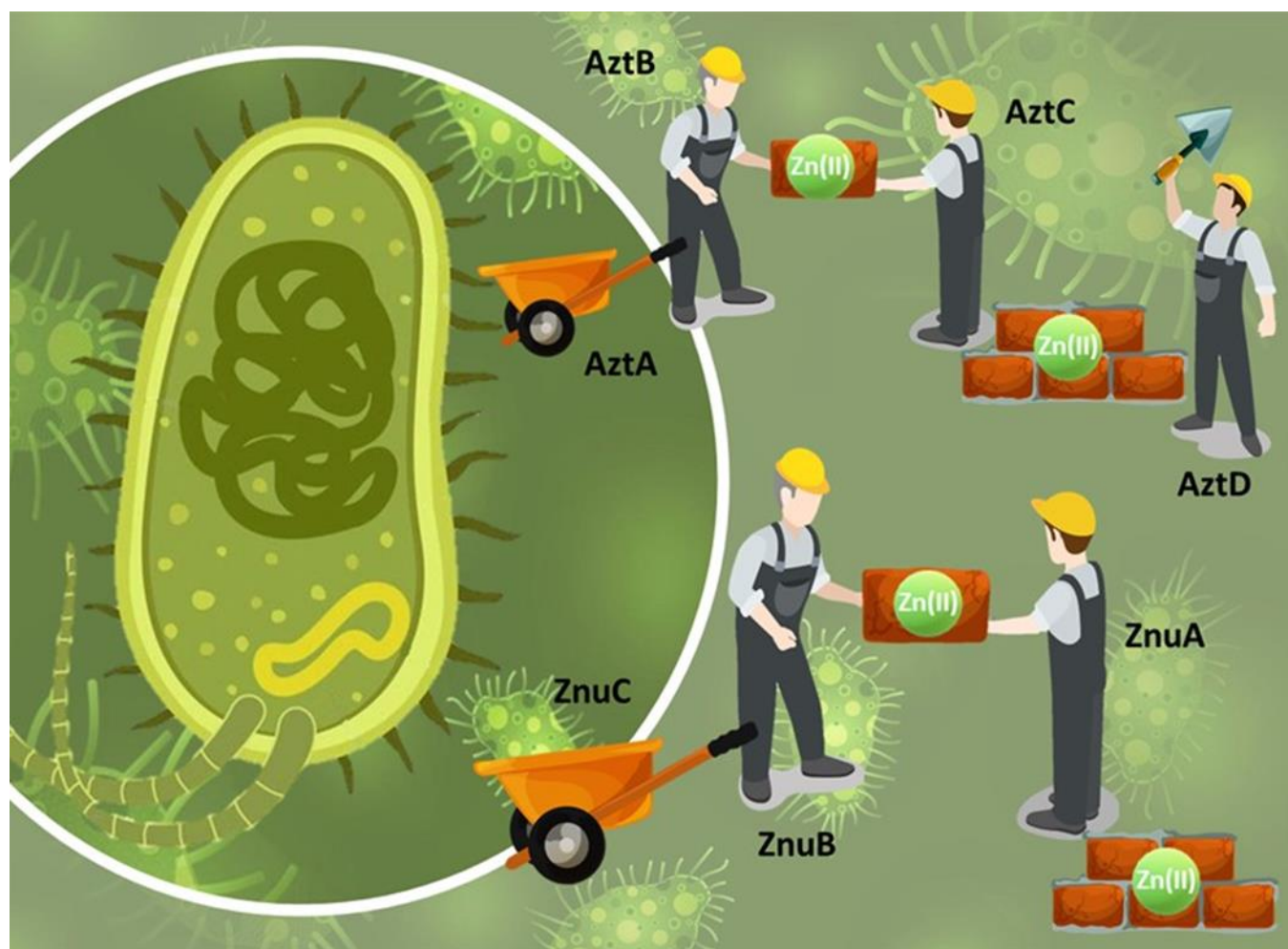


Figure 18 Schematic model of *Aspergillus fumigatus* Zn(II) scavenging from host cells. After invasion of the host cell, Asp2 is expressed and secreted. It binds Zn(II), either in the form of free Zn(II) or from Zn(II)-binding proteins of the host. Reassociation with the *Aspergillus fumigatus* cell surface and Zn(II) transportation into the cell occurs via a Asp2-ZrfC interaction

The more that the transport mechanism based on zincophores is similar to bacterial ABC transport systems and is well conserved also in other pathogens, such as *Candida albicans*, but is also present in Ascomycete and Basidiomycete species [410]. The Asp2 zincophore from *Aspergillus fumigatus* has similar properties to Pra1 from *Candida albicans* and shares 43% identity between the Asp2 and Pra1 amino acid sequences. Moreover, ZrfC transporter from *Aspergillus fumigatus* and Zrt1 from *Candida albicans* share 48% identity [423], [424]. *Candida albicans* is one of the few fungal species that constitutes the normal human microbiome and is a typical commensal of the oral cavity, gastrointestinal track, and urogenital tract [235], but it is also causing disseminated candidiasis, which is the third most common cause of nosocomial bloodstream infections [425]. *Candida albicans* secretes a zincophore (a scavenger protein), Pra1 [235], which sequesters Zn(II) from endothelial host cells (in the absence of free Zn(II) at alkaline pH) and returns to the fungal pathogen to deliver its Zn(II) via a physical interaction with the Zn(II) transporter, Zrt1 [235]. Pra1 was predicted to host two Zn(II)-binding coordination sites with additional multiple Zn(II)-binding histidine residues in the C-terminal part and released 4.6 moles Zn(II) per one mole protein, which means, that Pra1 can bind approximately three Zn(II) atoms [235]. The conducted research confirmed that the C-terminus part of the Pra1 zincophore binds Zn(II) with the highest affinity via a set of four histidine imidazoles: His288, His290, His292, and His298 [426]. *Candida albicans* encodes only two plasma membrane Zn(II) importers: Zrt1, which is specifically expressed at neutral and alkaline pH and supports its growth at those conditions, and Zrt2, whose expression is pH-independent and is

essential for its growth at acidic pH [427]. Thus, the major Zn(II) importer is Zrt2, whose is important for systemic candidiasis at later stages, while Zrt1 can support Zn(II) uptake specifically at neutral and alkaline pH [427]. The most probable Zn(II) binding site on the Zrt1 transporter is the region consisting of residues 151 to 170 [428]. Moreover, the Zn(II)-Zrt1 binding is more stable than Zn(II)-Pra1, which allows the Zn(II) transfer from Pra1 zincophore to Zrt1 transporter [428].

2. Zn(II) transport in *Paracoccus denitrificans*



Scheme 2 Schematic model of Zn(II) transport mechanism in *Paracoccus denitrificans*

One of the most studied *Paracoccus* species is *Paracoccus denitrificans*, which is an aerobe and mesophilic Gram-negative bacterium. It is a non-motile, denitrifying bacterium [429] known for reduction of nitrate and general metabolic versatility [430]. In contrast to most bacteria, it forms a unique and peculiar thin biofilm that consists of almost a monolayer of cells [431]. The interest in *Paracoccus denitrificans* is not only related to its electron transport chain but also to its metabolic flexibility. As mentioned, it is a model denitrifying organism that is important for soil fertility, greenhouse gas emissions, and waste and water treatment processes [432]. Despite being rather ubiquitous, *Paracoccus denitrificans* is found more often in environments that are subject to fluctuating conditions, like soil, sewage, or sludge [433]. In order to survive in such dynamic habitats, *Paracoccus denitrificans* encodes two Zn(II) ABC transporter systems.

The ATP binding cassette (ABC) transporter family is the most important zinc import system in many bacteria [277]. In gram-negative bacteria, however, a low-affinity Zn uptake

system – ZupT [270], which belongs to the ZIP protein family [434], also exists. Bacterial zinc transport requires zincophore-mediated zinc uptake [435], [436], P-type ATPase [437] and ZevAB [323]. The ATP binding cassette is minimally composed of a membrane-bound permease and cytoplasmic ATPase and contains three components: (i) the ZnuA periplasmic Zn(II) binding protein, that captures Zn(II) and delivers it to the ZnuB component; (ii) the ZnuB membrane protein, which transports Zn(II) across the cytoplasmic membrane; (iii) the ZnuC ATPase, that provides energy for metal ion transport across the membrane [303]. Some bacterial metal importers require the presence of solute binding proteins, called SBPs, which may be both periplasmic, membrane tethered or fused to the permease [438]. The function of the SPB protein is to bind the metal ions (with appropriate affinity and high specificity) and to deliver them to the permease, where they are imported into the cytoplasm [439] after ATP hydrolysis by the ATPase [440]. The substrate binding site of SBP is located between two structurally related α/β domains [439]. Their classification can be made based on their structure and according to the nature of the interdomain linker between α/β domains we can identify 7 clusters (A-F) [441]. The cluster A SBPs contain a long α -helix as the interdomain linker, and within this group we distinguish: (i) cluster A-I proteins, directly binding Zn(II), Mg(II) or Fe(II) [439], and (ii) cluster A-II proteins, which bind transition metal complexes [435]. Further analysis allowed cluster A-I proteins to be divided into three main groups [436], [442]. Group I is found in gram-positive bacteria and binds zinc with a 3His and 1Glu or 3His and 1H₂O coordination mode [436]. Group II proteins are found in gram-negative bacteria; they bind the metal with the same coordination environment of group I proteins. Both groups contain a flexible loop near the high-affinity binding site, but it differs in length and composition. Group III of cluster A-I proteins have no flexible loop; it is specific for manganese and iron ions and coordinates metal ions through a 2His, 1Asp and 1Glu or 3His and 1Asp [436], [439]. However, Zn(II) import is more complex and some pathogens such as *Listeria monocytogenes* [325], *Vibrio cholerae* [443] or *Streptococcus species* [444] express more than one zinc ABC transporter. Another example is *Paracoccus denitrificans*, which is a model organism for studies on zinc import with the use of ABC transporters because it encodes two zinc ABC transporter systems: (i) AztABCD and (ii) ZnuABC [439], which are transcriptionally regulated by proteins such as Zur (zinc uptake regulator) [430]. Homologues of these systems are also found in human pathogens like *Citrobacter koseri* or in some *Klebsiella pneumoniae* strains but were only characterized in the plant pathogen *Agrobacterium tumefaciens* [445]. Additionally, the AztC SBP and ZnuA SBP from *Paracoccus denitrificans* differ in both the length and the composition of a His-rich, flexible loop, which is located close to the zinc binding site [435]. The AztC loop contains 17 residues of which 3 are histidine residues and one is an aspartic acid residue (Figure 19).

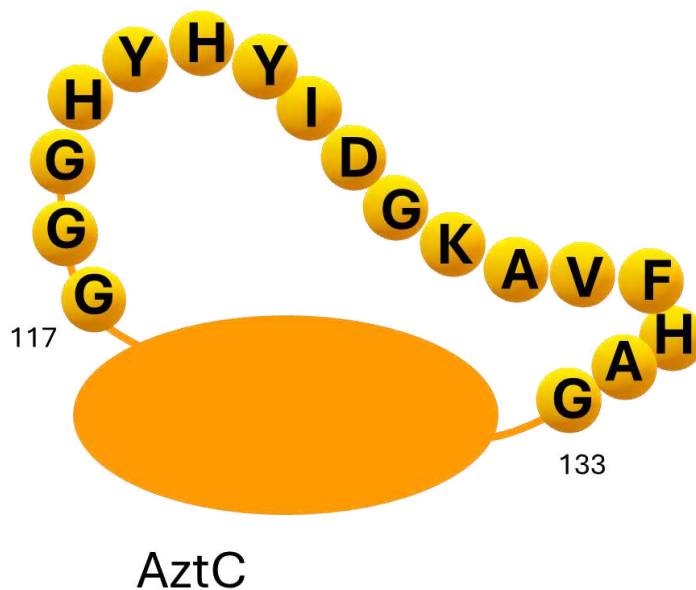


Figure 19 Schematic model of the AztC loop from *Paracoccus denitrificans*

The ZnuA loop contains 38 residues, 15 of which are His, 10 are Glu and 4 are Asp (Figure 20).

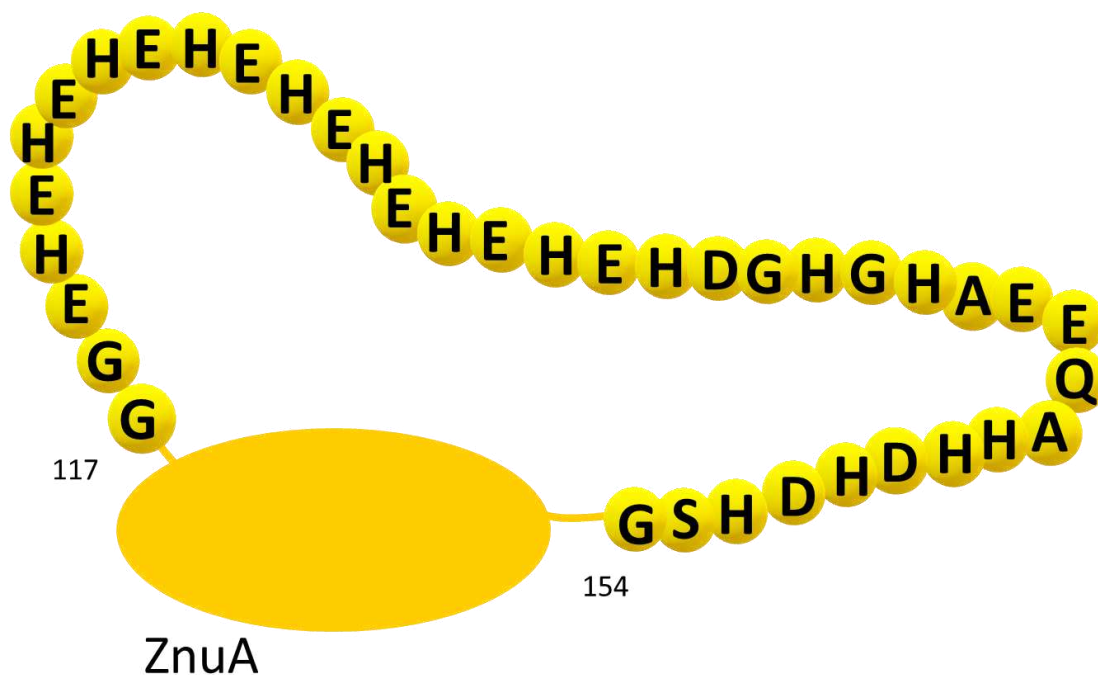


Figure 20 Schematic model of the ZnuA loop from *Paracoccus denitrificans*

The poly-His sequence located in the loop is characteristic for cluster A-I SBPs [446]. The *aztABCD* operon also encodes the AztD metallochaperone, which was identified as a periplasmic or cell surface zinc-binding protein [447] and can stoichiometrically transfer Zn(II) directly to the AztC [448], the solute binding protein for a zinc-specific ABC transporter system [447]. Neupane et al. identified a link between zinc and nitrogen metabolism, where zinc depletion leads to upregulation of genes involved in denitrification [430]. Zn(II) t occurs through an associative mechanism and requires the flexible loop with three conserved histidine residues

of AztC [449]. After Zn(II) is transferred, the labile AztC and AztD complex dissociates [447]. AztD SBP is not required for efficient Zn(II) import and pathogen growth, but it appears to be important for accumulation of Zn(II) ion from Zn(II)-limited environments [435]. The periplasmic Zn(II) binding proteins: ZnuA, AztC and AztD are highly conserved in carbapenem-resistant *Enterobacteriaceae* pathogens (CRE) [435]. As noted above, the model of a soluble metallochaperone is described quite well for several different organisms, like *Escherichia coli* [450] or *Salmonella enterica*, where ZinT protein performs a function analogous to AztD and transfers Zn(II) to ZnuA component of the ABC zinc transporter [438], [451]. Systems with similar functions are also present in fungi, for example Pra1 zincophore, which delivers Zn(II) to the Zrt1 transporter in *Candida albicans* or their orthologues Aspf2 zincophore, which delivers Zn(II) to ZrfC transporter in *Aspergillus fumigatus* [428]. The crystal structure of ZnuA from *Escherichia coli* contains two metal binding sites [452], the primary of which, His143, is situated close to the His-rich loop, which is located within residues 116-138 in the N-terminal domain; the second metal binding site is located about 12 Å from the main metal binding site [450]. The His-rich loop, together with His143, is proposed to play a significant role in Zn(II) acquisition [450], [453]. Additionally, the loop binds Zn(II) with the lower affinity than the primary site, His143, but it helps in Zn(II) acquisition and it may transfer Zn(II) to the primary metal binding site, or facilitate the interaction of ZnuA with ZnuB [454]. Studies on Zn(II) and Cu(II) complexes with ZnuA extracellular loop regions (Ac-GHFTVNPEIQPGAQRLHE-NH₂ and Ac-MKSIHGDDDDHDHAEKSDEDHHHGDFNMHLW-NH₂) provided the following conclusions: (i) the primary metal binding site, located close to the histidine rich loop (residues 116–138) and containing His143, is much more effective in zinc and copper binding at physiological pH than the secondary metal binding site (involving His224), (ii) both ligands have a nontypical α-helical structure, (iii) the primary metal binding site consists of four histidine residues, and the secondary one – of two histidine residues (iv) in both cases, loop structures are formed, (v) the more histidine residues in the studied region, the higher the pH at which amide nitrogens will participate in copper binding [450]. A simplified model for Zn(II) acquisition and homeostasis through ABC transporters in *Paracoccus denitrificans* is presented in Figure 21.

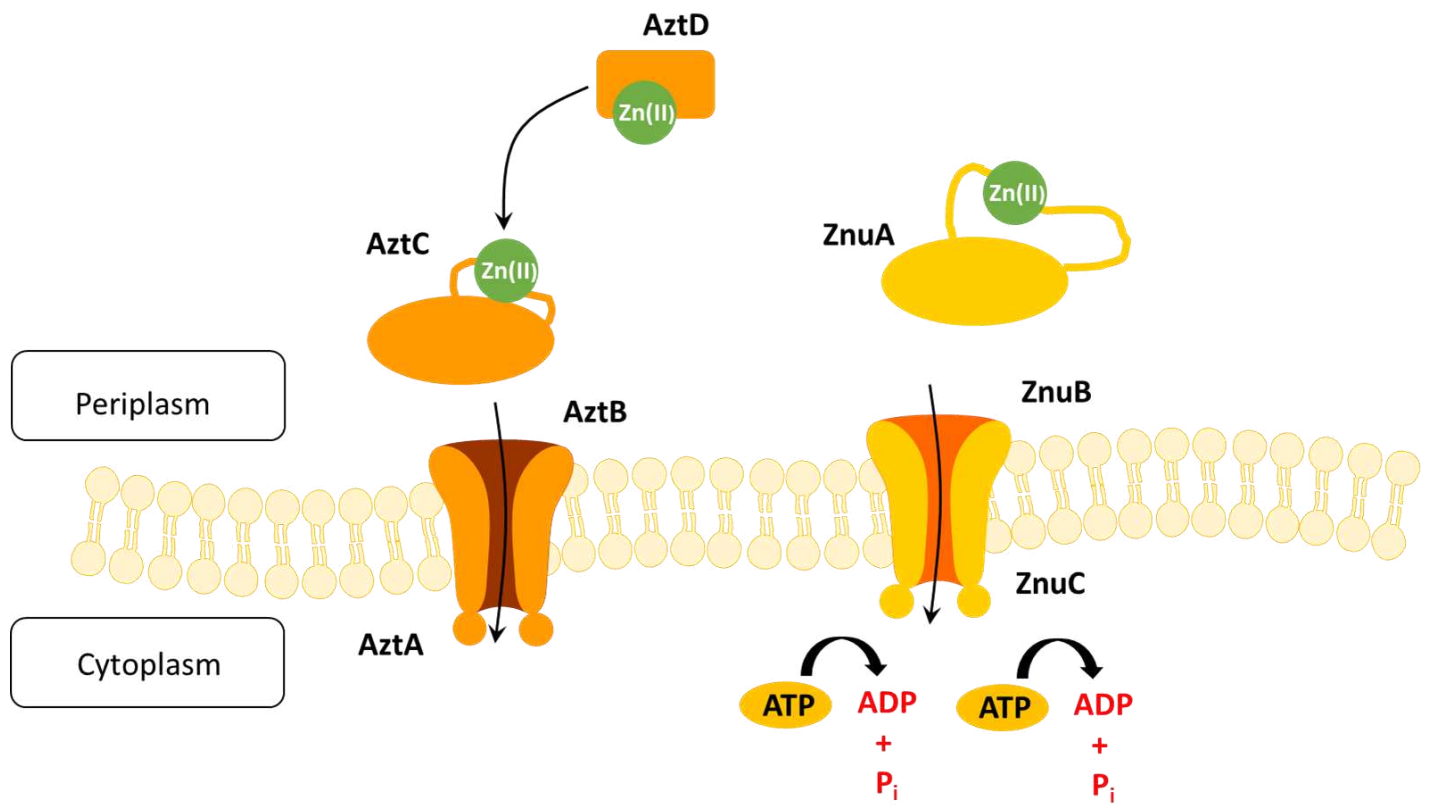
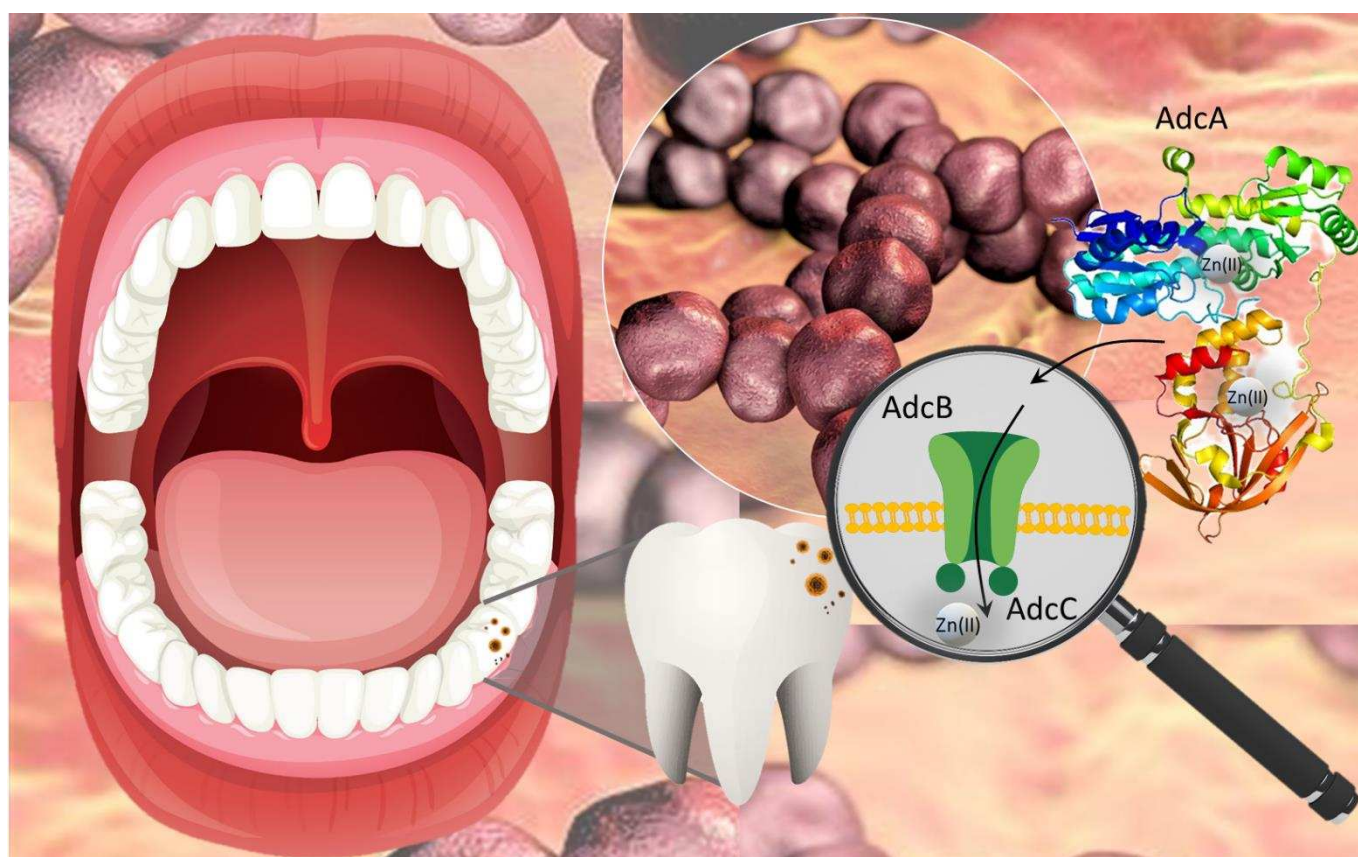


Figure 21 A simplified model for zinc acquisition through ABC transporters in *Paracoccus denitrificans*

3. Zn(II) transport in *Streptococcus mutans*



Scheme 3 Schematic model of Zn(II) transport mechanism in *Streptococcus mutans*

Streptococcus mutans is a Gram-positive pathogen, belonging to the family *Streptococcaceae* [455]. It is a key pathogen of dental caries [456] due to its ability to modify the architecture of the oral biofilm formed on hard surfaces of the tooth and environment [457], [458]. *S. mutans* does not act alone to cause dental caries but interacts with other organisms to assemble a cariogenic biofilm in the presence of a sugar-rich diet [457]. That resident of dental plaque is the producer of insoluble glucans [457], which changes the microenvironment for other acidogenic and aciduric bacteria to establish themselves [458], possibly at their own [457] and health-associated commensals expense [457]. Moreover, *S. mutans* is a causative agent of infective endocarditis, responsible for life-threatening infection of heart valves [459] and is strongly associated with the sclerosis multiplex (MS), especially in early childhood caries [460]. During its colonization, *S. mutans* have to respond and adapt to the changes in the environment, like for example oxidative and acid stress tolerance, carbohydrate utilization or intracellular metal ion homeostasis maintenance [461]. Human saliva is a unique body fluid that is critical for oral health maintenance and is produced by the salivary glands [462], [463], which owes its properties to organic and inorganic components, such as for example the divalent zinc ion [464].

Under physiological conditions, Zn(II) is an essential element for both eukaryotic and prokaryotic cells, where it can function as a structural or catalytic component of many proteins [465]. It is estimated that Zn(II) interacts with up to 10% of all human proteins [466], while in bacteria it is found in about 5% of them [154]. However, like in the case of other transition metal ion, the excess of Zn(II) might cause growth arrest and defects in virulence of microorganisms [461]. For this reason, the intercellular concentration of Zn(II) should be strictly regulated by bacteria for survival and virulence in the human oral cavity [467], [468], [469], [470]. Zn(II) is critical to the infectious process and the physiological salivary level of Zn(II) fluctuates from 0.01 to 0.2 ppm [461]. Below pH 6.0, most free zinc present in saliva is in the Zn(II) form, but the increasing pH drastically reduces its availability [471]. Moreover, the salivary Zn(II) level fluctuates throughout the day – between meals the zinc concentration is limited, whereas during mealtime its concentration increases [461]. Zn(II) is also present in the tooth enamel surface and dental plaque, where its concentration is much higher, reaching millimolar levels [472]. There are many different types of metal transport systems, and several of them are involved in Zn(II) homeostasis in streptococci [320] (Figure 22).

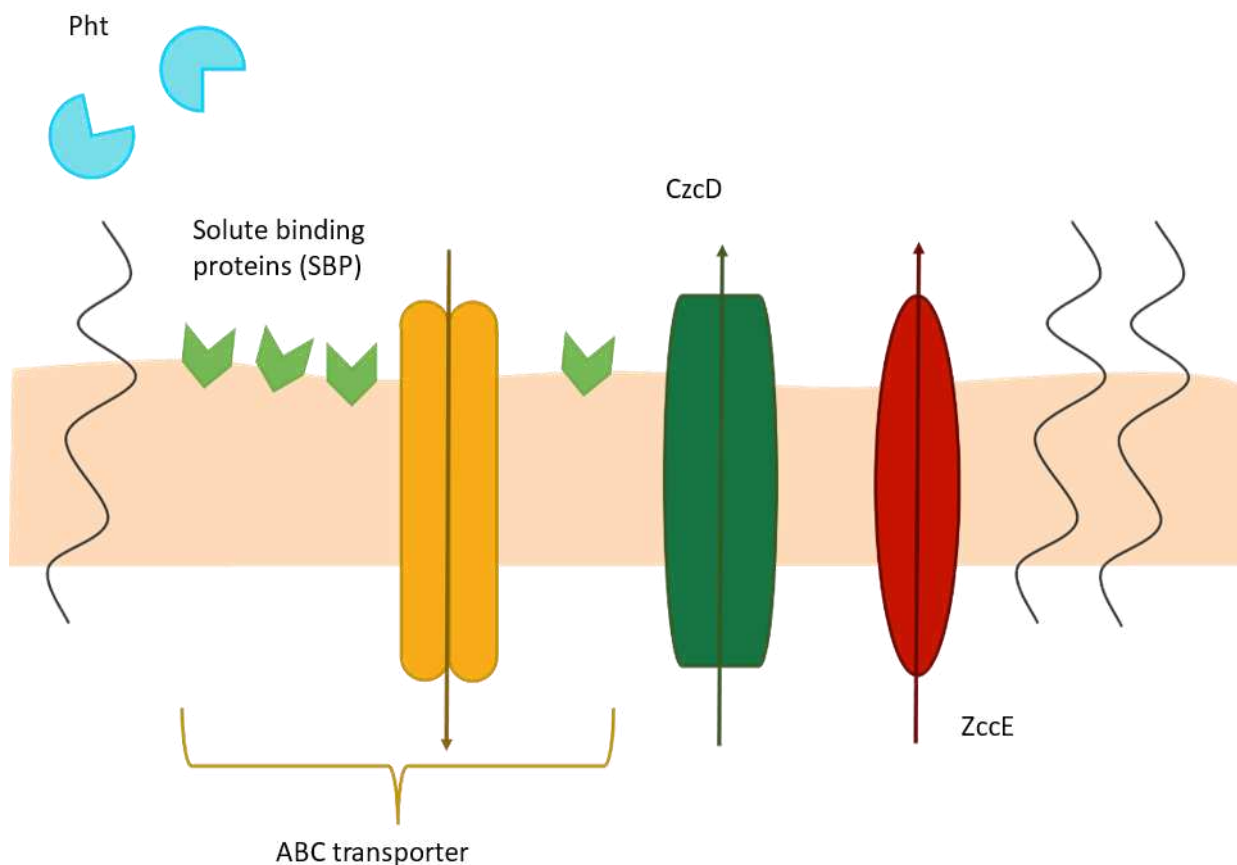


Figure 22 A summary diagram of all known types of transporters for Zn(II) in Streptococci with arrows indicating metal import or export: histidine triad proteins (Pht), ABC transporter, CDF pump: CzcD. The ZccE (P-type ATPase) is only found in *S. mutans*

Different from other pathogenic bacteria that encode multiple Zn(II) import systems, *S. mutans* encodes a single, highly conserved and high affinity zinc ABC-type importer known as AdcABC [473], [474]. ABC transporters consist of the AdcA, zinc-binding lipoprotein that localizes to the membrane via the secretory pathway in Gram-positive bacteria [475]; AdcB – a membrane

permease and AdcC – a cytoplasmic ATPase [476], [477], [478], [479] and their genes are regulated in response to environmental zinc availability by an adhesin competence repressor, AdcR, from the MarR (multiple antibiotic resistance regulator) family [480]. Moreover, *S. mutans* encodes a ZccE protein, which is a P-type ATPase, responsible for Zn(II) export [481], [482], [483]. Also, in contrast to most streptococcal genomes, which encode two copies of *adcA* (called *adcA* and *adcAll*), the genome of *S. mutans* encodes a single *adcA* gene [476], whose size is similar to the *adcBC* than to *adcAll* [482]. Mostly, the streptococcal *adcAll* is genetically coupled to the *pht* genes (metal-binding poly-histidine triad genes) and present in proteins that scavenge zinc, where it binds zinc outside the cell and then most probably shuttles it to the AdcA [484], [442], [485]. For this purpose, the polyhistidine triad proteins (Pht) contain multiple copies of the histidine triad motif (HxxHxH, where X may stand for any amino acid) [486]. Despite the AdcABC transporter is critical to the cariogenic potential of *S. mutans*, and zinc is a growth-limiting factor to producing the oral biofilm, the genome of *S. mutans* lacks *pht* homologs [482], [486] and *S. mutans* is the only streptococci species that does not express Pht proteins [487]. Wherefore the inactivation of *adcA* or *adcCB* prevents the growth of *S. mutans* [488], [489], [490].

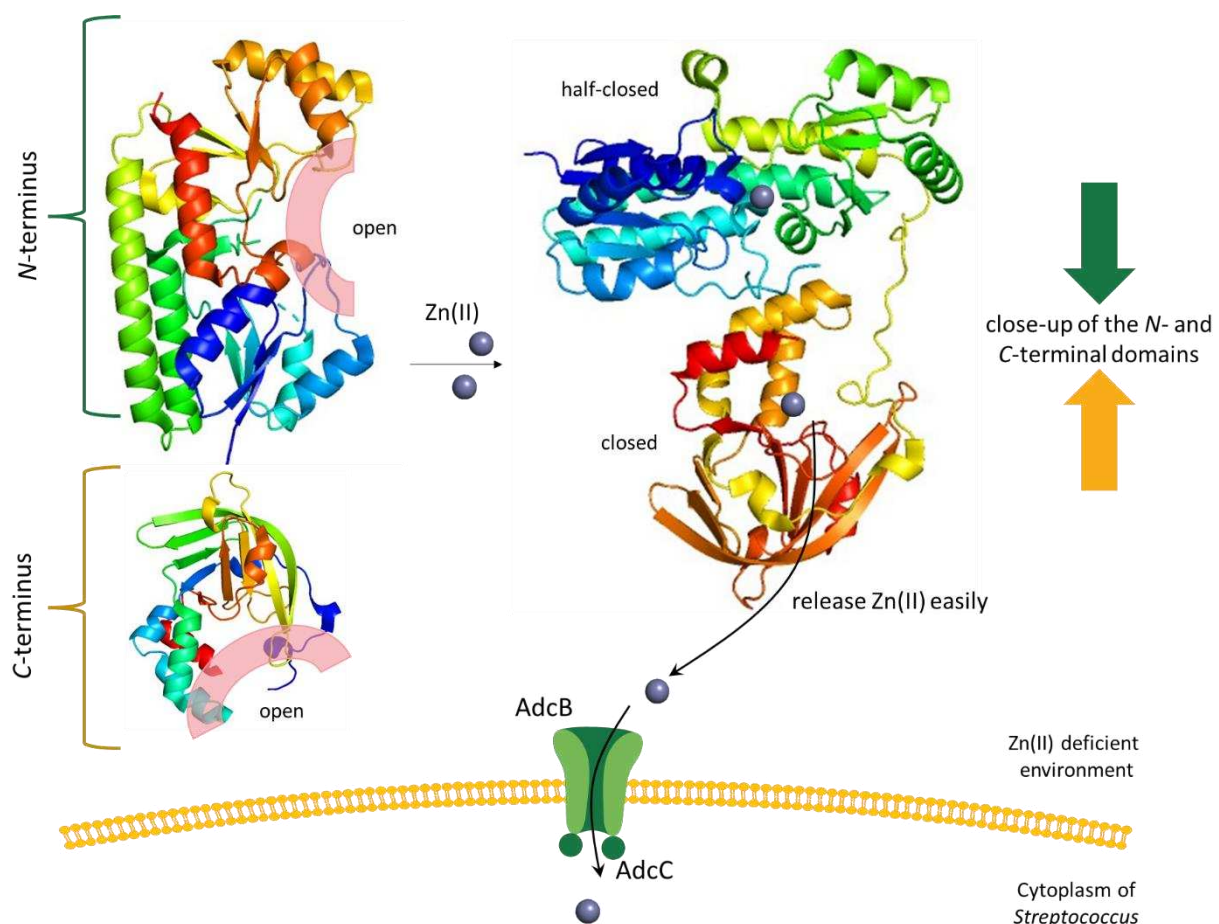


Figure 23 The model of Zn(II) uptake in *Streptococcus* via AdcA in a Zn(II)-deficient environment

For Zn(II) uptake, *Streptococcus* species synthesize the double-domain AdcA protein with two zinc-binding sites: the N-terminal and C-terminal domain (Figure 23). The N-terminal domain,

in the presence of Zn(II), quickly and strongly binds Zn(II) and then changes its conformation, which stabilizes the second domain, the C-terminal domain. A tight zinc-binding pocket facilitates zinc binding, and the relatively less stable binding and conformation ensure a rapid transfer of Zn(II) from AdcA to the transmembrane protein AdcB [491]. As in other streptococci, AdcA from *S. mutans* has two zinc-binding domains: the N-terminal domain, which consists of five histidine residues (H132, H134, H137, H138, H139), and the C-terminal domain, which contains three histidine residues (H457, H466, H468) [482]. Moreover, there are another three additional conserved histidine residues (H70, H144, H208) and one glutamic acid residue (E294) [491]. These high-affinity Zn(II) transport system is homologous to the ZnuA protein from *Escherichia coli* [450], ZnuA and AztC solute binding proteins from *Paracoccus denitrificans* [492] or zincophore based transport system, comprised of proteins with high Zn(II) affinity that are synthesized in the cytoplasm and then exported to bind Zn(II) [428], [426], [493], [494].

C. Coordination properties and biological significance of Zn(II)

Zn is the first-row d-block chemical element with an atomic number 30 and atomic weight of 65.39 [495] with a white-silvery appearance [496]. It is a strong, hard and ductile metal [497], stable in the +II oxidation state [265]. This renders it less reactive than the typical metals [495]. In all its compounds, Zn has an oxidation state of +II [495] and in nature, Zn(II) occurs mainly in the form of the mineral sphalerite, ZnS [495]. Due to their relative stability, transition metals (elements within the central block of the periodic table, that have variable outer shell electrons) are grouped in the Irving-Williams series (from least stable to most stable), where Zn(II) occupies the indicated place: Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II) [47].

The electronic configuration of Zn(II), d^{10} , displays different coordination arrangements and due to the filled d-shell, Zn(II) is spectroscopically silent. The most common coordination number of Zn(II) is four (the most common geometry: tetrahedral [498] or square planar geometry), five (five-coordinate trigonal pyramidal geometries) and six (octahedral geometry) [499] (Figure 24). The square planar geometry of Zn(II) complexes is most often observed in the case of porphyrins and phthalocyanines, whose ligands restrict the binding geometry [500]. Due to its flexibility, Zn(II) is capable of coordinating to six water molecules in aqueous solution [501]. The coordination number of three is not so common (trigonal planar geometry), while numbers: two are mainly observed with amide, silyl, thiophenolate, selenophenolate and alkyl donors [502], [503]), and seven and eight are unusual for Zn(II) and dependent on donor or ligand types [500]. For example, the coordination number of eight is observed in a tetraamide calixarene Zn(II) structure [504]. Trigonal planar geometry is common with alkyl, amide, phenolate and thiophenolate ligands [505] and is very much dependent on the steric bulk of the ligands [506]. The three-coordinate complexes have important properties as catalysts like in the ring opening of cyclic monomers such as lactides [507]. Four coordinate Zn(II) plays a structural role in zinc finger proteins and enzymes [500]. The histidine residue, due to the presence of imidazole coordinating group has a particular target for Zn(II) complex formation [500]. Moreover, amide and alkyl donors are often found in Zn(II) complexes [500] and thiolates

frequently form infinite lattice structures with tetrahedral Zn(II) centers [500]. Independently from the coordination environment, it has a zero ligand-field stabilization energy [496]. Zn(II) is borderline between a hard-soft ion, thus favors the complexation among a wide range of soft ligands such as S⁻ (cysteine sulphur atoms) and hard N⁻ (histidine nitrogen) and O⁻ (glutamate or aspartate oxygen) donor groups [508]. Those ligands usual donors with a frequency of His > Glu > Asp > Cys for all active sites containing a single, catalytic zinc atom [509]. The previously mentioned completely filled d shell makes diamagnetic Zn(II) a mute metal in the UV-Vis and EPR spectroscopy due to the lack of possibility of electronic d-d transitions. At the same time, NMR spectroscopy can be successfully used to study Zn(II) complexes and the abundant isotope of Zn(II) with nuclear spin is the quadrupolar ⁶⁷Zn (I = 5/2) [500].

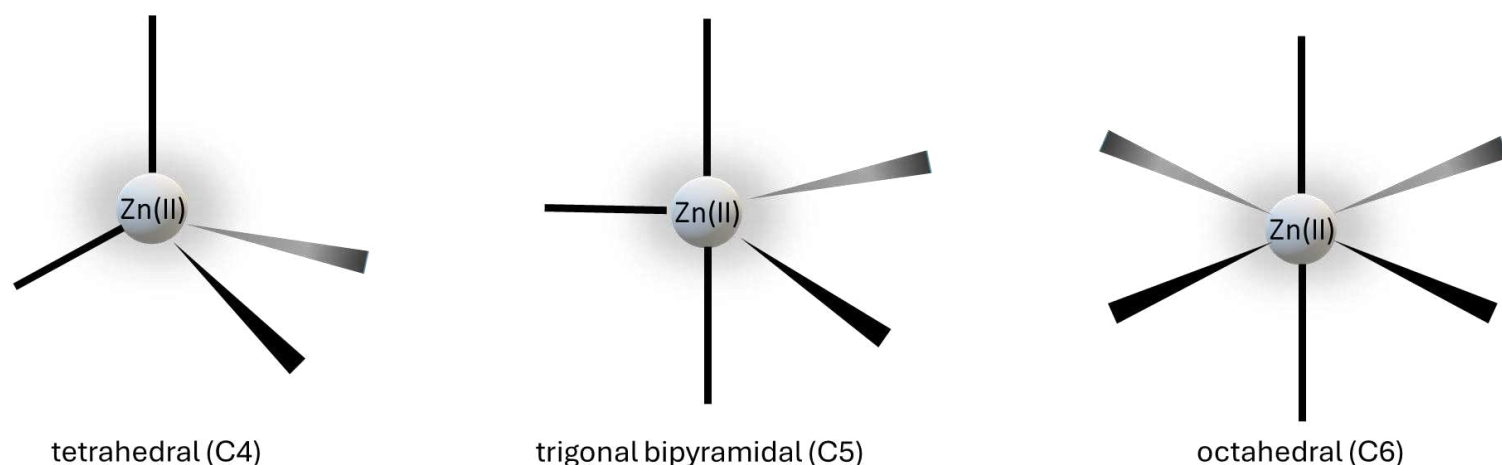


Figure 24 Typical geometries for 4, 5 and 6 coordinated Zn(II)

1. Biological significance of Zn(II)

Due to the fact that Zn(II) does not participate in redox reactions, it is a stable ion in a biological medium [183] and is hydrophilic and do not cross cell membranes by passive diffusion [510]. Zn(II) transport occurs both saturable and non-saturable components, depending on the Zn(II) concentrations [183]. However, Zn(II) exists primary in the form of complexes with proteins and nucleic acids [183]. Zn(II) is the second most common trace mineral (after Fe(II) in the cells) in the living organisms and is involved in numerous aspects of life. In human body Zn(II) plays different functions related to the metabolism (receptor function, insulin storage and secretion), immunity (antibody production), cardiovascular health (maintains blood pressure, improves mucociliary clearance), skin (regeneration, hair growth) and other sensory like night vision, taste, olfaction or inner ear function [511] and it is involved in homeostasis, oxidative stress, apoptosis via the activation of the caspase pathway [512] and ageing [510], membrane stability, wound healing or behavioral response [513], [514]. Moreover, within the normal Zn(II) concentration, it can directly interfere with caspase and endonuclease activity and thus suppress cell apoptosis [515]. Zn(II) also regulates both enzymatic activity and the stability of the proteins as an activator or as an inhibitor ion [514]. It is critical for the function of a number of metalloproteins, including members of hydrolase ligase, lyase or oxido-reductase family. Moreover, Zn(II) has co-activating functions with Cu(II)

in superoxide dismutase or phospholipase C [183]. It inhibits phagocytosis, reducing the release of oxyradicals and production of superoxide and hydrogen peroxide. The increase in ROS is apparently due to a G-protein coupled system activated by Zn(II) [183], but the Zn(II) role in protecting biological structures from damage by ROS may be due to several factors. These include also maintaining an adequate level of metallothioneins (ROS scavengers) [183]. ROS is any species that contains one or more unpaired electrons [516] and although Zn(II) is not classified as an antioxidant [183], it has stabilizing properties for sulfhydryls from oxidation (protects certain enzyme sulfhydryls, like tubulin, dihydroorotase or farnesyl transferase) [517]. Moreover, Zn(II) in various systems antagonizes the catalytic properties of transition metals that exhibit redox activity (such as iron and copper, which promote the formation of $\cdot\text{OH}$ from H_2O_2 in the Fenton reaction) [183] and can compete with Fe(II) or Cu(II) for certain specific types of binding sites [183] (Zn(II) can effectively compete for Cu(II) binding sites in several heme proteins [518]). Zn(II) is not stored in the body and the excess intakes results in reduced absorption and increased excretion [510]. The daily dietary Zn(II) recommendation is estimated at 15 mg/day [183] and its tolerable upper intake level is 25 mg/day [519], although the ingestion or administration of even very large amounts of Zn(II) and its compounds does not have adverse long-term consequences [510]. The Zn(II) concentration in plasma is about 15 $\mu\text{mol/l}$ and most of available Zn(II) is bound to albumin. The remaining part is bound to an α_2 -macroglobulin and amino acids [183]. In erythrocytes and leucocytes its concentration differs and is $\sim 1 \text{ mg}/10^6 \text{ cells}$ and $\sim 6 \text{ mg}/10^6 \text{ cells}$, respectively [520], [521] and the major regulator of zinc homeostasis in vertebrates by tuning both absorption and excretion is the gastrointestinal tract [49]. Zn(II)-deficiency increase the levels of lipid peroxidation in mitochondrial and microsomal membranes, but also the osmotic fragility of erythrocyte membranes. Moreover, Zn(II)-deficiency also produces impaired hemostasis due to defective platelet aggregation [183]. This results in retardation, anorexia, delayed sexual maturation, or Fe(II)-deficiency anemia [513] and its deficiency in the stomach increases gastric secretory volume, acid and pepsin and promotes or aggravates stress-induced gastric lesions and decrease in mast cell count [522]. Moreover, systemic Zn(II)-deficiency will promote or potentiate the development of gastric mucosal damage by increased mast cell histamine and acid-pepsin secretion [183], altered metabolism of androgens, oestrogens and progesterone [523]. The primary source of Zn(II) is a diet rich in eggs, fish, shellfish, red meat, grains, peanuts, fruits, dairy products and green leafy vegetables [524]. Zn(II) is involved in the processes of genetic stability and gene expression, like replication of DNA, transcription of RNA, DNA repair or programmed cell death [525]. Moreover, Zn(II) plays the key role in biological systems, particularly as structural components of a large number of proteins. Its role is attributed to the ability to bind to a wide range of tetrahedral sites, lack of redox chemistry and binding and exchange kinetics [500]. Zn(II) stabilizes the folded conformations of domains allowing interactions between proteins and other biological molecules such as DNA [465]. Zn(II) is a constituent of over 300 enzymes with much research into the coordination of Zn(II) to the protein backbone [526]. Nitric oxide-dependent apoptosis can be induced in motor neurons by Zn(II) deficient SOD [527]. Moreover, one form of hereditary hair loss or alopecia in humans is associated with a mutation in the gene encoding a single zinc finger transcription factor protein with limited expression in the brain and skin [528]. However, the excess amount of Zn(II) will

lead to some cytotoxic effects [529] because it can displace more weakly bound transition metals in the active sites of metalloenzymes [265].

D. Coordination properties and biological significance of Ni(II)

Nickel appears as a hard silver-white ferromagnetic metal with atomic number 28 and atomic weight 58.69 [530]. It possesses a common face-centered cubic structure [531] and is a good conductor of electricity and heat [496], is malleable, ductile and has superior strength and corrosion resistance [530]. It is ferromagnetic [532] and its most common oxidation state is +II, although it also exists in less common oxidation states between –I to IV [496]. Ni(II) forms complexes with the four, five and six coordination numbers and displays all the main structural geometries such as square planar, square pyramidal, tetrahedral, trigonal bipyramidal and octahedral [533]. The divalent Ni(II) complexes (d^8) are usually four-coordinated. They can be square planar (diamagnetic), or tetrahedral (paramagnetic), although these are much rarer. The five-coordinated complexes have trigonal-bipyramidal, square pyramidal or distorted geometries and they can have both high and low spin. Six-coordinated complexes are octahedral and have two unpaired electrons [534]. The most common Ni(II) geometries are presented in Figure 25. Ni(II) is able to induce the deprotonation of protein amide functions at neutral and basic pH [535]. Another metal binding sites for Ni(II) are the terminal amino nitrogen or carboxylate oxygen groups [536], [537]. Moreover, its bindings sites include also cysteine sulfurs or nitrogens from histidine imidazoles [538]. At basic pH uncoordinated Ni(II) precipitates in the form of $\text{Ni}(\text{OH})_2$ [534]. Ni(II) is an essential cofactor for some pathogen virulence factors and due to its low availability in host cells, pathogen must efficiently transport it [539], [540].

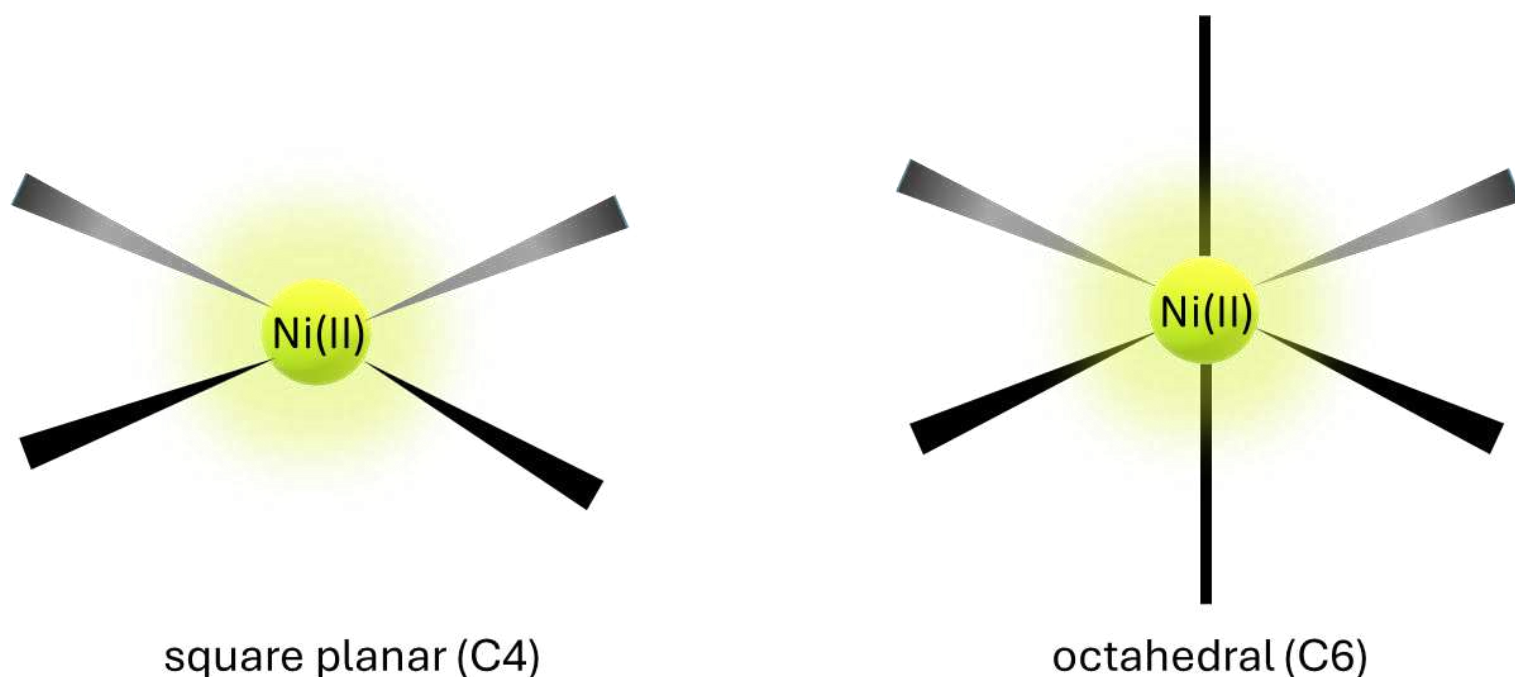


Figure 25 Typical geometries for 4 and 6 coordinated Ni(II)

1. Biological significance of Ni(II)

One of the fundamental differences between pathogens and mammals are Ni(II)-dependent enzymes. Mammals do not synthesize any Ni(II)-requiring proteins, however the intestinal microflora of mammals and fungi are comprised of many Ni(II)-utilizing members. It appears that Ni(II) is also required by archaea, plants [541] and higher animals, where the metal is important for bone health, cGMP signal transduction, carbohydrate and lipid metabolism, as well as for the utilization of gaseous molecules (O_2 , CO_2 , CO and NO) [542]. There are nine Ni(II)-dependent enzymes, both redox and nonredox Ni(II) enzymes [543], [544]. Ni(II) is found at less than 5 ppm in most human organs [545], [546] and compared to its levels in the heart, lung, brain and muscle, the amount of Ni(II) is less than 0.1% of human's Zn(II) in the liver and kidney [547]. In the human gastric environment Ni(II) is usually bound to proteins (like albumin) or to small molecules (like L-His) [548]. The Ni(II) homeostasis is maintained by a sophisticated trafficking system composed of different specialized proteins. They include: importers that acquire Ni(II) from the environment, Ni(II)-binding chaperones and mutation proteins, which deliver it to the active sites of the metalloenzymes, Ni(II)-storage proteins, that sequester Ni(II) from the cytoplasm and exporters, responsible for maintaining the appropriate Ni(II) concentration [549], [550].

Ni(II) is located in the centers of active enzymes and the most notable virulence-associated components are the Ni(II)-enzymes hydrogenases ([NiFe]-carbon monoxide dehydrogenase and [NiFe]-hydrogenase) and urease [551]. We also distinguish: Ni(II)-acireductone dioxygenase (ARD) [544], Ni(II)-superoxide dismutase (NiSOD) [552], and Ni(II)-glyoxalase I (GloI) [544], which play important roles in metabolic and detoxification [539] and lactate racemase (LarA), acetyl-coenzyme A synthase/decarbonylase (ACS) and methyl-coenzyme M reductase (MCR) [541], [543]. Those proteins coordinate Ni(II) via a variety of donor atoms with the most tempting coordination sites containing: cysteine sulfurs, histidine imidazoles and terminal amine group [538]. Ureases catalyze the hydrolytic decomposition of urea into ammonia and bicarbonate. It plays a dual role: products of this reaction can be used to neutralize the pH, what allow urease-containing organisms to survive even in an acidic environment (for instance *Helicobacter pylori*) and the ammonia produced serves as an important source of nitrogen for microorganisms, especially urinary tract pathogens [553]. Hydrogenases catalyze the conversion of molecular hydrogen into protons and electrons, and the reverse reaction [554]. The enzyme provides energy for the cell by this oxidation [555]. There are three classes of hydrogenases, which are defined based on the metal present in their active site: [NiFe], [FeFe] and [Fe] hydrogenases [539]. Among them several [NiFe] hydrogenases are the key point of the colonization and virulence in various organisms [556]. The next Ni(II)-dependent enzyme, Lar, catalyzes the interconversion between L- and D-lactic acid isomers [541]. ARD and GloI enzymes play important roles in the metabolism of their respective host. The acireductone dioxygenase is part of the methionine salvage pathway and during this pathway 5'-methylthioadenosine is converted into acireductone. It can be used as a substrate to generate two different products [557]. It has two different activities that depend on the cofactor (Fe(II) or Ni(II) [544], [558]). In the case of Ni(II)-depending enzyme, the reaction

products are: methylthiopropionate, carbon monoxide and formate (the substrates are the same: 1,2-dihydroxy-3-keto-5-methylthiopent-1-ene (acireductone) and O_2) [558]. The glyoxalase I (also called lactoylglutathione lyase) forms a part of a three-component system, which is responsible for the detoxification of α -ketoaldehydes such as methylglyoxal [541]. There are two distinct classes of GloI enzyme: a Zn(II)-dependent class and a Co(II)/Ni(II) dependent class [539]. The final product of this detoxification is the D-lactate [544]. ACS play an important role in the Wood-Ljungdahl pathway [541], which is found in acetogenes for energy conversation and autotrophic carbon assimilation [541]. This pathway can also act in the oxidative direction to produce reducing power from the oxidation of organic compounds [541]. In turn, the methyl-coenzyme M reductase plays an important role in carbon metabolism and is the key enzyme in the biological methane formation [559]. This enzyme catalyzes the reduction of methyl-S-coenzyme M (CH_3 -S-CoM) by coenzyme B (CoB) to yield methane [541]. The last Ni(II)-dependent enzyme, SOD, is a key contributor to oxidative stress resistance in bacteria [539]. Superoxide dismutases catalyze the dismutation of superoxide radicals ($O_2^{\cdot-}$) into molecular oxygen or hydrogen peroxide and are present in all domains of life [539]. There are three pronounced groups of SODs, which are based on amino acid sequence homology and preferred metal cofactors: Cu(II)/Zn(II)-SOD, Fe(II)-SOD and Mn(II)-SOD, and Ni(II)-SOD) [552]. In Table 2, catalytic reactions for Ni(II)-dependent enzymes are presented.

Table 2 Catalytic reactions for Ni(II)-dependent enzymes

Ni(II)-depend enzyme	Enzyme reaction
Redox enzymes	
[NiFe]-hydrogenase	$H_2 \leftrightarrow 2H^+ + 2e^-$
CO-dehydrogenase coenzyme	$CO + H_2O \leftrightarrow CO_2 + 2H^+ + 2e^-$
Acetyl-coenzyme A synthetase	$CO + CoA-S- + CH_3-CO_3 + FeSp \leftrightarrow CH_3C(O)-S-CoA + CO + FeSP$
Methyl-coenzyme M reductase	$CH_3-S-CoM + CoBSH \leftrightarrow CH_4 + CoBS-S-CoM$
Superoxide dismutase	$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$
Nonredox enzymes	
Urease	$(NH_2)CO_2 + H_2O \rightarrow CO_2 + 2NH_3$
Lactate racemase	$L\text{-lactate} \leftrightarrow D\text{-lactate}$
Glyoxalase I	$CH_3-CO-C(OH)-SG \rightarrow CH_3-CH(OH)-CO-SG$
Acireductone dioxygenase	$1,2\text{-dihydroxy-3-keto-5-methylthiopentane} + O_2 \leftrightarrow 3\text{-methylthiopropionate} + \text{formate} + CO$

To survive, pathogens must provide soluble Ni(II) [552], which is the key to the proper function of the Ni(II)-depending enzymes. In the host's environment, the availability of this metal is low (~ 0.5 nM) [546] and like other transition metals, Ni(II) needs to be first scavenged and then imported from the extracellular environment. So-called 'nickelophores' are involved in this transport (small molecules which can chelate Ni(II) before delivering it to the specific transporters) [539]. In Gram-negative bacteria, Ni(II) is transported across the outer membrane by the TonB/ExbB/ExbD machinery [539]. The transport is carried out by two proteins: FrpB4 and FecA3, which are also able to bind and transport Fe(II) [560]. TonB spans the periplasmic space and interacts with FrpB4 and FecA3 using its C-terminal domain. Moreover, it interacts

with a protein complex formed by ExbB and ExbD, which are located in the cytoplasmic membrane (using its *N*-terminus). The ExbB/ExbD/TonB complex transduces the H(I) motive force of the inner membrane into a conformational change of TonB, which induces Ni(II) transport by FrpB4 and FecA3 [561]. Additionally, it was proposed that at higher Ni(II) concentrations, this metal ion is transported passively through porins [562]. Moreover, two main types of high affinity transporters are used by bacteria to transport Ni(II) across the cytoplasmic membrane. These include ATP-binding cassette type transporters and the Ni(II)/Co(II) transporters [563]. The ATP-binding cassette transporters is divided into two subclasses: energy-coupling factor (ECF) transporters and canonical ABC-type importers [564] and is formed by NiuB, NiuD and NiuE proteins [540]. NiuB is a periplasmic soluble protein, which has high affinity for the [Ni(II)(L-His)₂] complex [565] and most probably bind NiuD. NiuD is a membrane protein located in the inner membrane that is activated by an ATPase – NiuE (a plasmatic membrane-associated ATPase). They interact on the cytoplasmic side of the membrane and are proposed to provide the energy required for Ni(II) transport [540]. The NiuE also causes the opening of the channel located at the dimer interface. It allows the [Ni(II)(L-His)₂] inner membrane crossing [540]. Most probably NiuD and NiuE form homodimers that form the typical quaternary assembly [566]. The NiuBDE system is active under acidic and neutral conditions, even at low Ni(II) concentration [565]. Ni(II) can pass into the cytoplasm also with the high-affinity Ni(II)-transport protein, NixA [540], which is required for the rapid acquisition of Ni(II) at acidic pH [540]. Ni(II) exporters are equally important, as they ensure that intracellular Ni(II) levels do not reach toxic concentrations [539] (like CznABC export pump, which play a critical role in Ni(II) homeostasis and stomach colonization and additionally exports Zn(II) and Cd(II) [550]). Ni(II) overload inside the cell is also prevented by efflux mechanisms and suppression of its transport factors. intracellular Ni(II) concentration and its storage for subsequent use in the production of Ni(II) enzymes depend on storage proteins. These include two histidine-rich proteins (called Hpn and Hpn-like), as well as chaperone proteins (HypA or UreE [567]), which deliver the metal ion to the active sites of urease and hydrogenase [544], [553] and also play a variety of different roles [539]. Hpn and HpnI proteins contain 47% and 25% of His residues in their sequences, respectively. Both are confined to the gastric colonizing types of *Helicobacter* [549] but differ in their occurrence. The Hpn is present in every gastric *Helicobacter* species, while HpnI is restricted to *Helicobacter pylori* and *Helicobacter acinonychis* [568]. Hpn is rich in potential Ni(II) binding sites, because it has an albumin-like N-terminal sequence, several His-rich motifs and two CC motifs [569]. Recombinant Hpn exists primarily as a 20-mer and each monomer bind five Ni(II) [570], while HpnI monomers bind two Ni(II) and forms multimeric structures with more than 20 subunits [571]. In both cases, Ni(II) is release under acidic conditions and most probably these proteins can supply Ni(II) in conditions where urease is also needed [539]. Other proteins, like NikR are crucial for the genic regulation [572]. The NikR regulator controls the expression of more than 10 genes in response to both conditions and Ni(II) concentration [573]. Moreover, the urease operon is additionally regulated by the Mua protein, which prevents the overexpression of urease when the Ni(II) concentration is high [574]. Another Co(II) and Ni(II) chelator and exporter is RcnA [575]. Its interactions with Ni(II) are still misunderstood. The scheme of Ni(II) homeostasis in bacteria is presented in Figure 26.

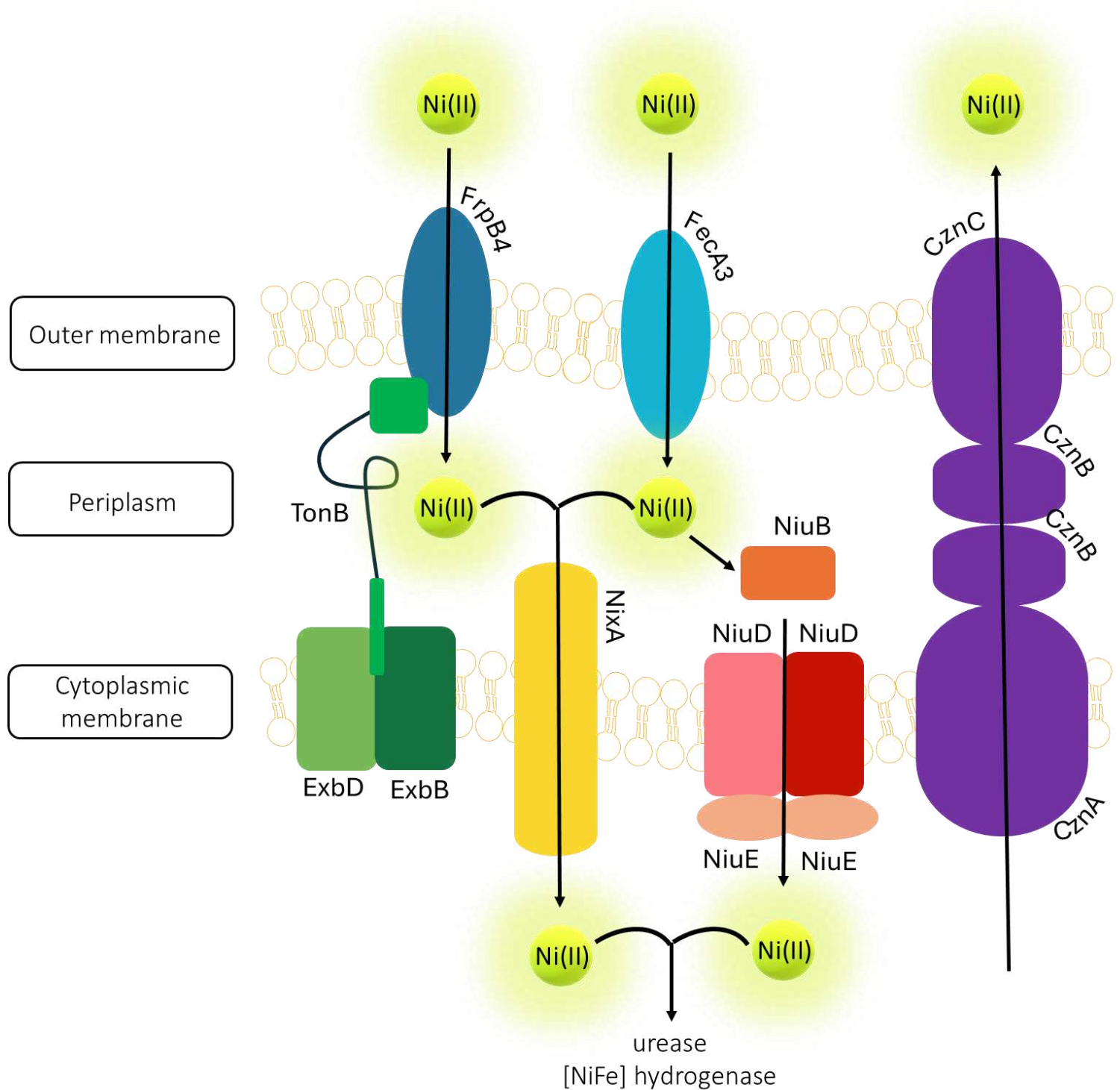


Figure 26 The scheme of Ni(II) homeostasis in bacteria

For mammals, Ni(II) is toxic and cause mutagenic effects after binding to the DNA [576]. However, for bacterial and fungal, Ni(II) is one of the crucial factors necessary to survival [534]. The host takes advantage of the fact and limits its amount to limit the development of the pathogen. The strategy is based on metal sequestering and is a key antibacterial mechanism used to inhibit initial infections or combat tissue-established ones [539] and uses the metal binding by the mammalian host mucosa in the previously mentioned process termed

'nutritional immunity' [577], [490]. With that Ni(II) restriction, no major effect on the host metabolism is expected, and it is suggested that Ni(II) sequestrations be a possible therapeutic approach to combat Ni(II)-requiring pathogens [534], [578], [562]. Ni(II)-depending enzymes do not play a direct role in the pathogenesis, but most probably they can be considered a 'health-related factor' [579].

IV. CHAPTER II: MAIN AIMS OF THE WORK

The main aim of this thesis is to understand the bioinorganic chemistry of chosen Zn(II) transporters from fungi (Asp2 zincophore and ZrfC transporter from *Aspergillus fumigatus*) and bacteria (ZnuA and AztC solute binding proteins from *Paracoccus denitrificans* and AdcA lipoprotein from *Streptococcus mutans*). The tested ligands that are the subject of this work are shown on Figure 27. In order to fulfill the main aim, it was necessary to find answers to the specific objectives:

- i) what are the structure, thermodynamic and binding sites of Zn(II) and Ni(II) complexes?
- ii) which of the tested unstructured fragments of zincophores and zinc transporters bind Zn(II) and Ni(II) with the greatest affinity?
- iii) is the transport of Zn(II) ions specific? Is Ni(II) able to displace Zn(II) from its binding site?

The obtained information will be the basis for the rational design of potential imaging agents and drugs that, when attached to metallophores, will be recognized by an appropriate bacterial or fungal transporter and smuggled into the pathogenic microorganism using the "Trojan Horse" strategy, allowing for more sensitive and specific methods of diagnosis and discrimination of invasive bacterial and fungal infections.

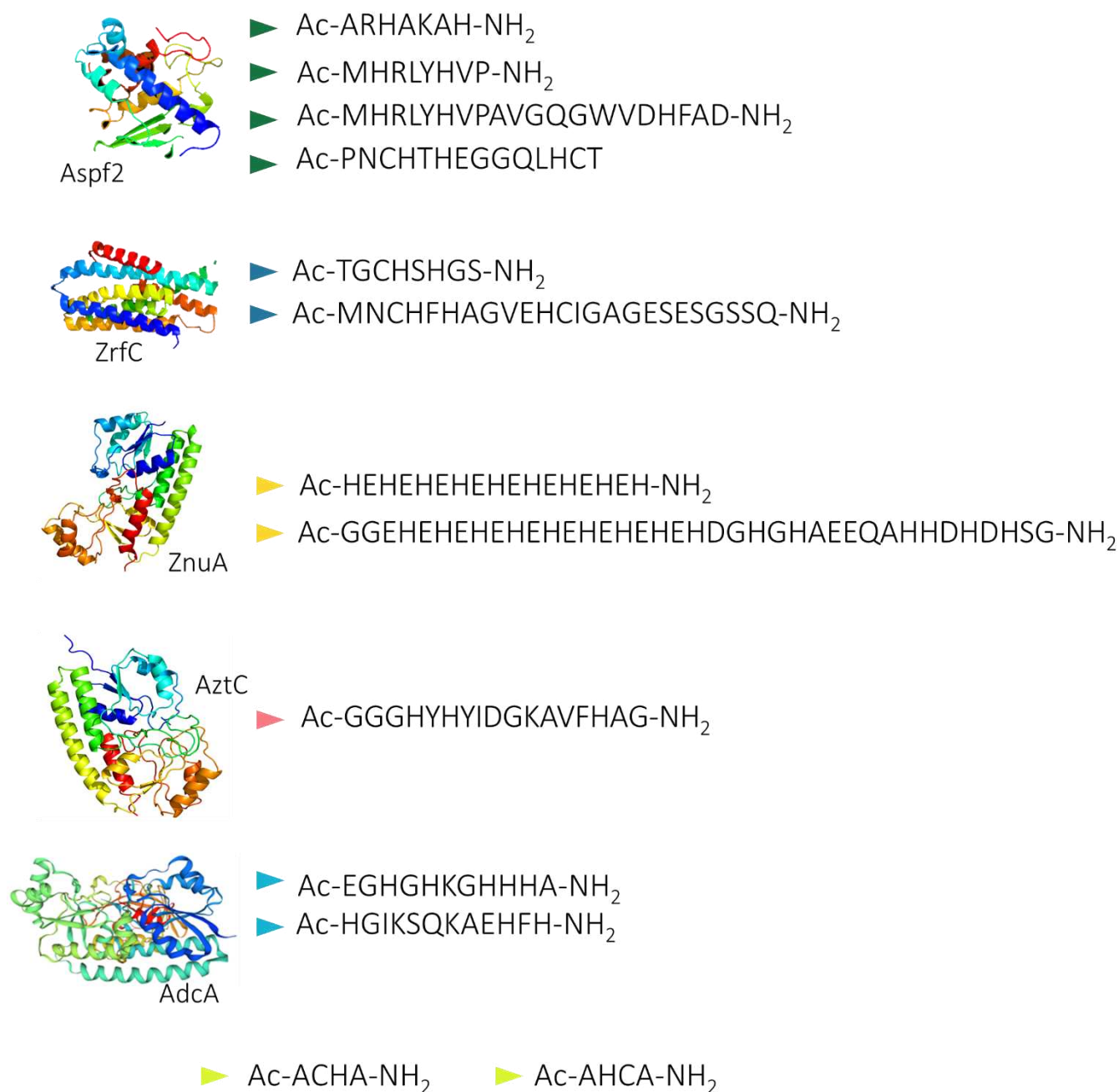


Figure 27 The schematic representation of the ligands investigated in this work

V. CHAPTER III: EXPERIMENTAL SECTION

A. Ligand synthesis

The peptides presented in Table 3 were commercially synthesized by KareBay Biochem (USA) and have a certified purity of 98%. They were used as received.

Tab 3 Amino acid sequences of unstructured fragments of zincophores and proteins involved in Zn(II) transport in *Aspergillus fumigatus*, *Paracoccus denitrificans* and *Streptococcus mutans* and model sequences

Name of protein	Sequence
<i>Aspf2, Aspergillus fumigatus</i>	Ac-ARHAKAH-NH ₂
	Ac-MHRLYHVP-NH ₂
	Ac-MHRLYHVPAVGQGWVDHFAD-NH ₂
	Ac-PNCHTHEGGQLHCT
<i>ZrfC, Aspergillus fumigatus</i>	Ac-TGCHSHGS-NH ₂
	Ac-MNCHFHAGVEHCIGAGESESGSSQ-NH ₂
<i>ZnuA, Paracoccus denitrificans</i>	Ac-HEHEHEHEHEHEHEHEH-NH ₂
	Ac-GGEHEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH ₂
<i>AztC, Paracoccus denitrificans</i>	Ac-GGGHYHYIDGKAVFHAG-NH ₂
<i>AdcA, Streptococcus mutans</i>	Ac-EGHGHKGHHHA-NH ₂
	Ac-HGKSQKAEHFH-NH ₂
Model sequences	Ac-ACHA-NH ₂
	Ac-AHCA-NH ₂

B. Reagents and experimental procedures

All reagents used for research as part of the doctoral thesis titled: Zincophore-based metal transport were of analytical purity. Triple distilled water, filtered using the ULTRA UV HYDROLAB set, was used to prepare aqueous solutions. Metal ion solutions were prepared from perchlorates of the appropriate metals: Zn(ClO₄)₂ (Sigma-Aldrich) and Ni(ClO₄)₂ (Sigma-Aldrich). The exact concentrations of the starting metal solutions were determined by atomic emission spectroscopy coupled with inductively coupled argon plasma (ICP-AES), using an Applied Research Laboratories spectrophotometer, model 3410 ICP (Fisons Instruments) in the department's Laboratory of Elementary Analysis.

Solid substances and solutions were weighed using a Sartorius analytical balance with an accuracy of ± 0.00001 g. All experiments were conducted using a class A measuring glass.

1. ESI-MS spectrometry

Mass spectrometry was used to confirm the mass of commercially synthesized ligands (based on the consistency of simulated and experimentally determined m/z values). Moreover, this method enabled the identification of complexes of the tested ligands with Zn(II) and Ni(II) and the determination of their stoichiometry. High-resolution mass spectra were obtained on a Bruker qTOF compact spectrometer (Bruker Daltonics, Germany). The mass spectrometer was operated in the positive ion mode. The instrumental parameters were as follows: dry gas: nitrogen, temperature: 453 K, and ion energy: 5 eV. The capillary voltage was optimized to the highest S/N ratio and it was 4800 V. The samples (metal : ligand in a 1 : 1 stoichiometry, [ligand]_{tot} = 10⁻⁴ M). The samples were prepared in a 1 : 1 methanol–water mixture. The variation of the solvent composition down to 5% of methanol did not change the species composition. The sample was infused at a flow rate of 3 $\mu\text{L min}^{-1}$. The instrument was calibrated externally with the Low Concentration Tuning Mix ESI-FOF (Agilent Technologies (SD ≤ 1 ppm)). Data were processed using the Bruker Compass DataAnalysis 4.1 program.

2. Potentiometry

Titration curves recorded in the pH range 2 - 11 were used to determine the protonation constants of the ligands and the stability constants of their complexes. Based on these parameters, pH-dependent distributions of complex forms and competition plots were created. Competition plots show the hypothetical situation in which equimolar amounts of all reagents were mixed. The stability constants for ligands and their Zn(II) and Ni(II) complexes were calculated from titration curves carried out of the pH range 2- 11 at 298 K in 4 mM HClO₄ and ionic strength 0.1 M NaClO₄ or 40 mM SDS. HClO₄ was prepared by diluting HClO₄ acid with an initial concentration of 70% and density $d = 1.67\text{g/cm}^3$. The total volume of the solution used was 3.0 cm³. The potentiometric titrations were performed using a Dosimat 800 Metrohm Titrator connected to a Metrohm 905 pH-meter and a Mettler Toledo pH inLab Science electrode. The thermostabilized glass cell was equipped with a magnetic stirring system, a microburet delivery tube and an inlet-outlet tube for argon. Solutions were titrated with 0.1 M carbonate-free NaOH. The electrodes were calibrated daily for hydrogen ion concentration through titrating HClO₄ with NaOH using a total volume of 3.0 cm³. The purities and the exact concentrations of the ligand solutions were determined using the Gran method. The exact concentration was determined using potentiometric titration using NaOH at a concentration of 0.1 M. The entire procedure was repeated at least three times. Potentiometric titrations were performed using a titrant of standard sodium hydroxide solution (Sigma Aldrich) at a concentration of 0.1 M, the concentration of which was determined with an accuracy of ± 0.00001 using a potentiometric standardized standard solution of potassium hydrogen phthalate (KHP) with a concentration of 4 mM and a constant ionic strength of 0.1 M NaCl. The ligand concentration was 0.5 mM. The Zn(II) and Ni(II) to ligand ratio was M(II):L 0.8 : 1. The HYPERQUAD 2006 [580] program was used for the stability constant calculations. The standard deviations were computed using HYPERQUAD 2006 [580] and referenced to random errors only. The constants for hydrolytic Zn(II) and Ni(II) species were used in these calculations. The speciation and competition diagrams were computed using the HYSS program [581].

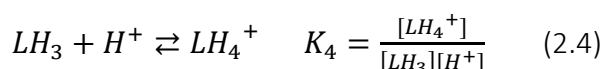
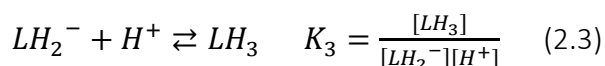
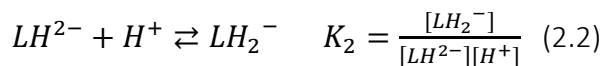
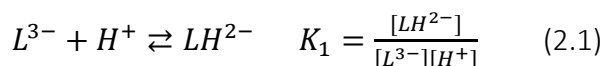
The obtained titration curves were analyzed in the GLEE program [582], where, based on the Gran method [583], in accordance with the modified Nernst equation (Equation 1), the current electrode parameters, such as E^0 (standard electrode potential) and SF (Slope factor), were calculated.

$$E^0 = E + SF(2.303RTF) \log[H^+] \quad (1)$$

where: E – equilibrium potential, R – gas constant [8.314 J/mol·K], F – Faraday constant [9.6485·10⁴ C/mol], T – absolute temperature [K], [H⁺] – hydrogen ion concentration. Ligand solutions were prepared by dissolving the tested peptides in a solution of 4 mM HClO₄ and ionic strength: 0.1 M NaClO₄ or 40 mM SDS to obtain a concentration of 0.5 mM. Ensuring constant ionic strength was necessary to make the system independent of the activity coefficients of individual solution components (Brønsted's principle) [584].

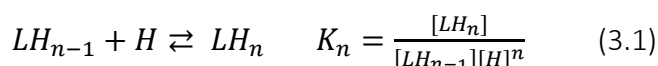
Protonation constants are equilibrium constants describing the reaction of adding a proton to a ligand. Therefore, they are the inverse of the acid dissociation constants, and their use

facilitates calculations regarding the complex formation reaction. They can be described by the following equations [584]:



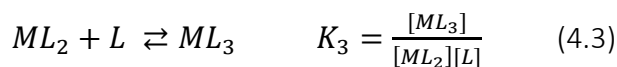
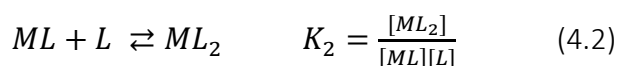
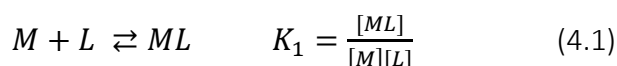
where: L refers to the ligand and H – to the proton.

The protonation constants $K_1 - K_4$ from equations 2.1 - 2.4 are referred to as the so-called subsequent protonation constants and concern the reaction of adding one proton. Equation 3.2 shows the summary (cumulative) protonation constant β_n , which is the product of all subsequent protonation constants. To simplify the notation, the charges have been omitted.



$$\beta = K_1 K_2 K_3 K_4 \dots K_n \quad (3.2)$$

Complex formation reactions can be described using simplified equations, omitting charges (Equations 4.1 - 4.4). These reactions usually proceed gradually, and each stage can be described using the so-called stability constants K , i.e., the equilibrium constants of the reaction between the metal (M), the ligand (L) and the complex being formed (ML_x) [584].



The constants marked in equations 4.1 - 4.4 as $K_1 - K_n$ are subsequent stability constants and concern the reaction of creating a given complex form, during which one ligand is added. The total (cumulative) stability constant for the complex has the form [584]:

$$\beta_n = \frac{[ML]_n}{[M][L]^n} = K_1 K_2 K_3 \dots K_n \quad \log \beta_n = \log K_1 + \log K_2 + \log K_3 + \dots + \log K_n \quad (5)$$

and refers to all stages of complex formation and, consequently, to all subsequent stability constants. In addition to the above-mentioned single-core complexes (with the general formula ML_n), multi-core complexes with the general formula M_pL_r can also be formed. Moreover, the complexes formed may have different degrees of ligand protonation, which leads to the

formation of complexes with $M_pH_qL_r$ stoichiometry. Then the cumulative life constant can be expressed by equation 6:

$$pM + qH + rL \rightleftharpoons M_pH_qL_r \quad \beta_{pqr} = \frac{[M_pH_qL_r]}{[M]^p[H]^q[L]^r} \quad (6)$$

To describe the protonation constants of the tested ligands and the stability constants of the complexes being formed, the negative logarithm of the cumulative stability constant, pK_{a_n} , is used:

$$pK_{a_n} = -\log K_{a_n} = \log \beta_{n+1} - \log \beta_n \quad (7)$$

The constants were determined by fitting experimental titration curves to theoretical curves using iterative least squares.

3. UV-Vis, CD and NMR spectroscopy

The use of UV-Vis absorption spectroscopy and circular dichroism (CD) was aimed at obtaining information about the coordination environment of the metal in the studied nickel complexes- donor atoms, and the geometry of the complex. The study of zinc complexes using these techniques is impossible due to the completely filled d-shell for Zn(II) ions and the resulting lack of possible d-d transitions. The secondary structure of the analyzed systems was also examined using circular dichroism. In order to precisely indicate binding sites in zinc and nickel complexes, NMR nuclear magnetic resonance spectroscopy was used. The absorption spectra were recorded on a Jasco-730 spectrophotometer, in the range 200- 800 nm, using a quartz cuvette with an optical path of 1 cm. Circular dichroism spectra were recorded on a Jasco J-1500 CD spectrometer in the 200 - 800 nm and 180 - 280 nm ranges, using a quartz cuvette with an optical path of 1 and 0.1 cm in the visible and near-UV range. The concentration of sample solutions used for spectroscopic studies was similar to those employed in the potentiometric experiment. The metal : ligand ratio was 1 : 1. All spectroscopic measurements were recorded in the pH range 2.0 - 11.0. The pH of the samples was adjusted with appropriate amounts of concentrated $HClO_4$ and $NaOH$ solutions. OriginPro 2016 [585] was used to process and visualize the obtained spectra. NMR spectra were recorded at 14.1 T on Bruker Avance III 600 MHz equipped with a Silicon Graphics workstation. The temperatures were controlled with an accuracy of ± 0.1 K. Suppression of the residual water signal was achieved by excitation sculpting, using a selective square pulse on water 2 ms long. The samples were prepared in 100% D_2O (99.95% from Merck) and in a 90% H_2O and 10% D_2O mixture. The proton resonance assignment was accomplished by 2D ^1H - ^1H total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy experiments, carried out with standard pulse sequences. Samples of the analyzed complexes were prepared by adding Zn(II) (in the M(II)/L molar ratio 1 : 1) and Ni(II) (in the M(II)/L molar ratio 1 : 10 (due to its possibly paramagnetic nature) to an acidic solution of a 1 mM ligand (pH 3.0) and the pH was then increased to 7.4. The NMR spectra recorded in the presence and in the absence of metal ions pointed out precise metal-binding sites and the combination of all used methods allowed to explain the coordination geometries. Total processing and analysis were performed using Bruker TOPSPIN 2.1 and Sparky programs [586].

The obtained spectroscopic spectra were interpreted on the basis of characteristic electronic transitions, the location and intensity of which informs about the number and type of donor atoms involved in the coordination of the metal ion under the measurement conditions (e.g. depending on pH) or - in the case of CD spectra in the range of 180- 280 nm - about the secondary structure of the tested system. The above spectroscopic parameters are summarized in Table 4 and Table 5.

Table 4 Charge transfer transitions in Ni(II) complexes (CD spectra) [587], [588], [589], [590]

Donor atom	$\lambda_{\max}$ [nm]
S ⁻	220 - 270, 320- 350
N ⁻	250 - 270
N _{im}	260 - 270

Table 5 Characteristic parameters of d-d transitions in Ni(II) complexes depending on their geometry [538], [591], [592]

Geometry of the complex	$\lambda_{\max}$ [nm]
Square-planar (diamagnetic)	420 - 600
Octahedral (paramagnetic)	380, 650

VI. CHAPTER IV: RESULTS AND DISCUSSION

A. Thermodynamic and structural properties of Zn(II) and Ni(II) complexes with unstructured fragments of the Aspf2 zincophore

A saprophytic and an opportunistic fungal pathogen, *Aspergillus fumigatus*, and the mechanism of its Zn(II) transport are presented in point B.1. of the Introduction (Zn(II) transport in *Aspergillus fumigatus*) and in the article [493]. To understand the transport of Zn(II) in *Aspergillus fumigatus*, I focus on Zn(II) and Ni(II) complexes of four Aspf2 zincophore regions (Ac-ARHAKAH-NH₂, Ac-MHRLYHVP-NH₂, Ac-MHRLYHVPVAVGQGWDHFAD-NH₂, and Ac-PNCHTHEGGQLHCT). The amino acid sequences of the Aspf2 zincophore are presented on Figure 28 and the probable Zn(II)-binding sites are highlighted in different colors. I explain their structural and thermodynamic properties, using a variety of complementary methods: mass spectrometry, potentiometry, NMR, UV-Vis and CD spectroscopy. The Aspf2 regions are chosen on the basis of three criteria:

- (i) having probable Zn(II)-binding residues,
- (ii) being unstructured,
- (iii) being evolutionally conserved sites,

and therefore, they are appropriate for our peptide-based approach. The structure was predicted by Phyre2, a remote homology recognition technique, capable of generating reliable protein models [593]. I also check the thermodynamic parameters of Ni(II) binding sites, keeping in mind that Ni(II) is a biologically relevant metal ion, essential for pathogens, and could theoretically compete with Zn(II) for the binding sites in its zincophore. It is important to keep in mind that in the full-length protein, the metal binding sites might likely occur in locally structured regions.

10	20	30	40	50
MAALLRLAVL	LPLAAPLVAT	LPTSPVPIAA	RATPHEPVFF	SWDAGAVTSF
60	70	80	90	100
PIHSSCNATQ	RRQIEAGLNE	AVEL ARHAKA	H ILRWGNESE	IYRKYFGNRP
110	120	130	140	150
TMEAVGAYDV	IVNGDKANVL	FRCDNPDGNC	ALEGWGGHWR	GANATSETVI
160	170	180	190	200
CDRSYTTRRW	LVSMCSQGYT	VAGSETNTFW	ASDL MHRLYH	VP AVGQGWVD
210	220	230	240	250
HF ADGYDEVI	ALAKSNGTES	THDSEALQYF	ALEAYAFDIA	APGVGCAGES
260	270	280	290	300
HGPDQGHDTG	SASAPASTST	SSSSSASGSG	ATTTPTDSPS	ATIDVP PNCH
310				
THEGGQLHCT				

Figure 28 Amino acid sequence of Aspf2 zincophore; probable Zn(II) binding sites are highlighted in different colors

1. Stoichiometry of Zn(II) and Ni(II) complexes with Aspf2 fragments

To confirm the stoichiometry of the formed complexes, I performed MS measurements. The results confirmed that all tested peptides are capable of forming Zn(II) and Ni(II) in a 1 : 1 (metal : ligand ratio) stoichiometry. Experimental peak assignments were compared with the simulated isotopic patterns, and they fit perfectly with the experimental ones.

a) Ac-ARHAKAH-NH₂

The detected MS signals correspond to equimolar Zn(II) complexes with Ac-ARHAKAH-NH₂ peptide ($m/z = 447.19$, $z = 2+$) (Figure 29A). In the case of Zn(II)-Ac-ARHAKAH-NH₂ mass spectra the prevailing signals correspond to the free ligand ($m/z = 831.46$, $z = 1+$ and $m/z = 416.23$, $z = 2+$), and sodium adducts of the peptide: with one sodium atom ($m/z = 853.44$, $z = 1+$ and $m/z = 427.23$, $z = 2+$) and two sodium atoms ($m/z = 438.22$, $z = 2+$). Signals which correspond to zinc complex sodium adducts are also visible ($m/z = 916.43$, $z = 1+$ and $m/z = 458.18$, $z = 2+$). In the Ni(II)-Ac-ARHAKAH-NH₂ spectra (Figure 29B), the signals with come from the free ligand ($m/z = 831.45$, $z = 1+$ and $m/z = 416.23$, $z = 2+$), its sodium adduct with one ($m/z = 853.44$, $z = 1+$) and two sodium atoms ($m/z = 438.22$, $z = 2+$), and nickel complex ($m/z = 887.37$, $z = 1+$ and $m/z = 444.19$, $z = 2+$) and its sodium adducts ($m/z = 455.19$, $z = 2+$) are observed.

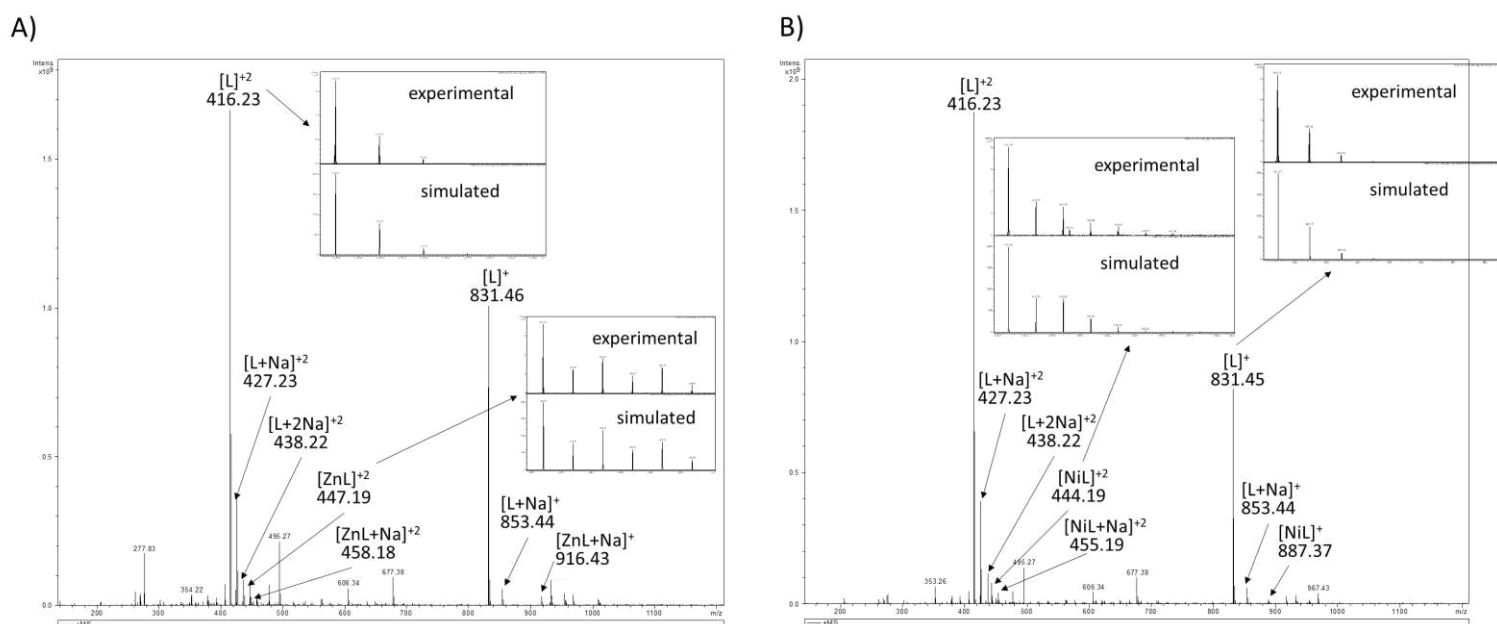
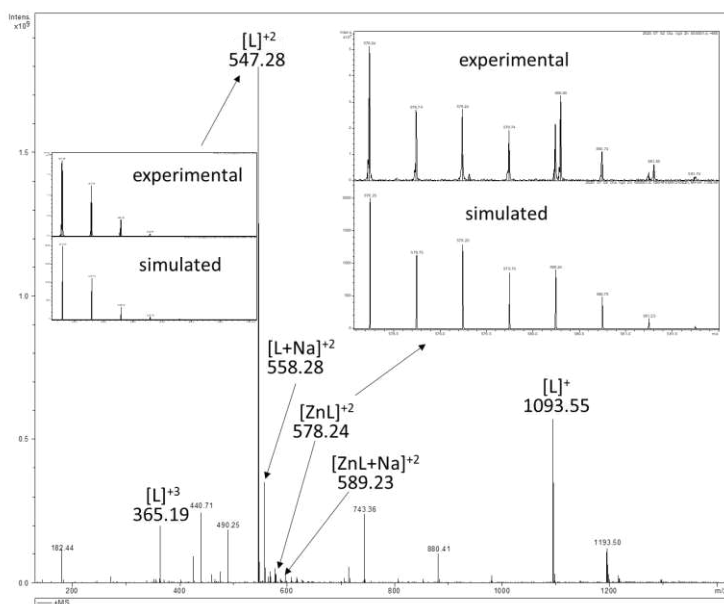


Figure 29 ESI-MS spectrum of A) Zn(II)-Ac-ARHAKAH-NH₂ and B) Ni(II)-Ac-ARHAKAH-NH₂

b) Ac-MHRLYHVP-NH₂

The signals $m/z = 578.24$, $z = 2+$ correspond to equimolar Zn(II) complexes with Ac-MHRLYHVP-NH₂ peptide (Figure 30A). In the Zn(II)-Ac-MHRLYHVP-NH₂ mass spectra, aside from the signals with come from the free ligand ($m/z = 1093.55$, $z = 1+$, $m/z = 547.28$, $z = 2+$ and $m/z = 365.19$, $z = 3+$), a sodium adduct ($m/z = 558.28$, $z = 2+$) and a zinc complexes sodium adduct ($m/z = 589.23$, $z = 2+$) is visible. In the Ni(II)-Ac-MHRLYHVP-NH₂ mass spectra (Figure 30B), beside the signals from the ligand ($m/z = 1093.55$, $z = 1+$, $m/z = 547.28$, $z = 2+$ and $m/z = 365.19$, $z = 3+$), sodium adducts of the ligand with one ($m/z=558.28$, $z=2+$) and two sodium atoms ($m/z = 569.27$, $z = 2+$), a nickel complex ($m/z = 575.24$, $z = 2+$) are visible.

A)



B)

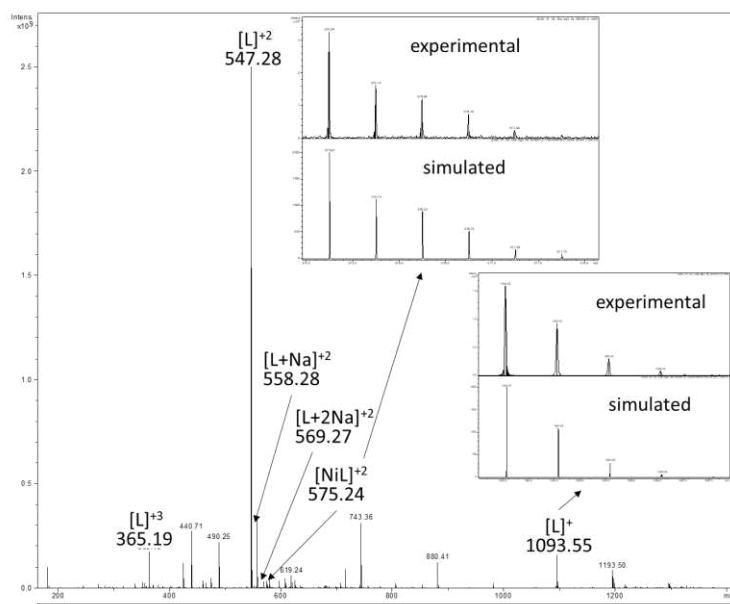


Figure 30 ESI-MS spectrum of A) Zn(II)-Ac-MHRLYHVP-NH₂ and B) Ni(II)-Ac-MHRLYHVP-NH₂

c) Ac-MHRLYHVPVGGQGWVDHFAD-NH₂

In the Zn(II)-Ac-MHRLYHVPVGGQGWVDHFAD-NH₂ mass spectra, signals which come from the free ligand ($m/z = 1189.05$, $z = 2+$ and $m/z = 793.04$, $z = 3+$) sodium adducts with one ($m/z = 1200.04$, $z = 2+$ and $m/z = 800.37$, $z = 3+$ and $m/z = 600.53$, $z = 4+$), two ($m/z = 1211.04$, $z = 2+$, $m/z = 807.70$, $z = 3+$ and $m/z = 606.03$, $z = 4+$) and three sodium atoms ($m/z = 815.03$, $z = 3+$ and $m/z = 611.52$, $z = 4+$) are visible. Moreover, signals correspond to its zinc complex ($m/z = 813.02$, $z = 3+$) and its adduct with one sodium atom ($m/z = 813.02$, $z = 3+$) are visible (Figure 31A). In the case of Ni(II)-Ac-MHRLYHVPVGGQGWVDHFAD-NH₂ (Figure 31B) the prevailing signal comes from the free ligand ($m/z = 793.09$, $z = 3+$). There is also a signal at $m/z = 1189.05$, $z = 2+$ and the signals from its sodium adducts with one ($m/z = 1200.04$, $z = 2+$, $m/z = 800.37$, $z = 3+$ and $m/z = 600.53$, $z = 4+$) and two sodium atoms ($m/z = 807.70$, $z = 3+$ and $m/z = 606.03$,

$z=4+$); a nickel complex ($m/z = 811.68$, $z = 3+$ and $m/z = 609.02$, $z = 4+$) and its sodium adducts ($m/z = 819.01$, $z = 3+$ and $m/z = 614.51$, $z = 4+$) are also observed.

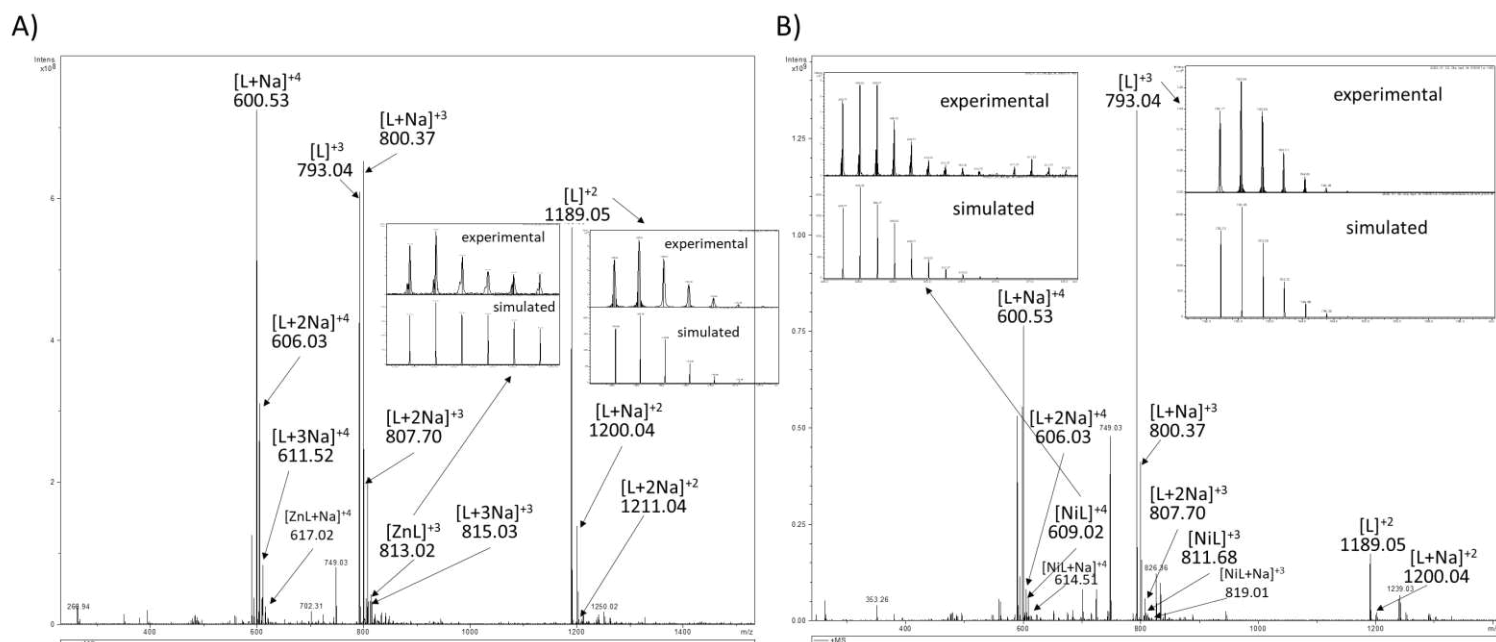


Figure 31 ESI-MS spectrum of A) Zn(II)-Ac-MHRLYHVPAGQGWDHFAD-NH₂ and B) Ni(II)-Ac-MHRLYHVPAGQGWDHFAD-NH₂

d) Ac-PNCHTHEGGQLHCT

In the Zn(II)-Ac-PNCHTHEGGQLHCT mass spectra (Figure 32A), the signal $m/z = 818.28$, $z = 2+$ correspond to Zn(II)-Ac-PNCHTHEGGQLHCT complex. Signals which correspond to the: free ligand ($m/z = 788.31$, $z = 2+$), sodium adducts of the ligand with one ($m/z = 799.30$, $z = 2+$ and $m/z = 533.21$, $z = 3+$), two ($m/z = 810.29$, $z = 2+$ and $m/z = 540.53$, $z = 3+$), three ($m/z = 821.29$, $z = 2+$ and $m/z = 547.86$, $z = 3+$) and four sodium atoms ($m/z = 555.19$, $z = 3+$) are visible. In the Ni(II)-Ac-PNCHTHEGGQLHCT spectra (Figure 32B), aside from the signal which comes the free ligand ($m/z = 788.31$, $z = 2+$ and $m/z = 525.88$, $z = 3+$), a sodium adduct with one sodium atom ($m/z = 799.30$, $z = 2+$ and $m/z = 531.21$, $z = 3+$) and two sodium atoms (m/z

= 540.54, $z = 3+$) and a nickel complex ($m/z = 816.27$, $z = 2+$ and $m/z = 544.52$, $z = 3+$) can be observed.

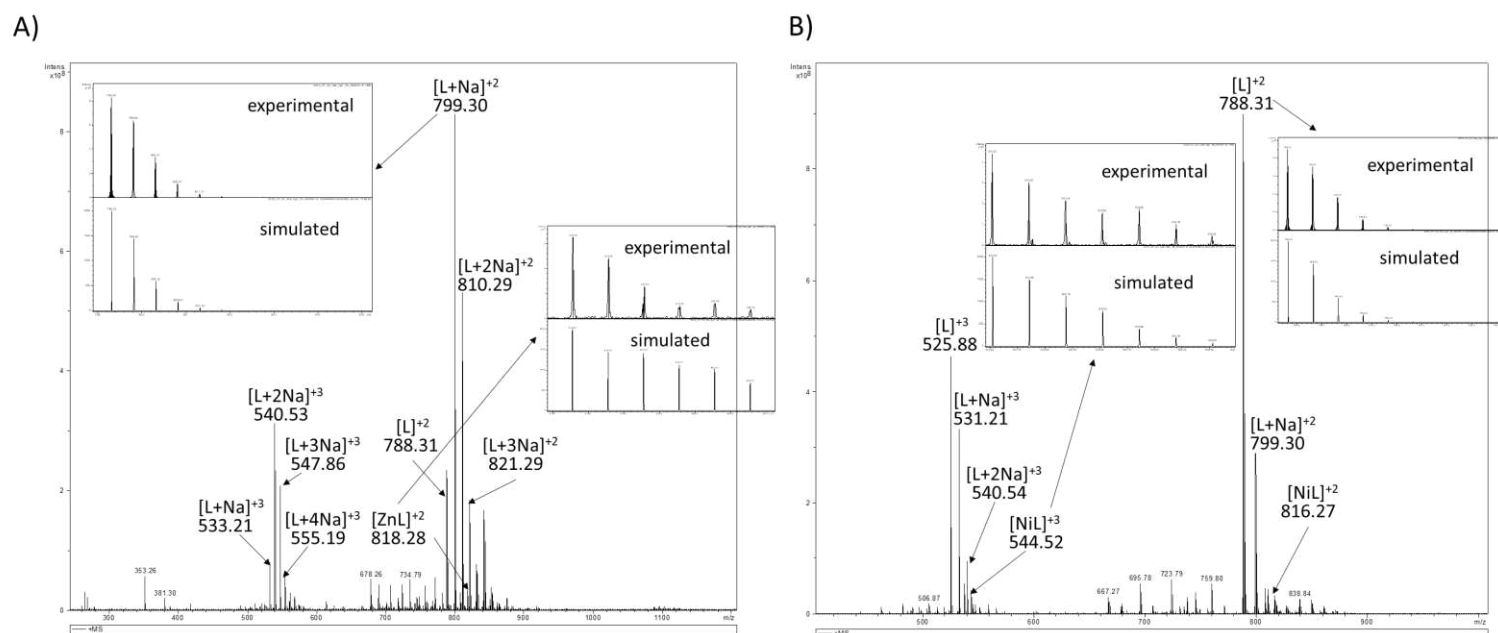


Figure 32 ESI-MS spectrum of A) Zn(II)-Ac-PNCHTHEGGQLHCT and B) Ni(II)-Ac-PNCHTHEGGQLHCT

2. Zn(II) and Ni(II) coordination modes in AspF2

a) Ac-ARHAKAH-NH₂

The Ac-ARHAKAH-NH₂ peptide acts like a typical LH₄⁴⁺ acid. The first two pK_a values (5.85, 6.72) come from the deprotonation of two imidazole groups of the histidine residues. The next constant (9.79) corresponds to the deprotonation of the lysine side chain group and the highest one (11.14) to the deprotonation of arginine. The results are reported in Table 6.

Table 6 Potentiometric data for the proton, Zn(II) and Ni(II) complexes of the, Ac-ARHAKAH-NH₂, Ac-MHRLYHVP-NH₂, Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂ and Ac-PNCHTHEGGQLHCT in a water solution of 4 mM HClO₄ with I = 0.1 M NaClO₄, T = 298 K for Ac-ARHAKAH-NH₂ and Ac-PNCHTHEGGQLHCT peptides or 4 mM HClO₄ with I = 0.04 M SDS, T = 298 K for Ac-MHRLYHVP-NH₂ and Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂ peptides. Standard deviations are presented in parentheses

Species	Ac-ARHAKAH-NH ₂			Ac-MHRLYHVP-NH ₂			Ac-MHRLYHVPVAVGQGWVDHFAD-NH ₂			Ac-PNCHTHEGGQLHCT		
	logβ	pK _a	residue	logβ	pK _a	residue	logβ	pK _a	residue	logβ	pK _a	residue
H ₆ L							48.90 (1)	4.72 (D)		42.23 (1)	4.24 (E)	
H ₅ L							44.18 (1)	6.34 (H)		37.99 (1)	5.73 (H)	
H ₄ L	33.50 (1)	5.85	(H)	36.32 (1)	7.22	(H)	37.84 (1)	7.88 (H)		32.26 (1)	6.54 (H)	
H ₃ L	27.65 (1)	6.72	(H)	29.10 (1)	8.33	(H)	29.96 (1)	8.60 (H)		25.72 (1)	7.23 (H)	
H ₂ L	20.93 (1)	9.79	(K)	20.77 (1)	9.56	(Y)	21.36 (1)	10.12 (Y)		18.49 (1)	9.00 (C)	
HL	11.14 (1)	11.14	(R)	11.21 (1)	11.21	(R)	11.24 (1)	11.24 (R)		9.49 (1)	9.49 (C)	

[illegible]

NiH ₄ L						41.49 (1)			36.28 (1)		
NiH ₃ L			34.41 (1)			34.99 (1)	6.50	(H)			
NiH ₂ L	24.76 (1)		27.17 (1)	7.24	(H)	27.72 (1)	7.27	(H)	25.11 (1)		
NiHL			19.78 (1)	7.39	(am)	16.62 (1)	11.1	(am)	17.98 (1)	7.13	(C)
NiL	7.64 (1)		10.39 (1)	9.39	(am)				10.55 (1)	7.43	(C)
NiH ₋₁ L	-0.98 (1)	8.62	(am)	0.61 (1)	9.78	(am)	-0.81 (1)		1.63 (1)	8.92	(am)
NiH ₋₂ L	-10.85 (1)	9.87	(K)						-8.53 (1)	10.16	(am)
NiH ₋₃ L	-22.23 (1)	11.38	(R)	-19.78 (1)			-22.49 (1)		-19.04 (1)	10.51	(am)

(1) $\text{Zn(II)-Ac-ARHAKAH-NH}_2$

In the case of Ac-ARHAKAH-NH₂, the first observed complex, ZnH₂L⁴⁺, with a maximum at pH 7, involves two histidine residues in binding (Table 6). The loss of the next two protons leads to the ZnL²⁺ form and is most likely related to the deprotonation of subsequent water molecule bound to the central zinc ion. In the ZnH₋₁L⁺ complex, another water molecule bound to the central zinc ion loses a proton. ZnH₋₂L deprotonation comes from a lysine side chain, which does not take part in the coordination and the last form (ZnH₋₃L⁻) corresponds to the deprotonation of the arginine side chain, which also does not participate in binding (Figure 33).

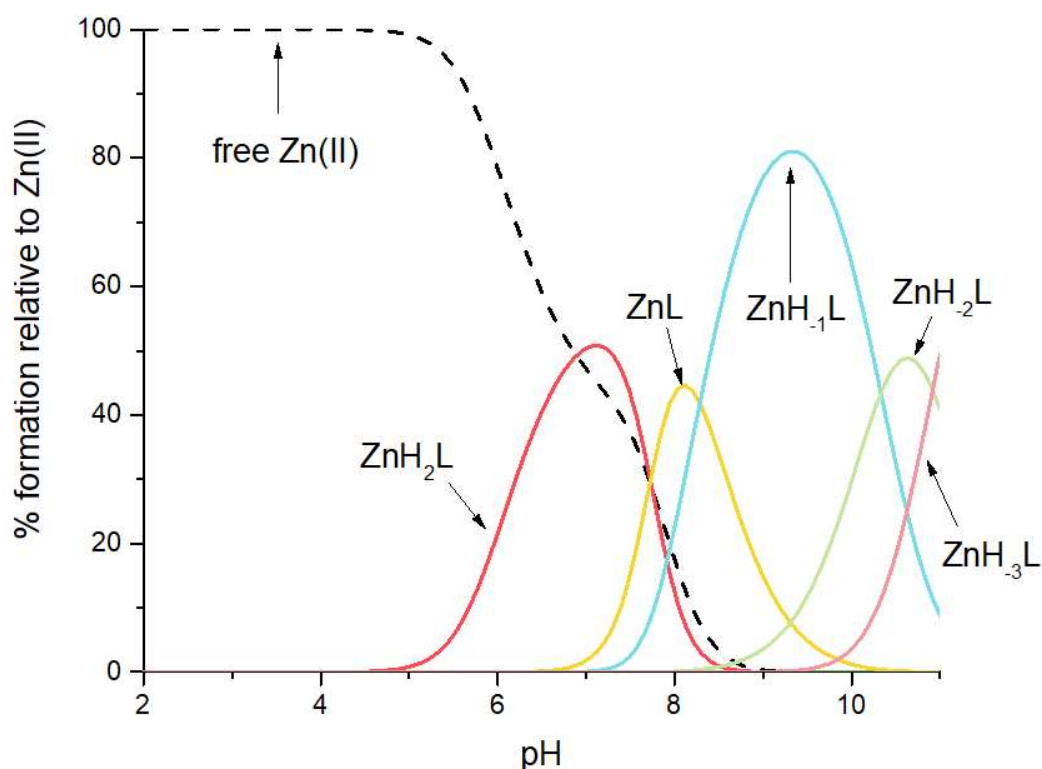


Figure 33 Species distribution diagram for the formation of Zn(II) complex with the Ac-ARHAKAH-NH₂ Aspf2 fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

(2) Ni(II)-Ac-ARHAKAH-NH₂

The first Ni(II) complex observed at acidic pH for Ac-ARHAKAH-NH₂ is NiH₂L⁴⁺, with a maximum at pH 7.5 (Table 6 and Figure 34) and here, most likely, the Ni(II) ion is anchored to two imidazole groups of histidine. The next form, NiL²⁺, dominates in the solution around pH 8.7 and engages an amide nitrogen in metal coordination. CD spectra (Figure 35) confirm this finding with pronounced d-d bands at 430 and 520 nm, typical of amide involving square-planar nickel-peptide complexes and a UV-Vis charge transfer band near 275 nm, which can be assigned to N_{im} → Ni(II) and N⁻ → Ni(II) charge transfer transitions (Figure 36) [587]. The loss of the next proton leads to the NiH₋₁L⁺ form, with a maximum at pH 9, and it is associated with the deprotonation of a second amide nitrogen; the earlier mentioned CD bands increase their intensity. Eventually, at basic pH, a square-planar nickel complex is formed, in which Ni(II) is bound to one histidine imidazole and three amide nitrogens, in a {N_{im}, 3N⁻} binding mode; one of the imidazoles coordinated at acidic pH was replaced with an amide. In the NiH₋₂L form, the comparable pK_a value of the lysine group in the complexed ligand (pK_a = 9.87) with respect to the free one (pK_a = 9.79), (Table 6), strongly suggests that this group is not involved in binding. The last complex form, NiH₋₃L⁻, which appears above pH 10, comes from the deprotonation of arginine residue, which also does not participate in binding (complexed ligand - pK_a = 11.38, free one - pK_a = 11.14).

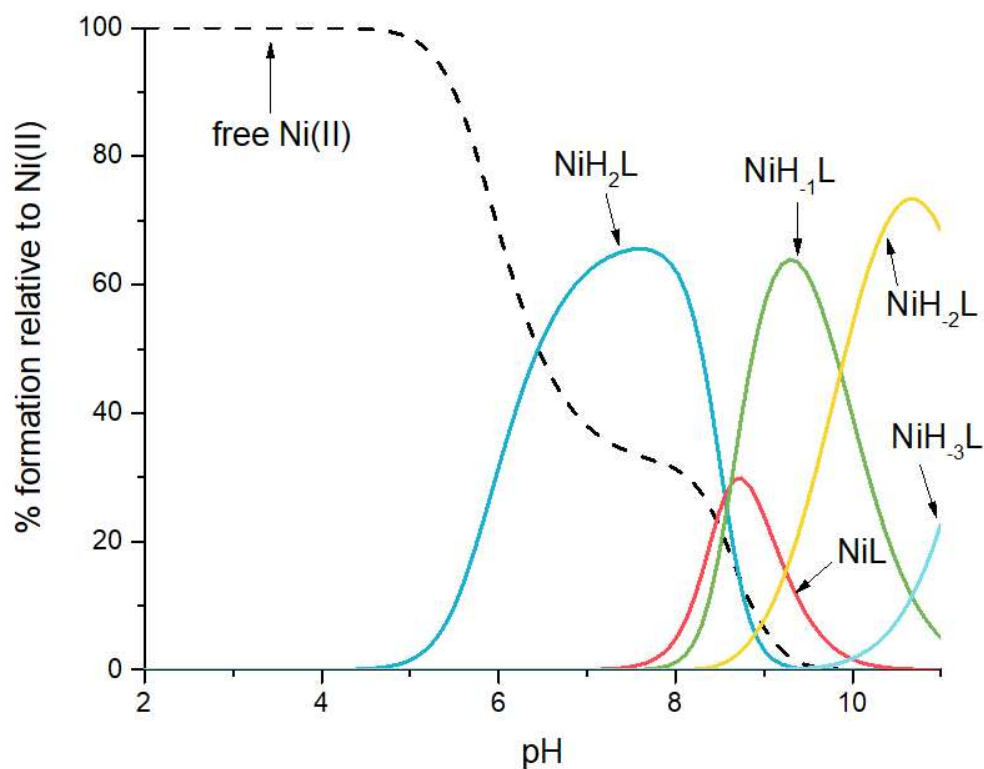


Figure 34 Species distribution diagram for the formation of Ni(II) complex with the Ac-ARHAKAH-NH₂ Aspf2 fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

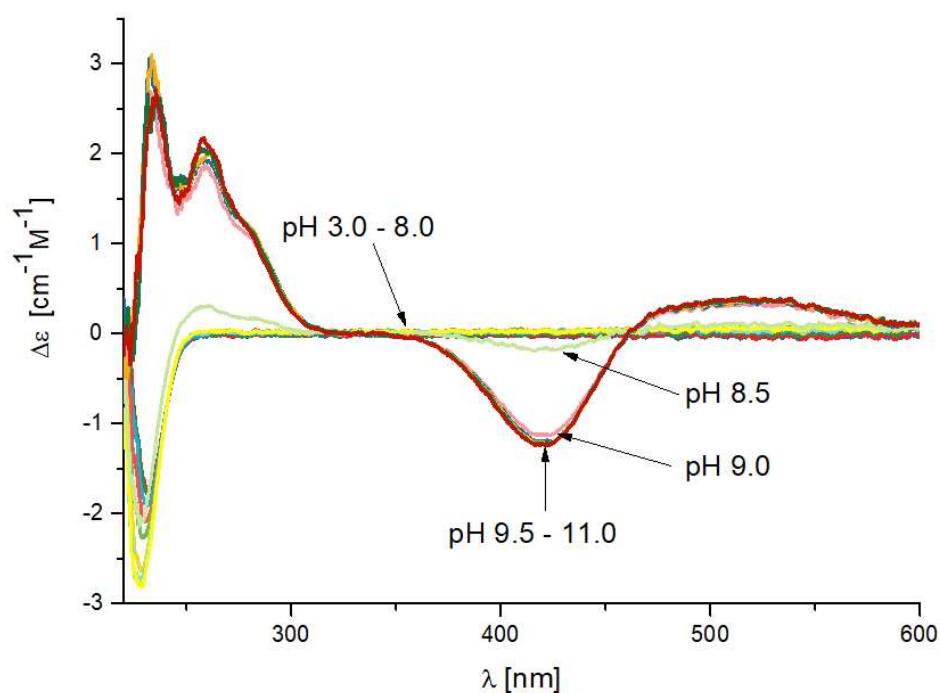


Figure 35 CD spectra of Ni(II) complex with the Ac-ARHAKAH-NH₂ fragment from Aspf2 protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

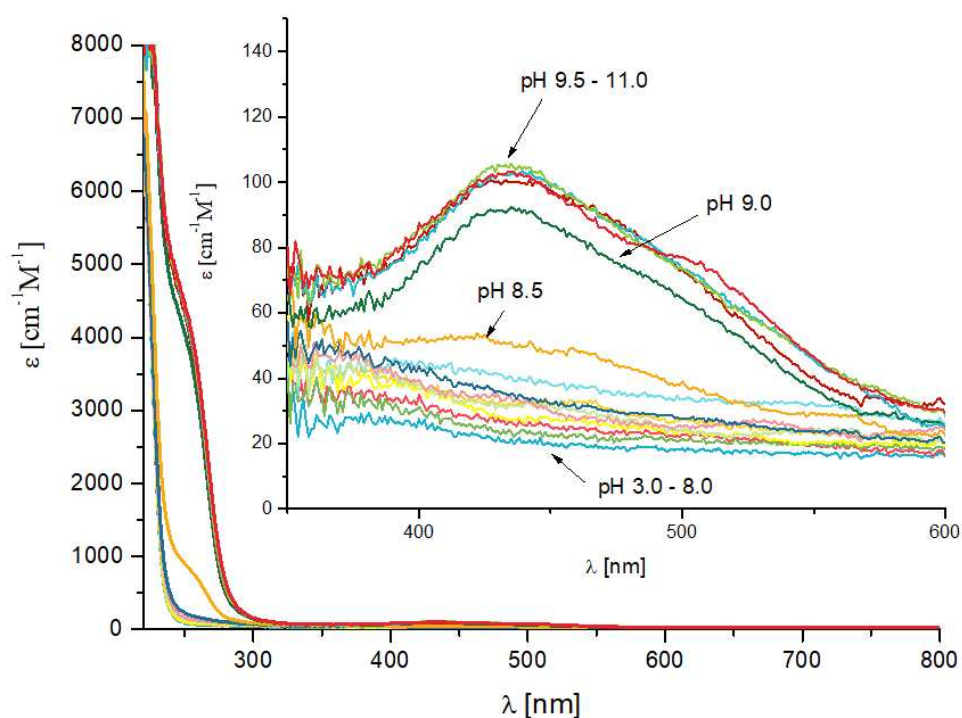


Figure 36 UV-Vis spectra of Ni(II) complex with the Ac-ARHAKAH-NH₂ fragment from Aspf2 protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

b) Ac-MHRLYHVP-NH₂

The Ac-MHRLYHVP-NH₂ peptide has four protonation constants. The first two (7.22, 8.33) can be assigned to the deprotonation of two histidine imidazole groups (Table 6). The next constant, with a pK_a of 9.56 is related to the deprotonation of tyrosine, and the most basic one (11.21) corresponds to the deprotonation of arginine.

(1) Zn(II)-Ac-MHRLYHVP-NH₂

The first observed Zn(II) complex of Ac-MHRLYHVP-NH₂, ZnH₂L³⁺, has a maximum at around pH 8 and involves two histidine residues in binding (Figure 37). The loss of the next two protons, leading to ZnHL²⁺ and ZnL⁺ forms, is most likely related to the deprotonation of two water molecules bound to the central zinc ion. At higher pH values, the coordination mode does not change and the loss of the next two protons is related to the deprotonation of the tyrosine and arginine side chains, which do not participate in binding (Table 6).

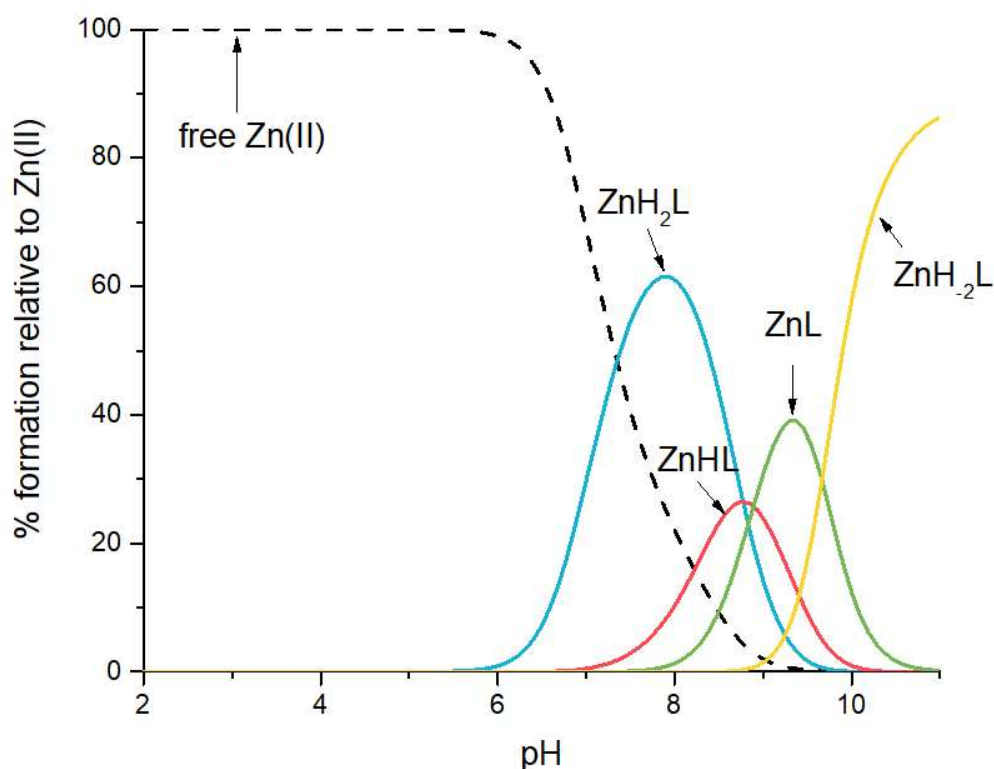


Figure 37 Species distribution diagram for the formation of Zn(II) complex with the Ac-MHRLYHVP-NH₂ Aspf2 fragment, T = 298 K, I = 0.04 M SDS, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

(2) Ni(II)-Ac-MHRLYHVP-NH₂

Six complex forms with Ni(II) for the Ac-MHRLYHVP-NH₂ peptide were detected (Figure 38). In the NiH₃L⁴⁺ species, the Ni(II) ion is anchored to one of the histidine imidazoles. The loss of the next proton leads to the formation of the NiH₂L³⁺ form and the lowered pK_a value of the histidine group in the complexed ligand (pK_a = 7.24) with respect to the free one (pK_a = 8.33), strongly suggests that the second histidine is involved in binding. The NiHL²⁺ form dominates in the solution at pH around 8.5 and it is associated with the coordination of an amide nitrogen, as indicated by UV-Vis band above pH 10.0 with a maximum at 445 nm (Figure 39). In the next two complexes, NiL and NiH₋₁L⁻, two further amides are involved in Ni(II) binding [and replaced one of the imidazoles in the coordination sphere of Ni(II)]; the CD bands with a negative Cotton effect at 430 nm and a positive Cotton effect at 520 nm gradually increase their intensity (Figure 40), indicating that also in this case, at basic pH, a square-planar nickel complex with a {N_{im}, 3N⁻} binding mode is formed. The last complex form, NiH₋₃L²⁻, appears above pH 10 and is connected with the deprotonation of the arginine residue, which does not take part in coordination.

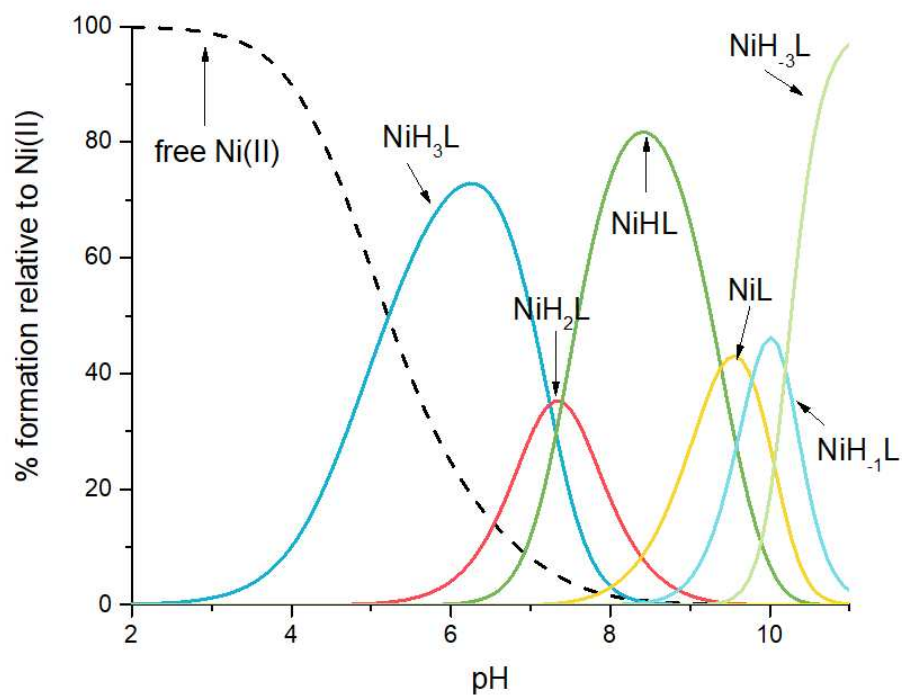


Figure 38 Species distribution diagram for the formation of Ni(II) complex with the Ac-MHRLYHVP-NH₂ Asp2 fragment, T = 298 K, I = 0.04 M SDS, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

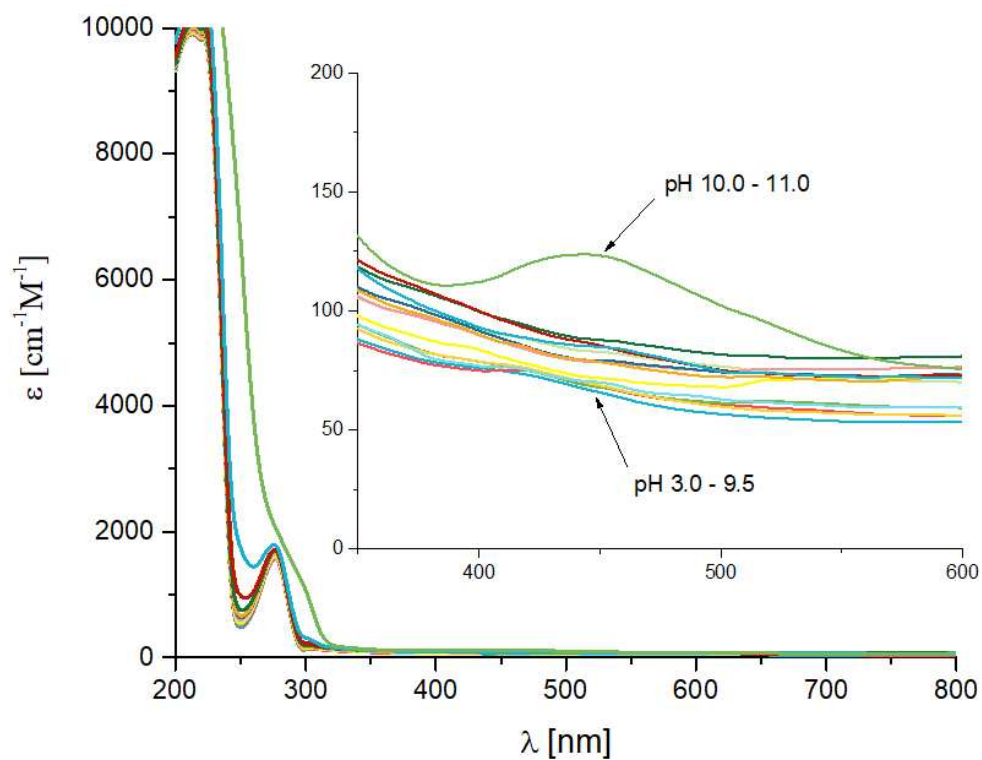


Figure 39 UV-Vis spectra of Ni(II) complex with the Ac-MHRLYHVP-NH₂ fragment from Asp2 protein in the range

200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

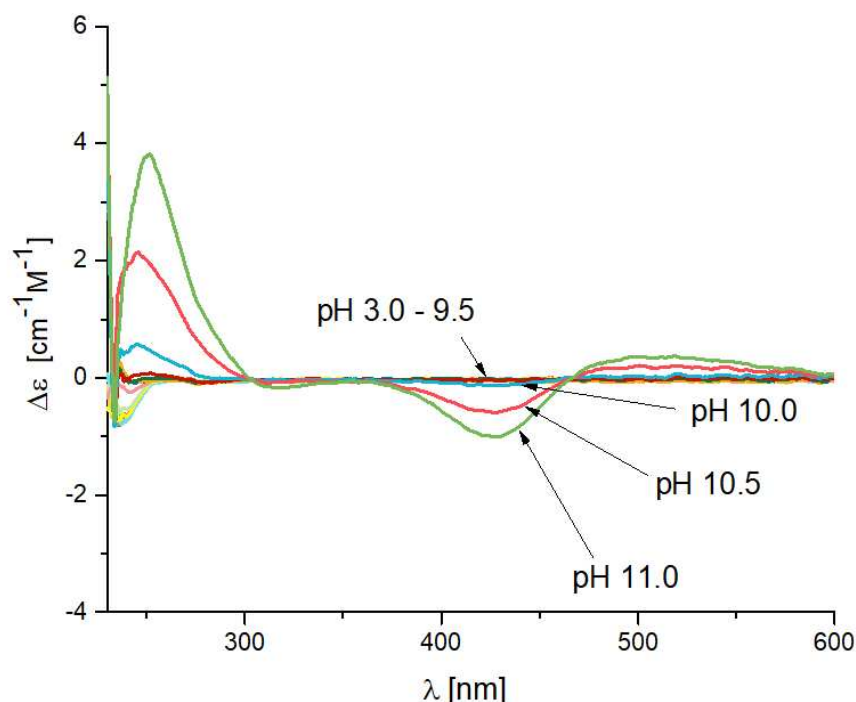


Figure 40 CD spectra of Ni(II) complex with the Ac-MHRLYHVP-NH₂ fragment from Asp2 protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

c) Ac-MHRLYHVPVGGQGWVDHFAD-NH₂

The Ac-MHRLYHVPVGGQGWVDHFAD-NH₂ peptide behaves as an LH₆³⁺ acid with the deprotonating groups corresponding to one glutamic acid side chain group (4.72), three histidine imidazole groups (6.34, 7.88, 8.60), tyrosine (10.12), and arginine (11.24).

(1) Zn(II)-MHRLYHVPVGGQGWVDHFAD-NH₂

The first complex observed for Ac-MHRLYHVPVGGQGWVDHFAD-NH₂ is ZnH₅L⁴⁺, with a maximum at pH around 5; here, most likely the Zn(II) ion is anchored to one of the aspartic acid side chains. The next three forms, ZnH₄L³⁺, ZnH₃L²⁺ and ZnH₂L⁺, are related to the coordination of subsequent histidine residues. A significant decrease of the pK_a values (Table 6) suggests that all histidine are involved in Zn(II) coordination. The last observed form, ZnHL, corresponds to the deprotonation of the tyrosine side chain, which does not participate in binding (Figure 41 distribution). The deprotonation of arginine in the complex form lies beyond the measuring scope of the electrode.

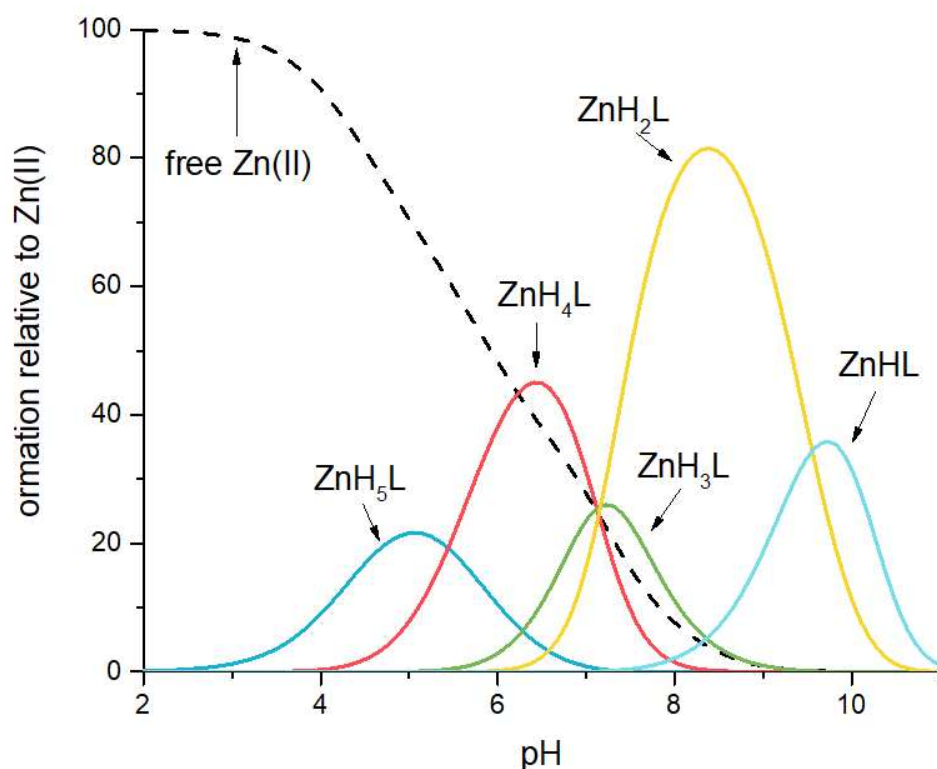


Figure 41 Species distribution diagram for the formation of Zn(II) complex with the Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂ Asp2 fragment, T = 298 K, I = 0.04 M SDS, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

(2) Ni(II)-Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂

The first detected Ni(II)-Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂ complex is NiH₄L³⁺, with a maximum around pH 6 and here, most likely, the Ni(II) ion is anchored to an imidazole of one of the histidine side chains (Figure 42). The loss of one proton leads to the NiH₃L³⁺ form, with a maximum at pH 7. The lowered histidine pK_a value in the complexed ligand (pK_a = 6.50) with respect to the free one [pK_a = 7.88, (Table 6)], strongly suggests that this residue is also directly involved in binding. The next form, NiH₂L⁺, is associated with the coordination of a third histidine residue (complexed ligand- pK_a = 7.27, free one – pK_a = 8.60), as indicated by the UV-Vis band with a maximum at 275 nm (Figure 43) that can be related to the N_{im} → Ni(II) charge transfer transition [587]. The increase of pH leads to the NiHL form, with a maximum at pH around 9.4, which is associated with the coordination of an amide nitrogen. Two further amides are bound in the NiH₋₁L²⁻ form, which appears above pH 10. The maximum absorption of the d-d band with a positive Cotton effect at 515 nm and a negative one at 425 nm in the CD spectra (Figure 44) and the d-d transitions at 445 nm in the UV-Vis spectra (Figure 43) indicate the formation of the square-planar nickel complex that involves one histidine imidazole and three amide nitrogens in metal binding.

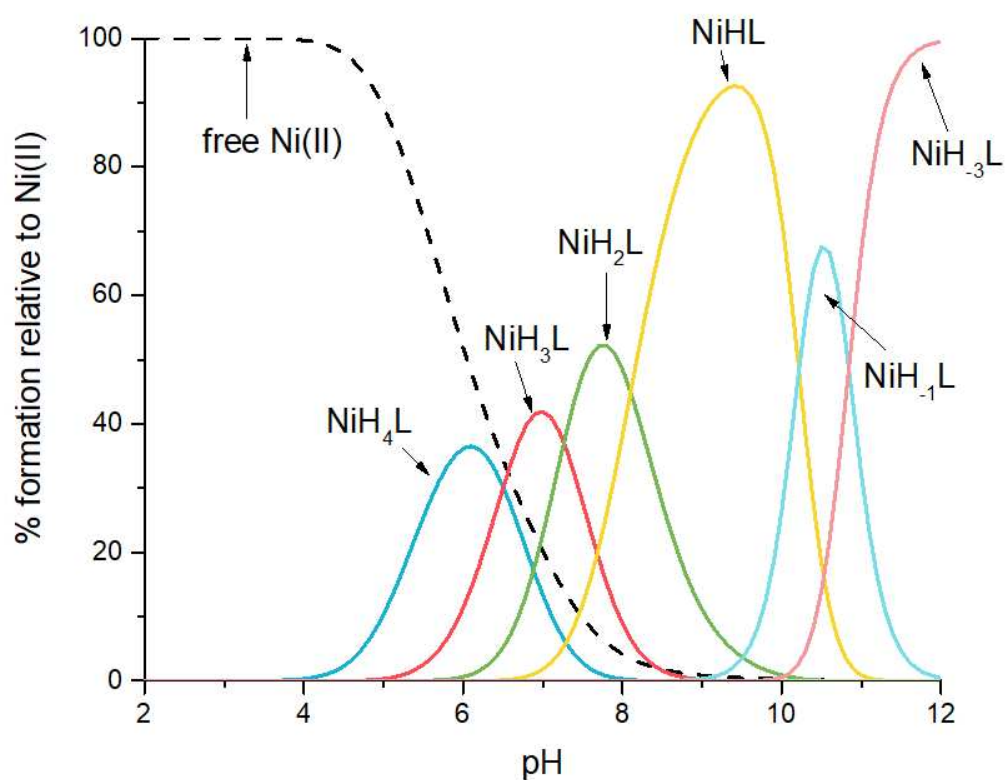


Figure 42 Species distribution diagram for the formation of Ni(II) complex with the Ac-MHRLYHVPVAVGQGWDHFAD-NH₂ Asp2 fragment, T = 298 K, I = 0.04 M SDS, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

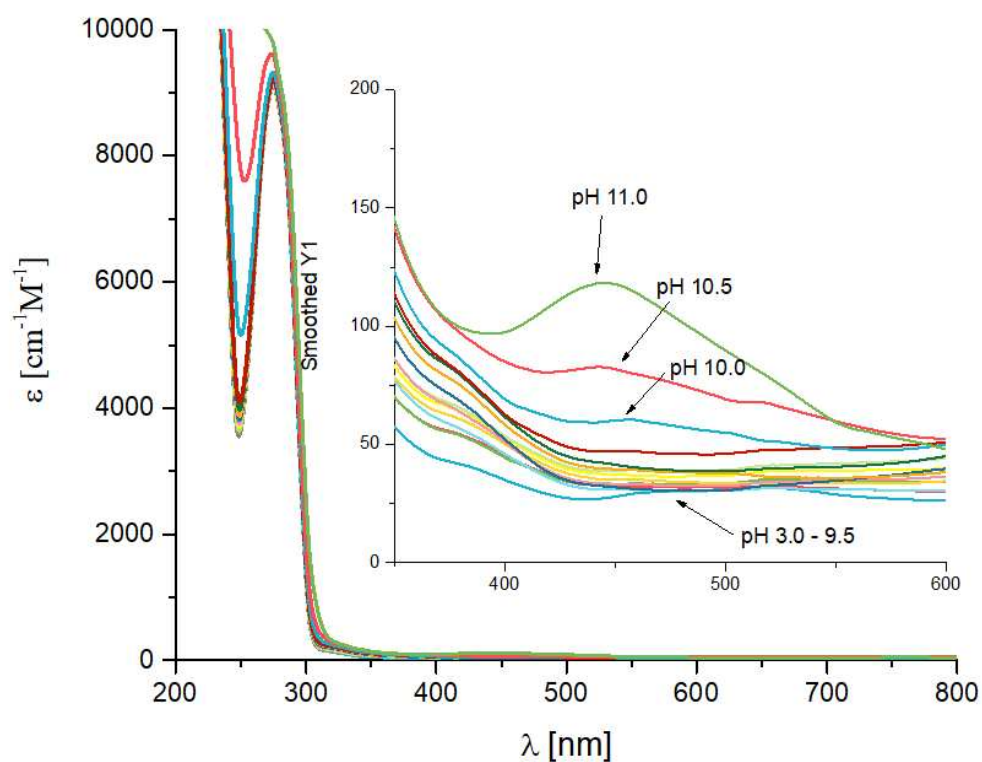


Figure 43 UV-Vis spectra of Ni(II) complex with the Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂ fragment from Aspf2 protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

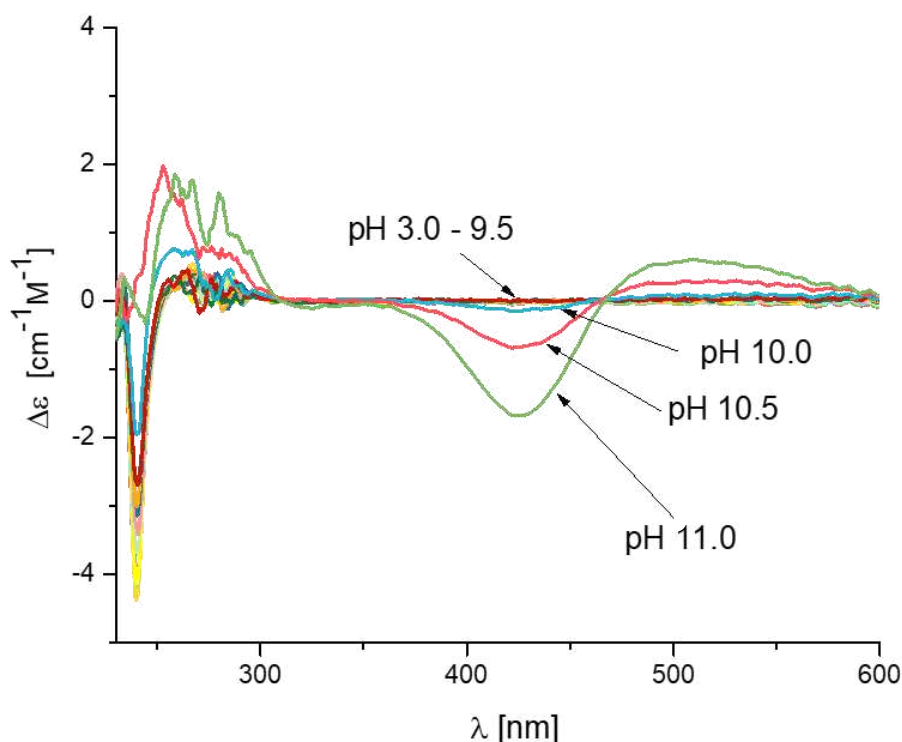


Figure 44 CD spectra of Ni(II) complex with the Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂ fragment from Aspf2 protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

d) Ac-PNCHTHEGGQLHCT

In the Ac-PNCHTHEGGQLHCT peptide, potentiometric measurements were able to detect seven protonation constants. The first pK_a value (3.08) comes from the deprotonation of the C-terminal carboxylic group, and the next one corresponds to the deprotonation of the carboxylic side chain of Glu (pK_a = 4.24). The next three constants (5.73, 6.54, 7.23) are related to the deprotonation of three His imidazole groups and the last two (9.00, 9.49) correspond to the deprotonation of two cysteine side chains (Table 6).

(1) Zn(II)-Ac-PNCHTHEGGQLHCT

Zinc coordination to Ac-PNCHTHEGGQLHCT starts already below pH 5 and the first complex that can be detected is ZnH₃L⁺. Most probably, in this form, one imidazole of histidine and one cysteine thiolate already participate in binding. In the next form, ZnH₂L, with a maximum at pH 6.5, the second imidazole nitrogen takes part in binding, judging by the complex pK_a value (5.35), which is lower than the one for the free ligand (6.54) (Figure 45). The loss of the next proton leads to the ZnHL⁻ form, with the maximum at pH 7. A significant decrease of the pK_a value calculated for the complexed (5.56) and the free imidazole (7.23) suggests that this histidine is also involved in Zn(II) binding. In the next form, ZnL²⁻, the lowered pK_a value for

the cysteine residue in the complexed ligand compared to the free one suggests that also this residue is bound, and the coordination mode at this point is $\{2S^-, 2N_{im}\}$ (Figure 46). This is further confirmed by UV-Vis spectroscopy at pH 7.4, where a pronounced band around 225 nm appears, which can be assigned to a $S^- \rightarrow Zn(II)$ charge transfer transition (Figure 47) [594]. These results are in good agreement with the NMR data (Figure 48), which show that, after the addition of an equimolar amount of Zn(II) to the ligand, both signals assigned to the $H_\alpha-H_\beta$ correlations of cysteines broaden to baseline, which strongly suggests their involvement in binding. After the addition of Zn(II), we observe shifts in the positions of signals from residues which do not directly participate in binding- the correlations of Thr $H_\beta-H_\gamma$, Leu $H_\alpha-H_\beta$ and $H_\alpha-H_\gamma$, Asp $H_\alpha-H_\beta$ and Pro $H_\alpha-H_\beta$ and $H_\delta-H_\beta$. The resonances of these residues are affected because they sense a different chemical environment after the metal binding to histidine residues, which are in their close proximity. The most basic form, $ZnH_{-1}L^{3-}$, is related to the deprotonation of a water molecule. The results are reported in Table 6.

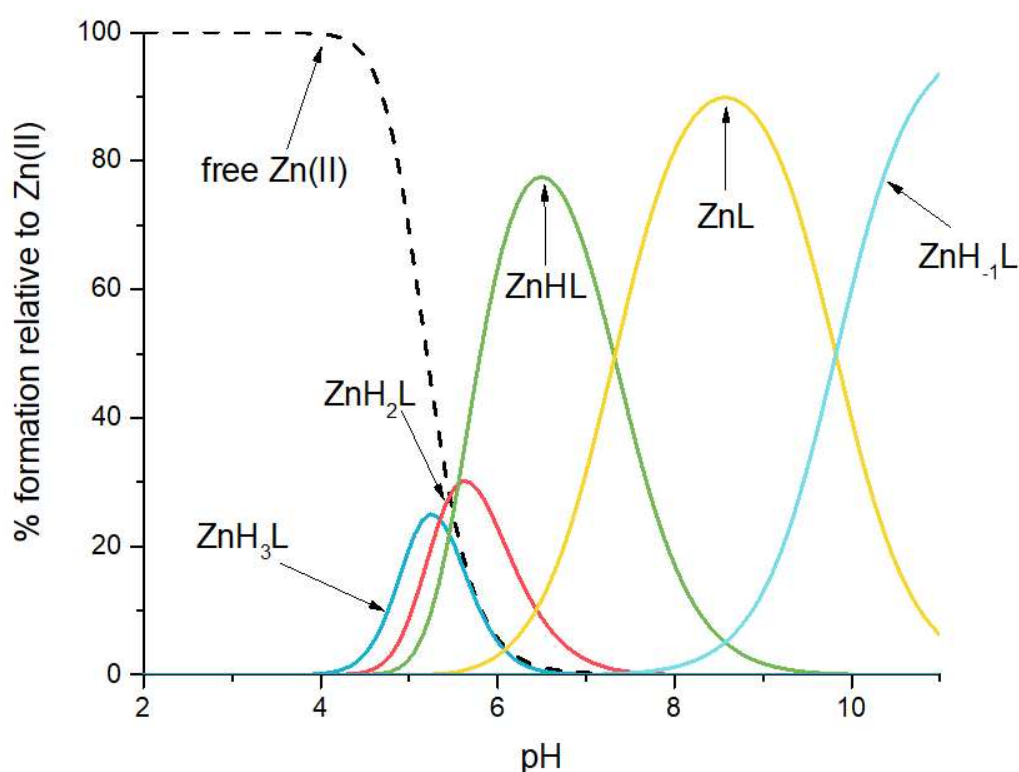


Figure 45 Species distribution diagram for the formation of Zn(II) complex with the Ac-PNCHTHEGGQLHCT Aspf2 fragment, $T = 298\text{ K}$, $I = 0.1\text{ M NaClO}_4$, $[L] = 0.0005\text{ M}$, $Zn(II)/L$ molar ratio = 1 : 1

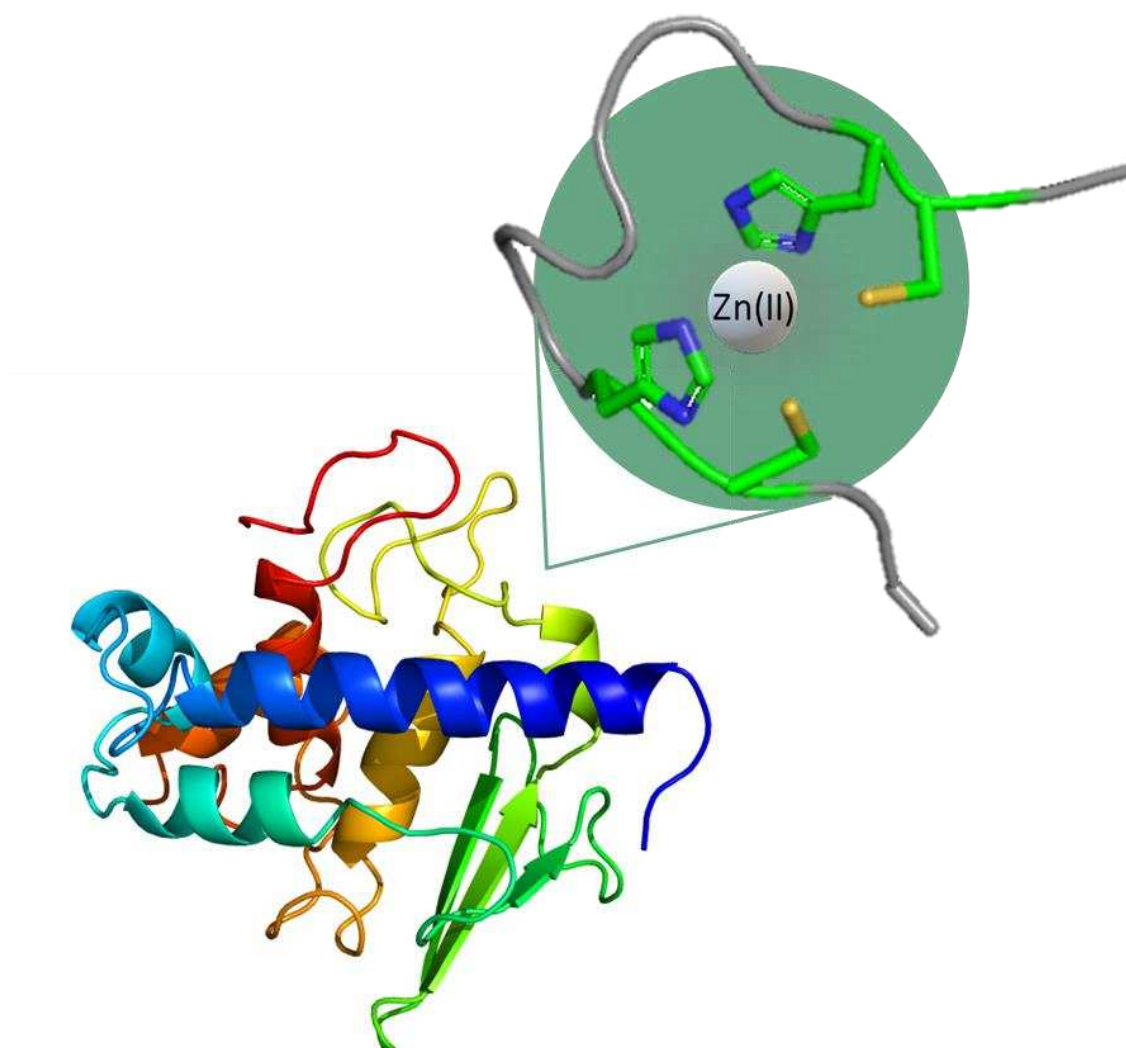


Figure 46 Proposed binding mode for Zn(II)-Ac-PNCHTHEGGQLHCT fragment of AspF2 protein at pH 7.4 (form - {2N_{im}, S⁻} binding mode). The AspF2 structure is based on a Phyre2 simulation [593] and the figure was generated by PyMOL [595]

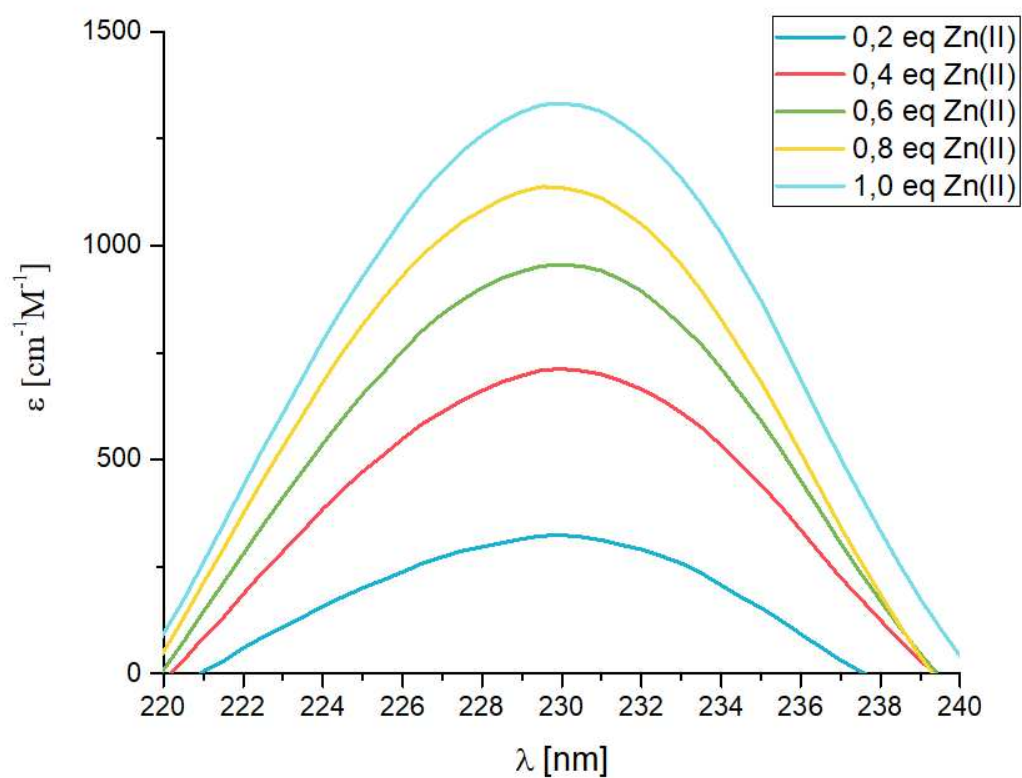


Figure 47 UV-Vis spectra of Zn(II) complexes of the Asp2 protein fragment (Ac-PNCHTHEGGQLHCT) at the pH 7.4, titrated with Zn(II) ions at a step of 0.2 molar equivalents. Final conditions: [Zn(II)] : [Ac-PNCHTHEGGQLHCT] = 1 : 1. Spectra were recorded at 298 K, in the range 220- 240 nm and with the optical path 0.1 cm

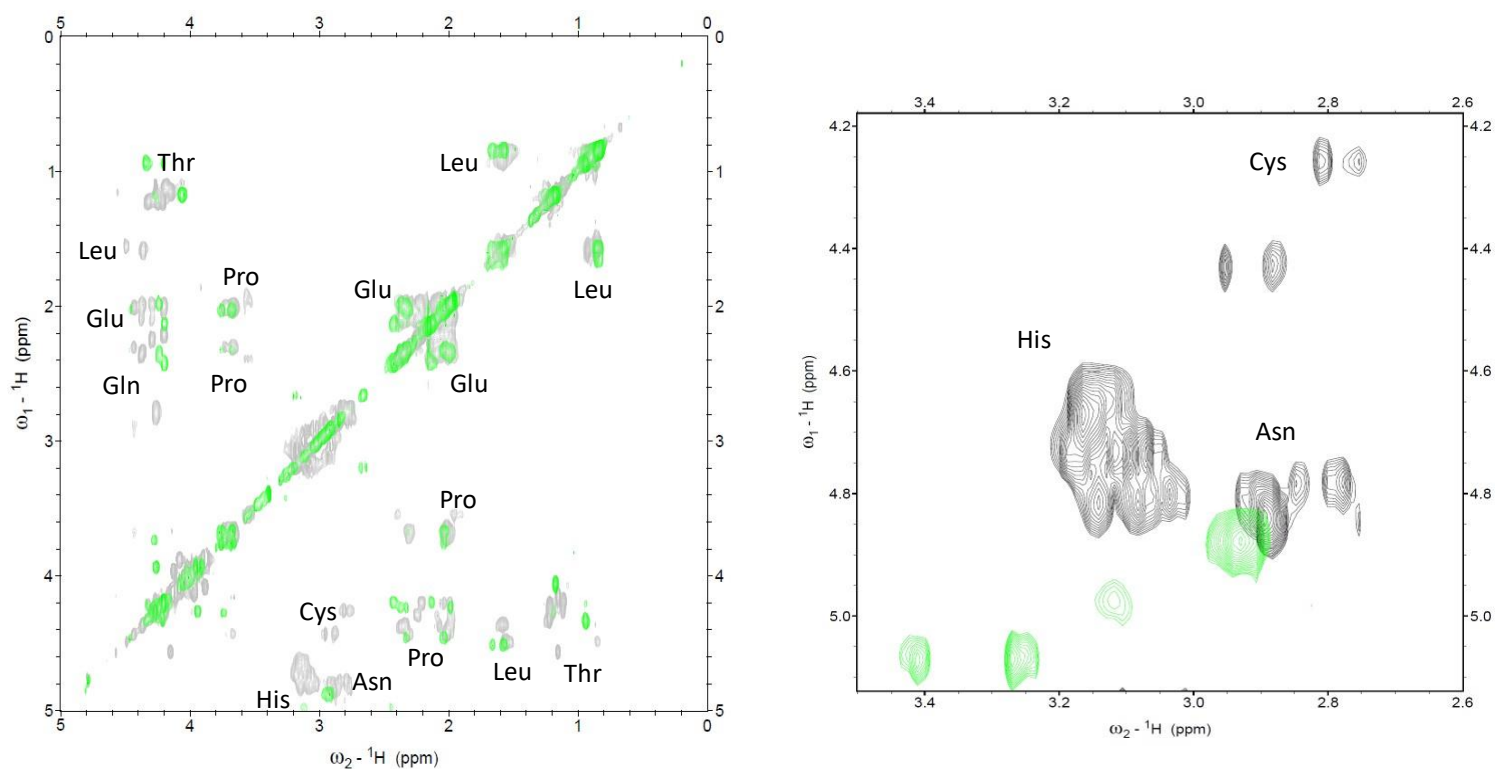


Figure 48 TOCSY spectra of 3 mM Ac-PNCHTHEGGQLHCT Asp2 fragment at pH 7.4, in the absence (gray) and presence of 1 Zn(II) equivalent (green)

Ac-PNCHTHEGGQLHCT starts to bind Ni(II) at pH around 4 and the first detected complex is $\text{NiH}_4\text{L}^{2+}$, in which Ni(II) is anchored to a histidine imidazole. Two further imidazoles are most probably bound in the NiH_2L form, with a maximum at around 6.5 (Figure 49). The next complex, NiHL , dominates in solution around pH 7. At this pH, a pronounced CD band at 356 nm appears (Figure 50) that is related to a $S \rightarrow \text{Ni(II)}$ transition, which, together with the lowered Cys pK_a value with respect to the free ligand ($\text{pK}_a = 7.13$ versus $\text{pK}_a = 9.00$) indicates thiolate binding to the central Ni(II) ion [596]. Loss of the next proton leads to the formation of NiL^- that dominates in the solution around pH 8 and is related to the deprotonation and coordination of the second cysteine residue, which might replace one of the imidazoles. The lowered pK_a value for the cysteine residue in the complexed ligand (7.43) in comparison to the free one (9.49) strongly suggests its participation in binding. NMR data recorded at pH 7.4 further prove this finding, showing a severe broadening of the $\text{H}_\alpha\text{-H}_\beta$ correlations of both Cys residues in the presence of Ni(II) (Figure 51). In general, square-planar Ni(II)-peptide complexes are yellow and have no free electron on the nickel ion (nickel is diamagnetic), whereas octahedral complexes are blue and paramagnetic. At pH 7.4, we were expecting a mixture of paramagnetic and diamagnetic species in equilibrium (NiH_2L , NiHL^- , NiL^{2-}) and hence only a 0.1 equivalent of Ni(II) ions was used. As in the case of Zn(II) ions, broadening may be a result not of a paramagnetic effect, but of the dynamics and fast exchange of the system. The loss of the next three protons leads to the formation of the $\text{NiH}_{-1}\text{L}^{3-}$, $\text{NiH}_{-2}\text{L}^{4-}$ and $\text{NiH}_{-3}\text{L}^{5-}$ forms; all three deprotonations correspond to the binding of three subsequent amide nitrogens (Table 6). UV-Vis (Figure 52) and CD spectra confirm this finding with pronounced bands with a negative Cotton effect at 445 nm and a positive Cotton effect at 525 nm, which are typical of amide involving square-planar nickel-peptide complexes.

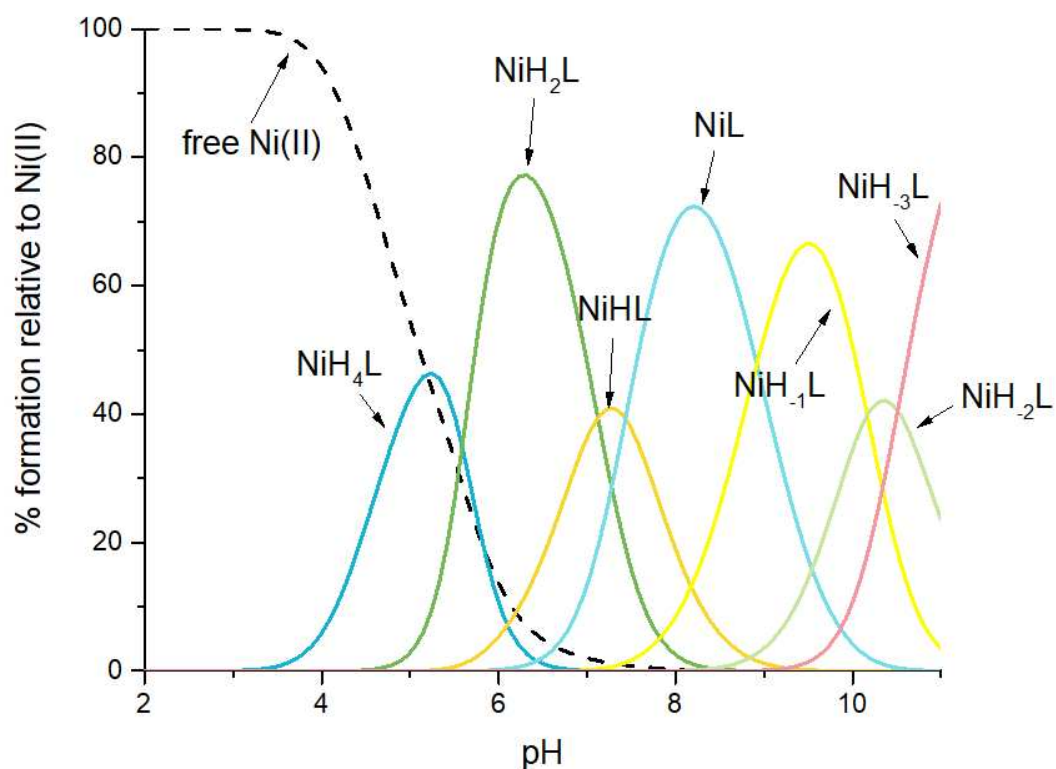


Figure 49 Species distribution diagram for the formation of Ni(II) complex with the Ac-PNCHTHEGGQLHCT Asp2 fragment, $T = 298\text{ K}$, $I = 0.1\text{ M NaClO}_4$, $[L] = 0.0005\text{ M}$, $\text{Ni(II)/L molar ratio} = 1 : 1$

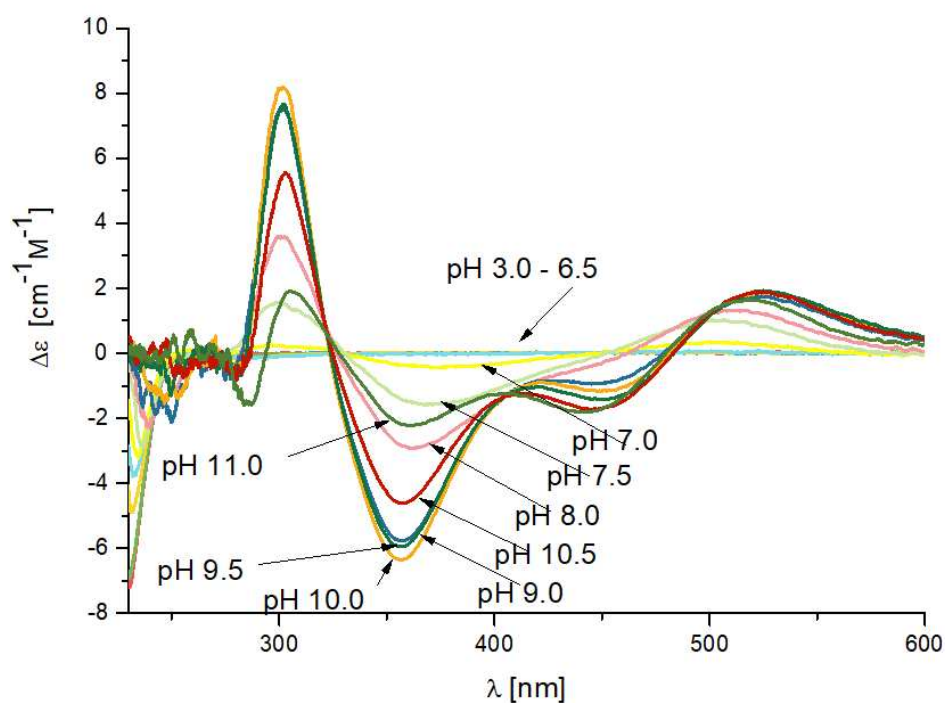


Figure 50 CD spectra of Ni(II) complex with the Ac-PNCHTHEGGQLHCT fragment from Asp2 protein in the range $240 - 800\text{ nm}$ an pH range $2.5 - 10.5$, $T = 298\text{ K}$, optical path = 1 cm , $[L] = 0.0005\text{ M}$, $\text{Ni(II)/L molar ratio} = 1 : 1$

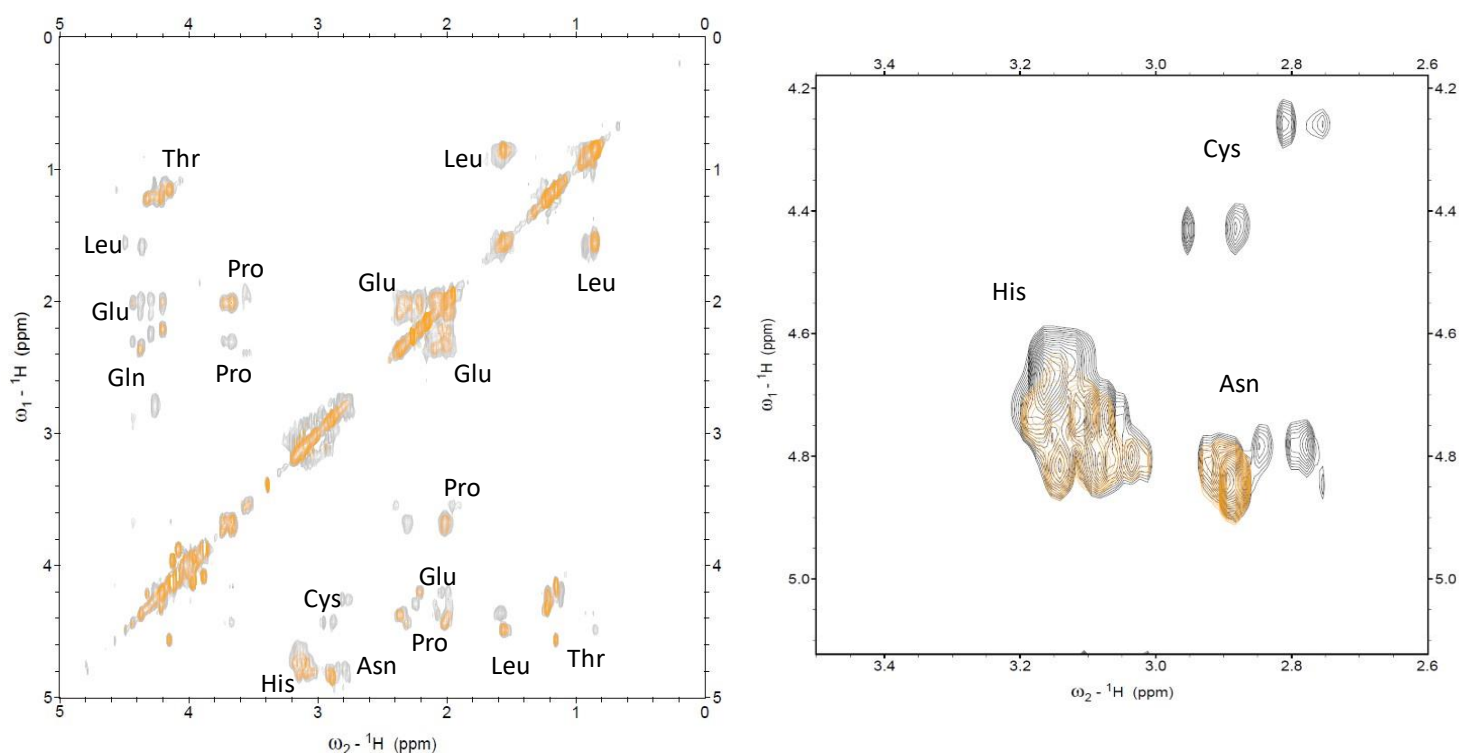


Figure 51 TOCSY spectra of 3 mM Ac-PNCHTHEGGQLHCT Asp2 fragment at pH 7.4, in the absence (gray) and presence of 0.1 Ni(II) equivalent (orange)

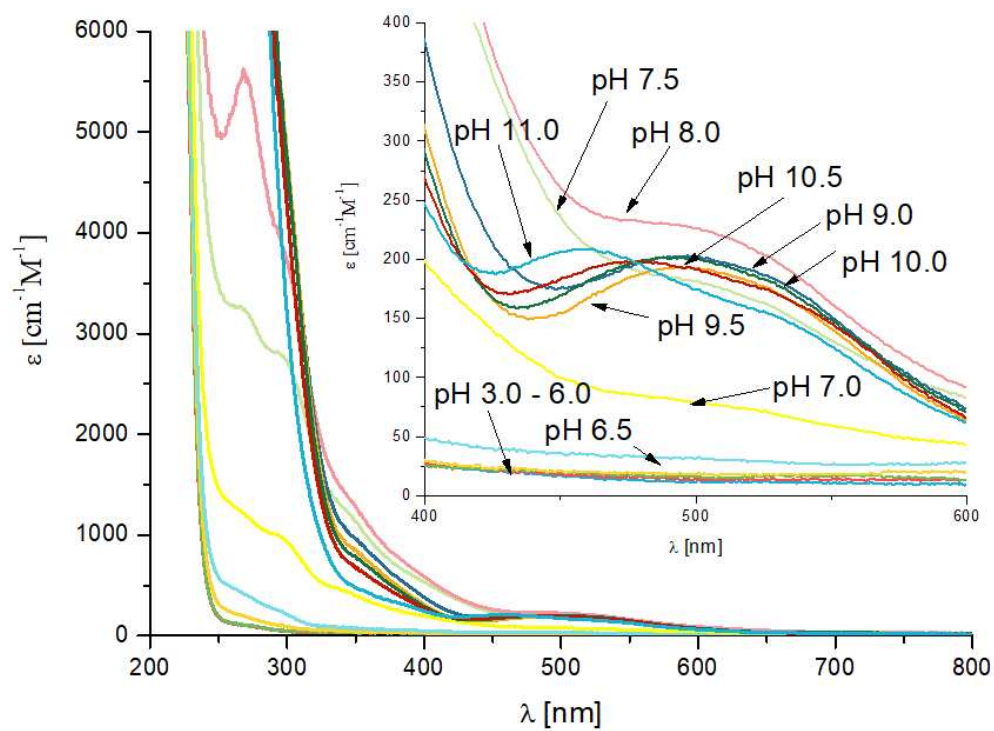


Figure 52 UV-Vis spectra of Ni(II) complex with the Ac-PNCHTHEGGQLHCT fragment from Asp2 protein in the

range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

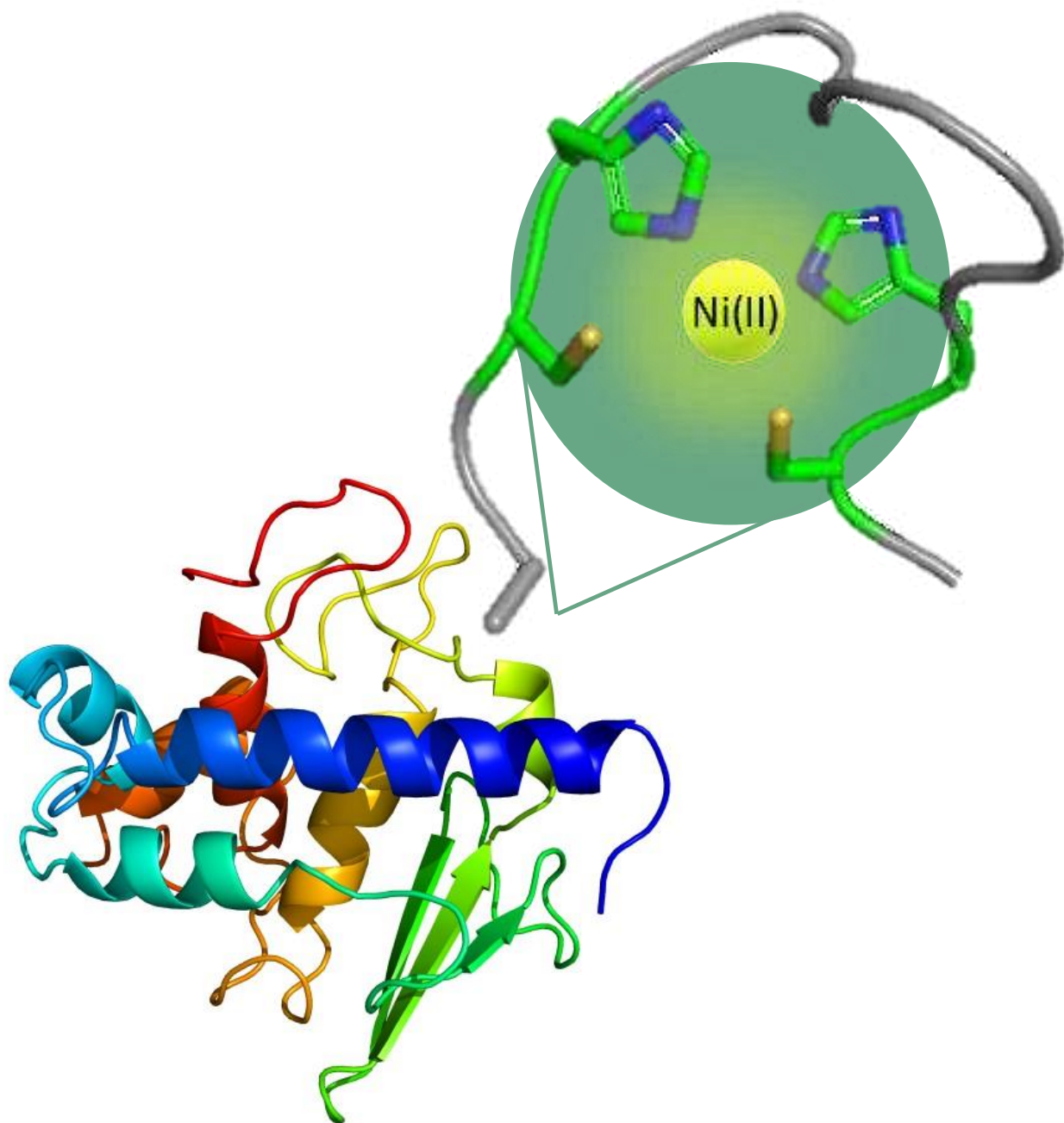
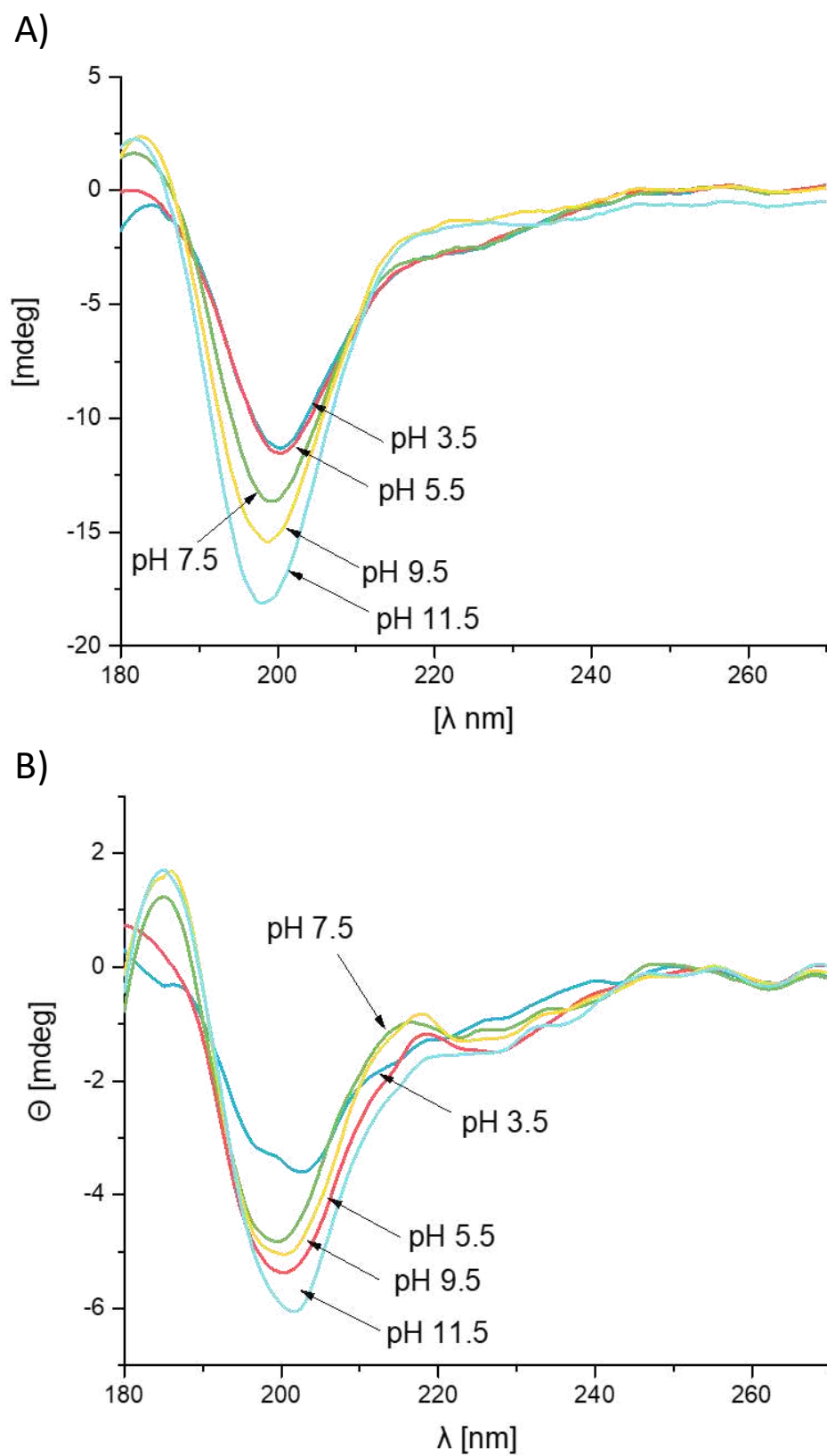


Figure 53 Proposed binding mode for Ni(II)-Ac-PNCHTHEGGQLHCT fragment of Asp2 protein at pH 7.4 (form - {2N_{im}, S⁻} binding mode). The Asp2 structure is based on a Phyre2 simulation [593] and the figure was generated by PyMOL [595]

3. Secondary structure analysis

I evaluated the secondary structures for the most probable binding site in the Asp2 zincophore (Ac-PNCHTHEGGQLHCT). The presence of Zn(II) does not influence the secondary

structure of the tested ligand, but at pH 7.5, Ni(II) induces the formation of a partial poly proline II (PPII) like structure (Figure 54)



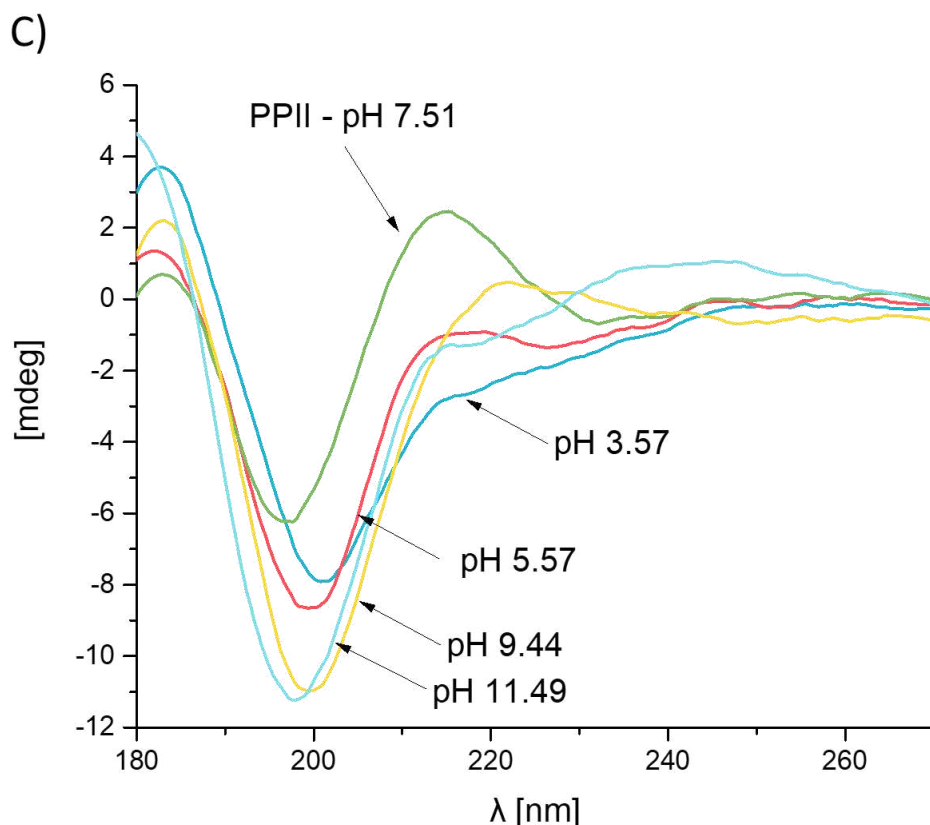


Figure 54 CD spectra of A) Ac-PNCHTHEGGQLHCT and its complexes with B) Zn(II) and C) Ni(II), PPII- polyproline II helix; optical path 0.1 mm, Zn(II) and Ni(II)/L molar ratio=1:1.

4. Comparison of the stability of Zn(II) and Ni(II) complexes

In order to establish which AspF2 zincophore region binds Zn(II) with the highest affinity, basing on the potentiometric data, we simulated a hypothetical situation, in which equimolar amounts of Zn(II) and all studied AspF2 regions are mixed—this approach allows a direct comparison of the calculated constants at different pH values (Figure 55). Above pH 6, the Ac-PNCHTHEGGQLHCT C-terminal AspF2 fragment becomes the primary zinc-binding site. At around physiological pH (pH 7.4), more than 95% of metal is coordinated to this sequence, being bound to two histidine imidazoles and two cysteine thiolates. The participation in the binding of Zn(II) by the remaining analysed sequences (Ac-ARHAKAH-NH₂, Ac-MHRLYHVP-NH₂ and Ac-MHRLYHVPVAGQGWDHFAD-NH₂) is quite small. The differences in the binding affinity of Zn(II) are due to differences in the coordination modes of these ions.

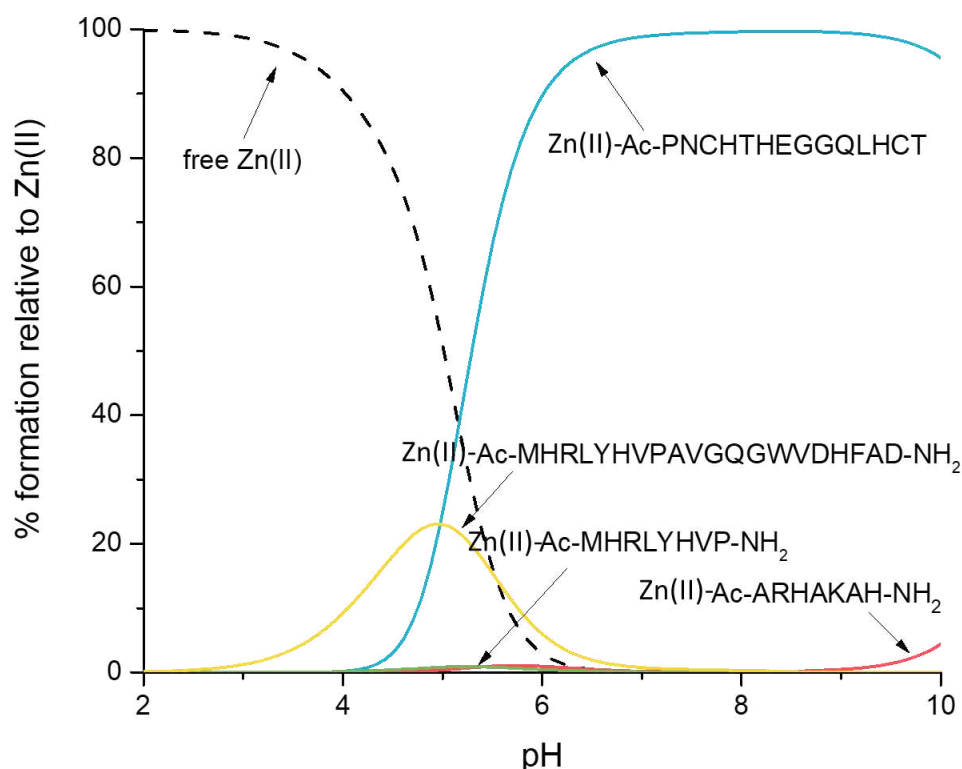


Figure 55 A competition plot between Asp2 fragments: Ac-ARHAKAH-NH₂, Ac-MHRLYHVP-NH₂, Ac-MHRLYHVPAGQGWDHFAD-NH₂, Ac-PNCHTHEGGQLHCT, and Zn(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 6. Conditions: 298 K, I = 0.1 M NaClO₄ or I = 0.04 M SDS, [Zn(II)] = [Ac-ARHAKAH-NH₂] = [Ac-MHRLYHVP-NH₂] = [Ac-MHRLYHVPAGQGWDHFAD-NH₂] = [Ac-PNCHTHEGGQLHCT] = 0.001 M.

At acidic pH, the Asp residue present in the Ac-MHRLYHVPAGQGWDHFAD-NH₂ peptide becomes the anchoring site for Zn(II) (its pK_a is lower than the pK_a of Glu in the Ac-PNCHTHEGGQLHCT peptide). In our hypothetical simulation, at pH 5, about 23% of the available Zn(II) would be bound to Ac-MHRLYHVPAGQGWDHFAD-NH₂, but at pH 7.4, less than 1% of this metal would choose to bind this sequence (at physiological pH, 3 His imidazoles and one carboxylic side chain are the Zn(II)-binding sites). The highest affinity of Zn(II) to the Ac-PNCHTHEGGQLHCT peptide fragment is due to the coordination mode similar to that of zinc fingers, where two His imidazoles and two Cys thiolates participate in the metal binding.

The thermodynamically most stable Zn(II)-binding site in Pra1, a zincophore from *Candida albicans*, is also situated in its evolutionally conserved C-terminal region; however, in this case, the Zn(II) coordination environment consists of four His imidazoles [426]. This candidal zincophore is able to pass the metal over to the Zrt1 zinc transporter [428], and most likely, the C-terminal part of Pra1 can be selectively recognized by Zrt1, which gives hope that the Pra1 C-terminus could be used as a specific targeting molecule to enable precise drug delivery when linked to standard therapeutics or to antimicrobial peptides [597].

In comparison with the Zn(II)-binding Pra1 zincophore region from *Candida albicans*, the *Aspergillus fumigatus* C-terminal Asp2 region has a much higher affinity for Zn(II); at pH 6, 60%,

and at pH 7.4- more than 80% of this metal would be bound to this sequence in a hypothetical situation, in which equimolar amounts of Zn(II) and both C-termini would be present (Figure 56). The comparison of these stabilities also shows that the {2N_{im}, 2S⁻} binding mode present in the Ac-PNCHTHEGGQLHCT fragment from *Aspergillus fumigatus* is more stable than the {4N_{im}} one detected in the Ac-SHCHTHADGEVHC zincophore region from *Candida albicans*.

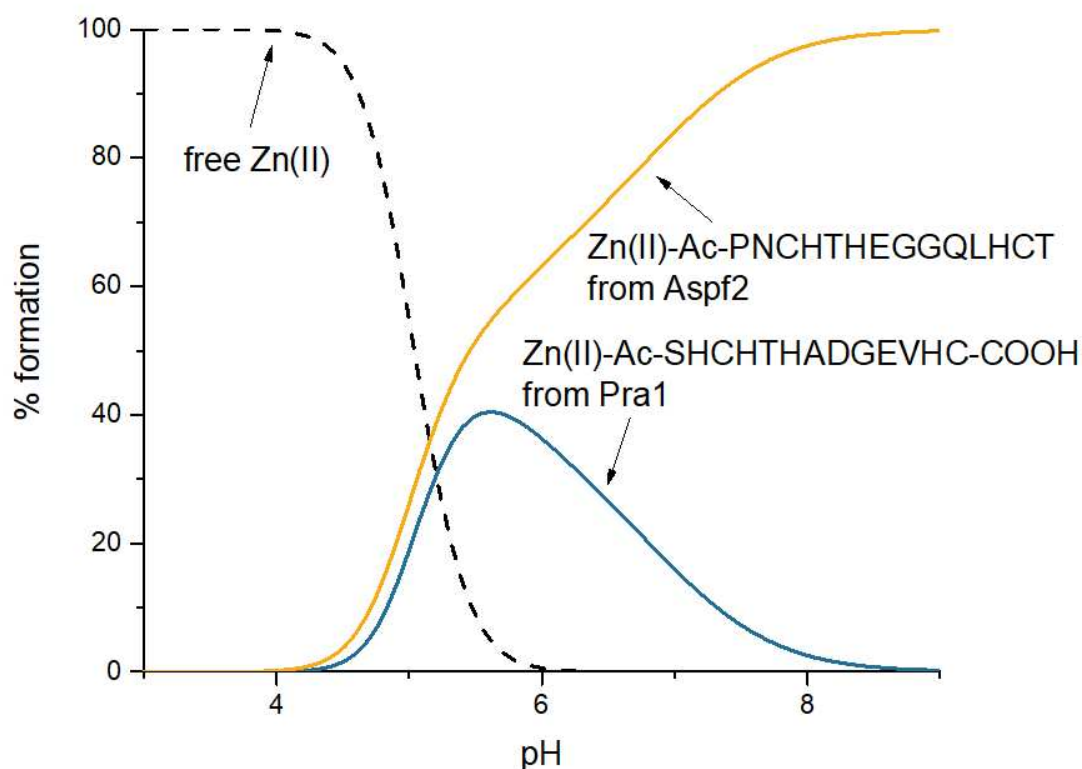


Figure 56 A competition plot between Aspf2 fragment: Ac-PNCHTHEGGQLHCT, Pra1 fragment: Ac-SHCHTHADGEVHC and Zn(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 6 and [426]. Conditions: 298 K, I = 0.1 M NaClO₄, [Zn(II)] = [Ac-PNCHTHEGGQLHCT] = [Ac-SHCHTHADGEVHC] = 0.001 M.

The Aspf2 region, which binds Ni(II) with the highest affinity, is, as in the case of Zn(II), the C-terminal part of the zincophore, Ac-PNCHTHEGGQLHCT. At pH 7.4, the coordination of the nickel ions by Ac-PNCHTHEGGQLHCT involves two histidine imidazoles and two cysteine thiolates. At basic pH, the nickel coordination mode to Ac-PNCHTHEGGQLHCT changes, most likely to a {3N⁻, S⁻} one, with the C-terminal part of this zincophore remaining the strongest Ni(II)-binding site. The strength of nickel binding may also be influenced by the secondary structure of the peptide. Interestingly, at pH 7.4, Ni(II) binding to Ac-PNCHTHEGGQLHCT induces the formation of a partial poly proline II (PPII) like structure (Figure 54CX), while the structures of this complex at other pH values and the structures of its Zn(II) complex are not well defined (Figure 54B), although at pH 7.4, both Zn(II) and Ni(II) occupy the same binding site. NMR results also suggest differences in the folding of the Zn(II) and Ni(II) peptide complexes- we observe differences in the position of signals for the residues remote from the binding site, such as on

glutamine H_α-NH correlations, which may further suggest differences in the folding of the peptide complexes.

The remaining three Asp2 fragments analyzed in this work do not show high affinity for Ni(II). At acidic pH, the Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂ fragment with one aspartic acid residue, binds Ni(II) with the highest affinity, most likely due to the presence of the Asp residue. The shorter Asp2 regions (Ac-ARHAKAH-NH₂ and Ac-MHRLYHVP-NH₂) have an even lower affinity toward Ni(II) ions (Figure 57).

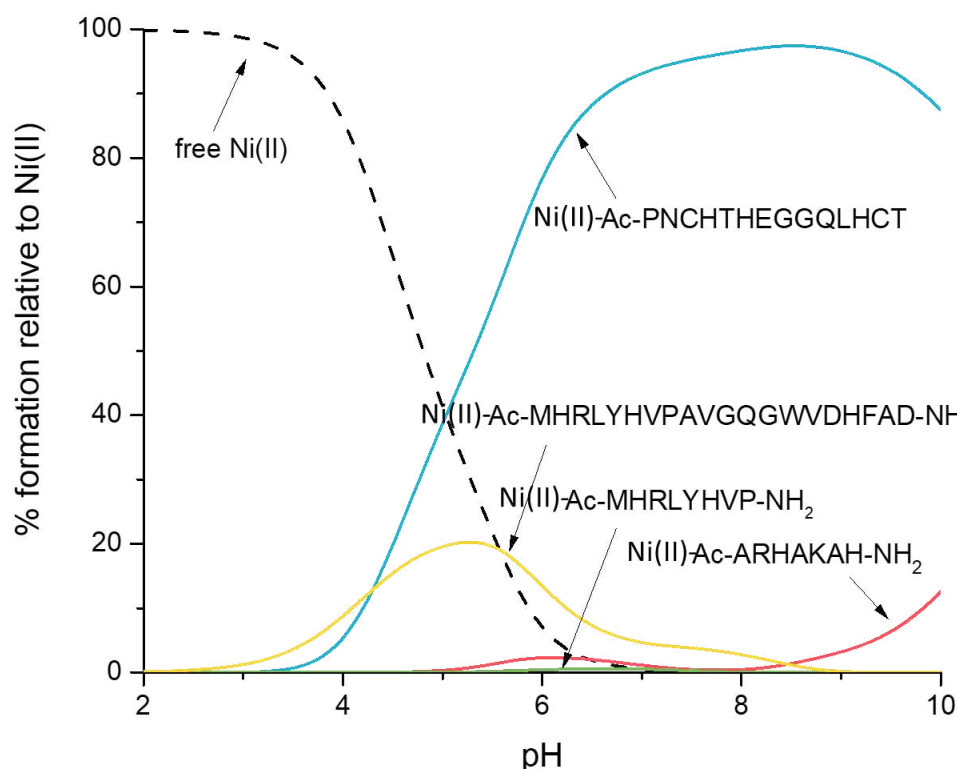


Figure 57 A competition plot between Asp2 fragments: Ac-ARHAKAH-NH₂, Ac-MHRLYHVP-NH₂, Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂, Ac-PNCHTHEGGQLHCT, and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 6. Conditions: 298 K, I = 0.1 M NaClO₄ or I = 0.04 M SDS, [Ni(II)] = [Ac-ARHAKAH-NH₂] = [Ac-MHRLYHVP-NH₂] = [Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂] = [Ac-PNCHTHEGGQLHCT] = 0.001 M.

The Asp2 zincophore strongly prefers to bind Zn(II) over Ni(II); even in a theoretical situation in which equimolar amounts of both metals would be available, at pH 7.4, about 85% of the C-terminal part of the zincophore would bind Zn(II) (Figure 58). From the biological point of view, this suggests a Zn(II) specificity of Asp2; from the point of view of coordination chemistry, it shows that the {2N_{im}, 2S⁻} binding mode, typical for zinc fingers, is indeed more tempting for Zn(II) than for Ni(II) ions [598]. To additionally prove which metal ion is more strongly bound to the C-terminal fragment of the Asp2 protein, we performed a CD-spectroscopic titration of the Ni(II)-Ac-PNCHTHEGGQLHCT species with Zn(II) at pH 7.4 with a step of 0.1 molar equivalents

and observed a stepwise displacement of Ni(II) with Zn(II) ions [naturally, for Zn(II), a d^{10} metal, no d-d bands are visible] (Figure 59), which further confirms that Zn(II) is able to displace Ni(II) from its binding site.

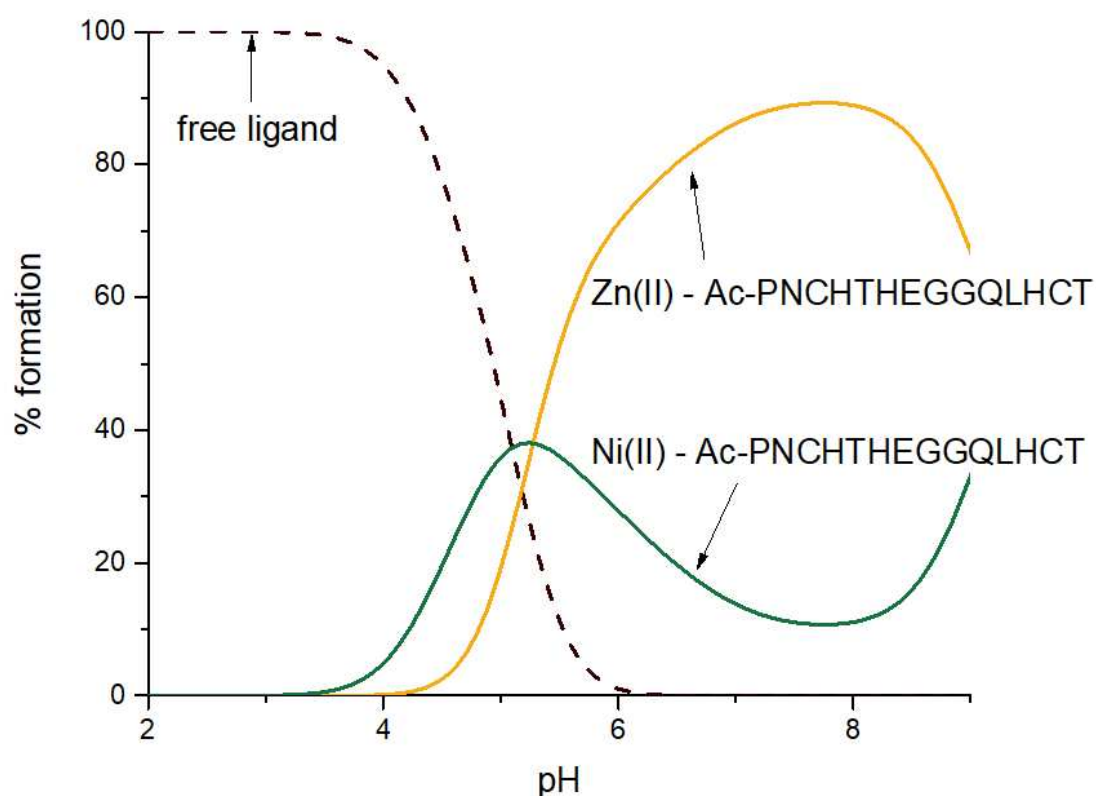


Figure 58 A competition plot between Aspf2 fragment Ac-PNCHTHEGGQLHCT, Zn(II) and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of the three reagents are mixed. Calculations are based on binding constants from Table 6

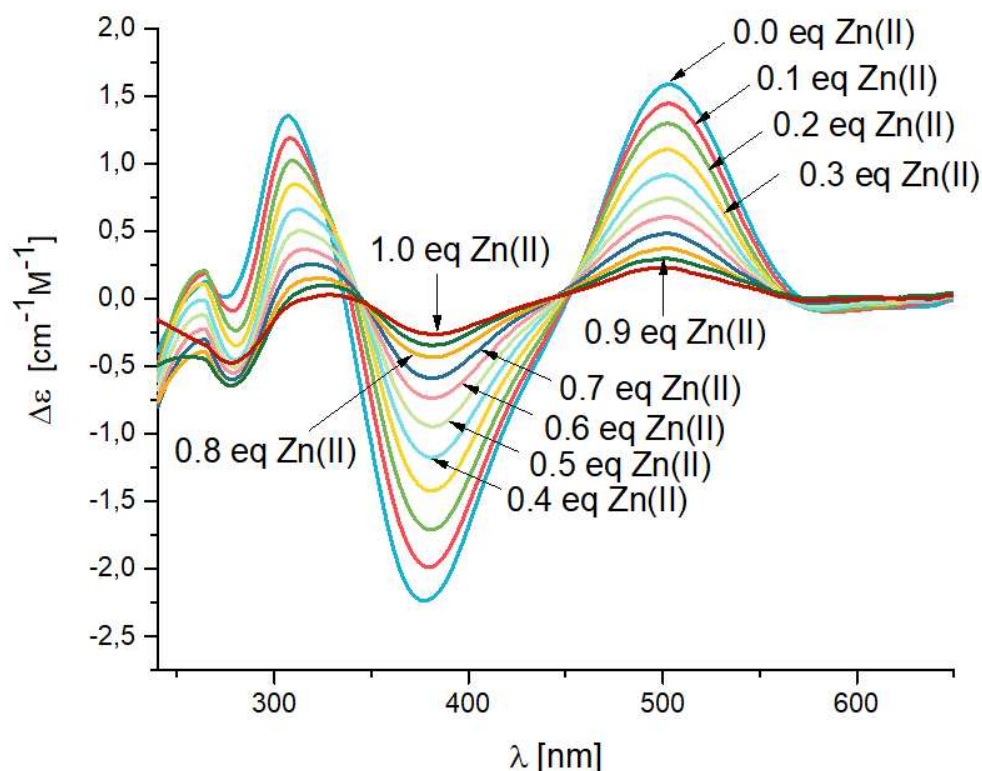


Figure 59 CD spectra of Ni(II) complexes of the Aspf2 protein fragment (Ac-PNCHTHEGGQLHCT) at the pH 7.4, titrated with Zn(II) ions at a step of 0.1 molar equivalents. Final conditions: [Zn(II)] : [Ni(II)] : [Ac-PNCHTHEGGQLHCT] = 1 : 1 : 1. Spectra were recorded at 298 K, in the range 240 - 650 nm and with the optical path 1 cm

What about the other potential metal-binding sites? In a theoretical situation in which equimolar amounts of Zn(II) and Ni(II) would be available for Ac-ARHAKAH-NH₂, Ac-MHRLYHVP-NH₂ and Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂ peptide fragments at pH 7.4, all sequences would bind Ni(II) with higher affinity than Zn(II) (Figure 60) (however with much lower affinities than the C-terminal site). From the chemical point of view, it shows that a binding mode involving histidine residues is more tempting for Ni(II) than for Zn(II) ions. The general lower affinity for both metal ions in these sequences in comparison to the C-terminal one, Ac-PNCHTHEGGQLHCT, is due to the absence of cysteine residues in the analyzed fragments, because the cysteine residues play an important role as anchoring groups for Zn(II) and Ni(II), with which they form very stable complexes with binding modes similar to that of zinc fingers. The obtained results are in agreement with what could have maybe been expected from the Irving-Williams series - experiments carried out with small chelating agents show that the HS-CH₂-CH₂-NH₂ ligand binds Zn(II) a little stronger than Ni(II), while the H₂N-CH₂-CH₂-NH₂ one has a stronger affinity toward Ni(II) [599]. Taken together, the data show that the mixture of thiolates and amines show good selectivity for Zn(II) ions.

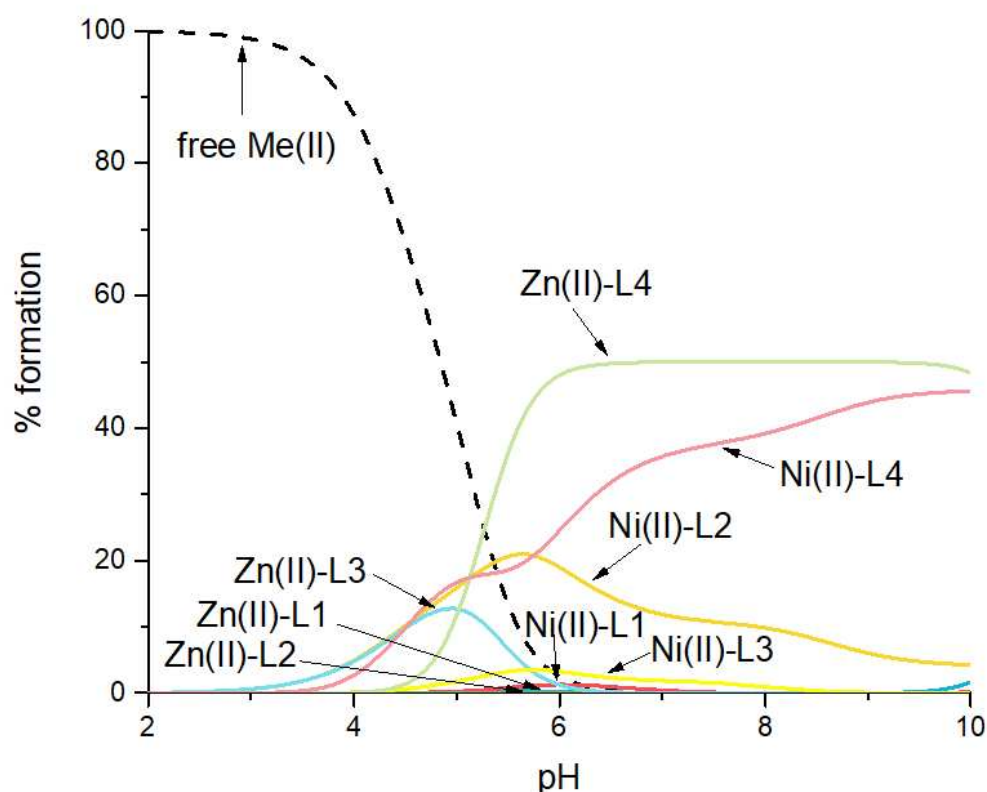


Figure 60 Competition plot between AspF2 fragments; where: L1- Ac-ARHAKAH-NH₂; L2- Ac-MHRLYHVP-NH₂; L3- Ac-MHRLYHVPVAGQGWDHFAD-NH₂; L4- Ac-PNCHTHEGGQLHCT; Zn(II) and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 6

The low affinity of the Ac-ARHAKAH-NH₂, Ac-MHRLYHVP-NH₂ and Ac-MHRLYHVPVAGQGWDHFAD-NH₂ sequences for Zn(II) in the whole pH range indicates that there is a low probability that AspF2 bind the two metal ions in different sites. Additionally, competition plots comparing the binding strength of Zn(II) and Ni(II) for the remaining sequences indicate that at physiological pH, Zn(II) are bound with a very low affinity compared to Ni(II). However, after binding the metal ion in the primary binding site, the conformation of the native protein may slightly change, and the availability of the other binding sites may differ from the affinity obtained for model sequences.

5. Conclusions

Zn(II) is crucial for the virulence and survival of *Aspergillus fumigatus* in the host organism. I characterized four potential regions of AspF2 zincophore from *Aspergillus fumigatus*, using mass spectrometry, potentiometry, UV-Vis, CD and NMR spectroscopy. The most probable Zn(II)-binding site on the AspF2 zincophore is its C-terminal region, Ac-PNCHTHEGGQLHCT. Moreover, at pH 7.4, the same C-terminal part of the zincophore binds Ni(II) via an analogous coordination mode {2N_{im}, 2S⁻}, but with a smaller affinity than Zn(II). The

PNCHTHEGGQLHCT region of the zincophore may probably serve as a targeting molecule that is able to (analogously to the *Candida albicans* Pra1 zincophore) bring an appropriately bound therapeutic to the proximity of *Aspergillus fumigatus*, specifically passing over the Zn(II) ion to the ZrfC transporter.

B. Thermodynamic and structural properties of Zn(II) and Ni(II) complexes with unstructured fragments of ZrfC transporter

A saprophytic and an opportunistic fungal pathogen, *Aspergillus fumigatus*, and the mechanism of its Zn(II) transport are presented in chapter B.1. of the Introduction (Zn(II) transport in *Aspergillus fumigatus*) and in the article [494]. To understand the transport of Zn(II) in *Aspergillus fumigatus*, I focus on Zn(II) and Ni(II) complexes of two ZrfC transporter regions (Ac-TGCHSHGS-NH₂ and Ac-MNCHFHAGVEHCIGAGESESGSSQ-NH₂). The amino acid sequences of the ZrfC transporter are presented in Figure 61. The probable Zn(II)-binding sites are highlighted in color. I explain their structural and thermodynamic properties, using a variety of complementary methods: mass spectrometry, potentiometry, UV-Vis spectroscopy and CD spectroscopy. As in the case of the zincophore fragments, also ZrfC regions are chosen on the basis of three criteria:

- (i) having probable Zn(II)-binding residues,
- (ii) being unstructured,
- (iii) being evolutionally conserved sites,

and therefore, they are appropriate for our peptide-based approach. The structure was predicted by Phyre2, a remote homology recognition technique, capable of generating reliable protein models [593]. I also check the thermodynamic parameters of Ni(II) binding sites, keeping in mind that Ni(II) is a biologically relevant metal ion, essential for pathogens, and could theoretically compete with Zn(II) for the binding sites in its transporter. It is important to keep in mind that in the full-length protein, the metal binding sites might also occur in locally structured regions.

10	20	30	40	50
MAKALLLSL	LGGLGLAHAQ	YVTYTGCHNH	GSTEYCFGPD	GKETPFPTPT
60	70	80	90	100
ESASRTVPVT	VAAAAAATTT	SSADASAVTG	CHSHGSDVFC	IDGDGNEVQV
110	120	130	140	150
ILPSTPTGEL	PAQYTGCHSH	GSETFCMDPE	GNDVQIVGEE	GSTEGSSSSG
160	170	180	190	200
SSGESQSGSK	EGMNCHFHAQ	VEHCIGAGES	ESGSSQKSCG	LRTRDYDVPL
210	220	230	240	250
RIGTLFVVLV	TSSIGVFLPM	LLVKLPsAKI	NGVVSTVIKQ	FGTGVLSTA
260	270	280	290	300
FVHLYTHANL	MFTNECLGEL	EYEATTSAVV	MAGIFLSFLF	EYIGHRIILA
310	320	330	340	350
RATRCASPCP	EQTGDMSPSS	TSKELPASQP	PPPPPQQQQQ	QPPTLAALGH
360	370	380	390	400
HHGPPLDPTN	PNTKLSVLVM	EAGVVFHSIL	IGLTLVVAGD	SFYKTLLVVI
410	420	430	440	450
VFHQFFEGLA	LGARIAMLPG	PLLGSKALMA	GTFAVITPIG	MAIGLGVLSH
460	470	480	490	500
FNGNDQSTLV	ALGTLDALSA	GILVWVGLVD	MWARDWVMDG	GEMMNARLSI
510	520			
VAVGGFSLIA	GMVLMGVLGK	WA		

Figure 61 Amino acid sequence of ZrfC transporter; probable Zn(II) binding sites are highlighted in different colors

1. Stoichiometry of Zn(II) and Ni(II) complexes with ZrfC fragments

To confirm the stoichiometry of the formed complexes, I performed MS measurements. ESI-MS proved that the tested peptides were extra pure products and that they form mononuclear complexes with Zn(II) and Ni(II). The assignment peaks were compared to simulated isotopic patterns, which fit perfectly with the experimental data.

a) Ac-TGCHSHGS-NH₂

The signal $m/z = 826.34$, $z = 1+$ correspond to the free ligand for Ac-TGCHSHGS-NH₂. The signals: $m/z = 848.32$, $z = 1+$ correspond to the Ac-TGCHSHGS-NH₂ adduct with sodium atom. In the mass spectra of Zn(II)-Ac-TGCHSHGS-NH₂ beside the signals from the ligand and its sodium adduct, we observe a signals which comes from the zinc complex ($m/z = 885.25$, $z = 1+$) and its adducts with potassium atom ($m/z = 926.30$, $z = 1+$) (Figure 62A). In the case of Ni(II)-Ac-TGCHSHGS-NH₂ (Figure 62B) the signal ($m/z = 882.24$, $z = 1+$) correspond to the equimolar Ni(II) complex.

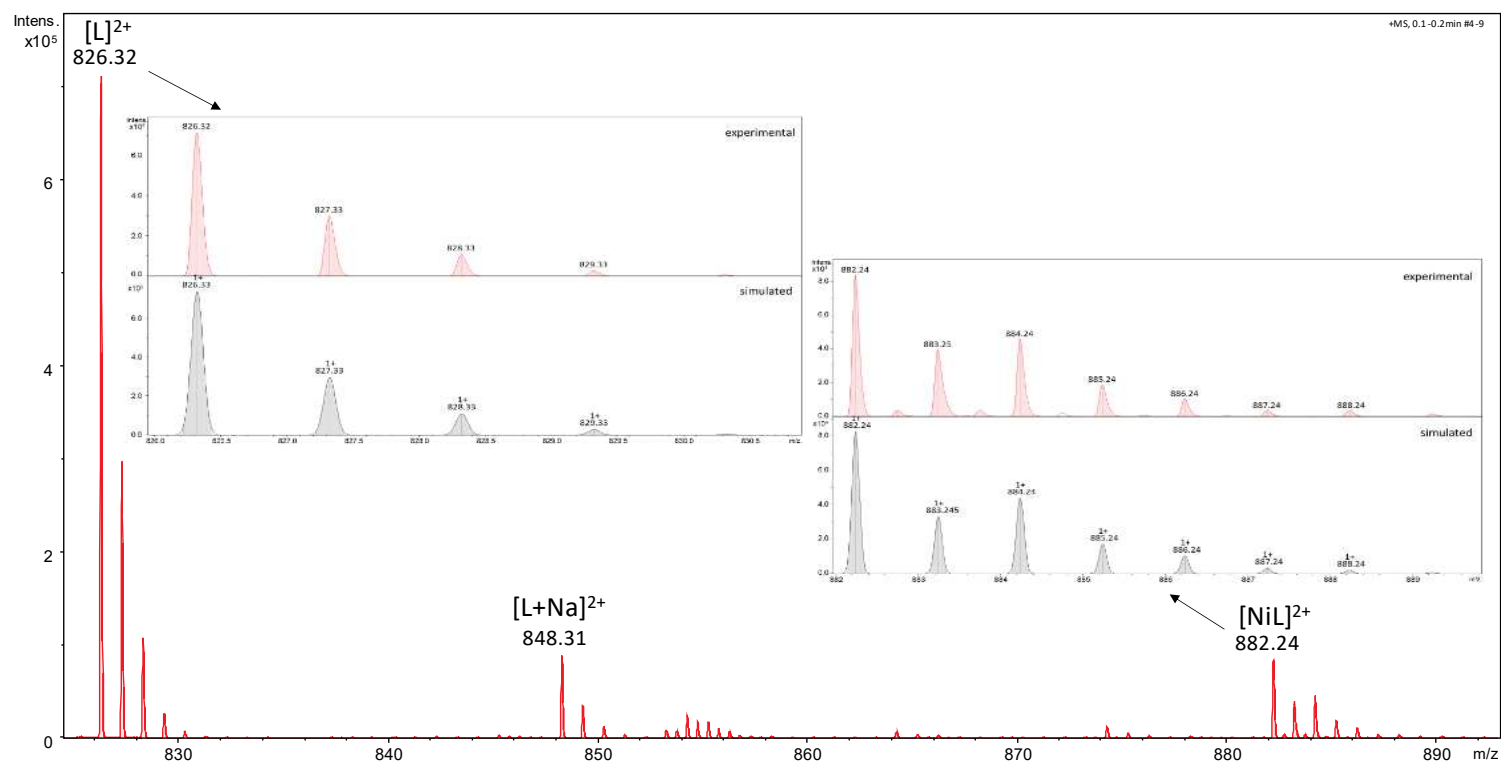
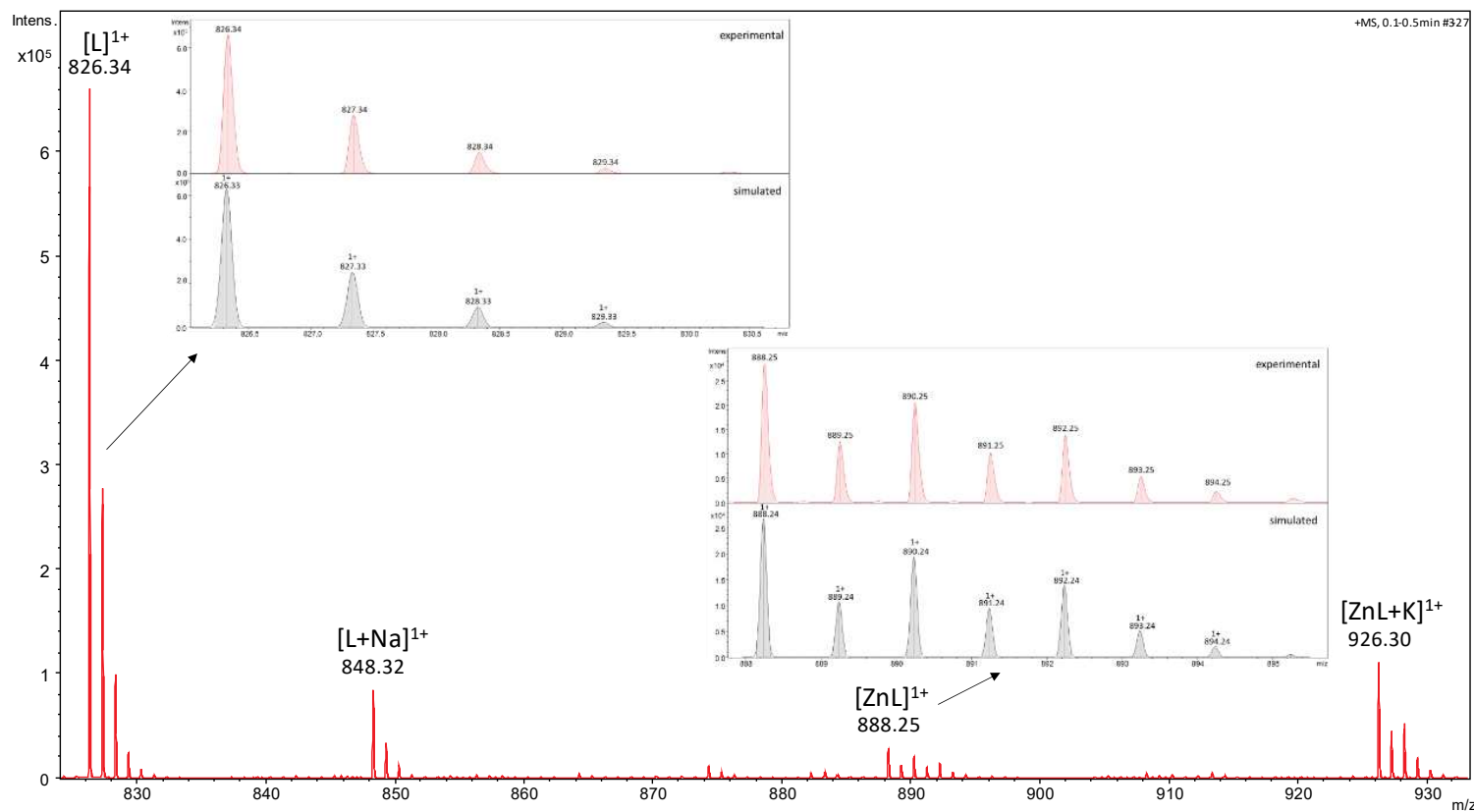


Figure 62 ESI-MS spectrum of A) Zn(II)-Ac-TGCHSHGS-NH₂ and B) Ni(II)-Ac-TGCHSHGS-NH₂

b) Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂

The signal $m/z = 1258.52$, $z = 2+$ correspond to the free ligand for Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂. In the case of Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ mass spectra the signals: $m/z = 1269.52$, $z = 2+$ correspond to ligand adduct with sodium atom and the signals: $m/z = 1277.51$, $z = 2+$ correspond to ligand adduct with potassium atom. In the Zn(II)-Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ spectra, aside from the signals which comes from the free ligand and its adducts, a equimolar Zn(II) complex ($m/z = 1289.98$, $z = 2+$), its sodium adduct ($m/z = 1300.97$, $z = 2+$), potassium adduct ($m/z = 1308.97$, $z = 2+$) and sodium and potassium adduct ($m/z = 1320.95$, $z = 2+$) can be observed (Figure 63A). In the spectra of the same ligand with Ni(II) (Figure 63B), the signals can be assigned to the nickel complex ($m/z = 1286.99$, $z = 2+$) and a nickel complex with a sodium adduct ($m/z = 1297.98$, $z = 2+$).

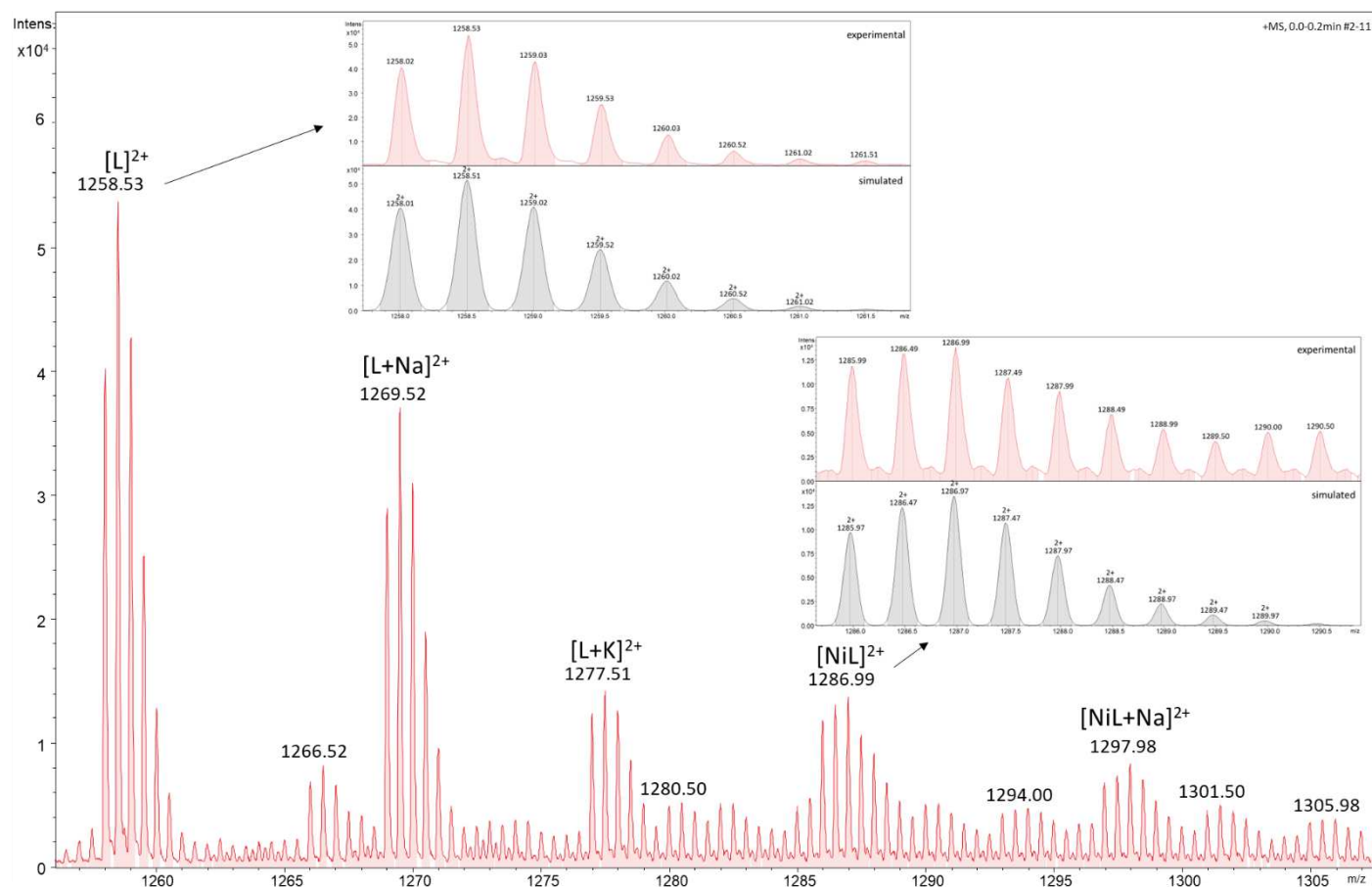
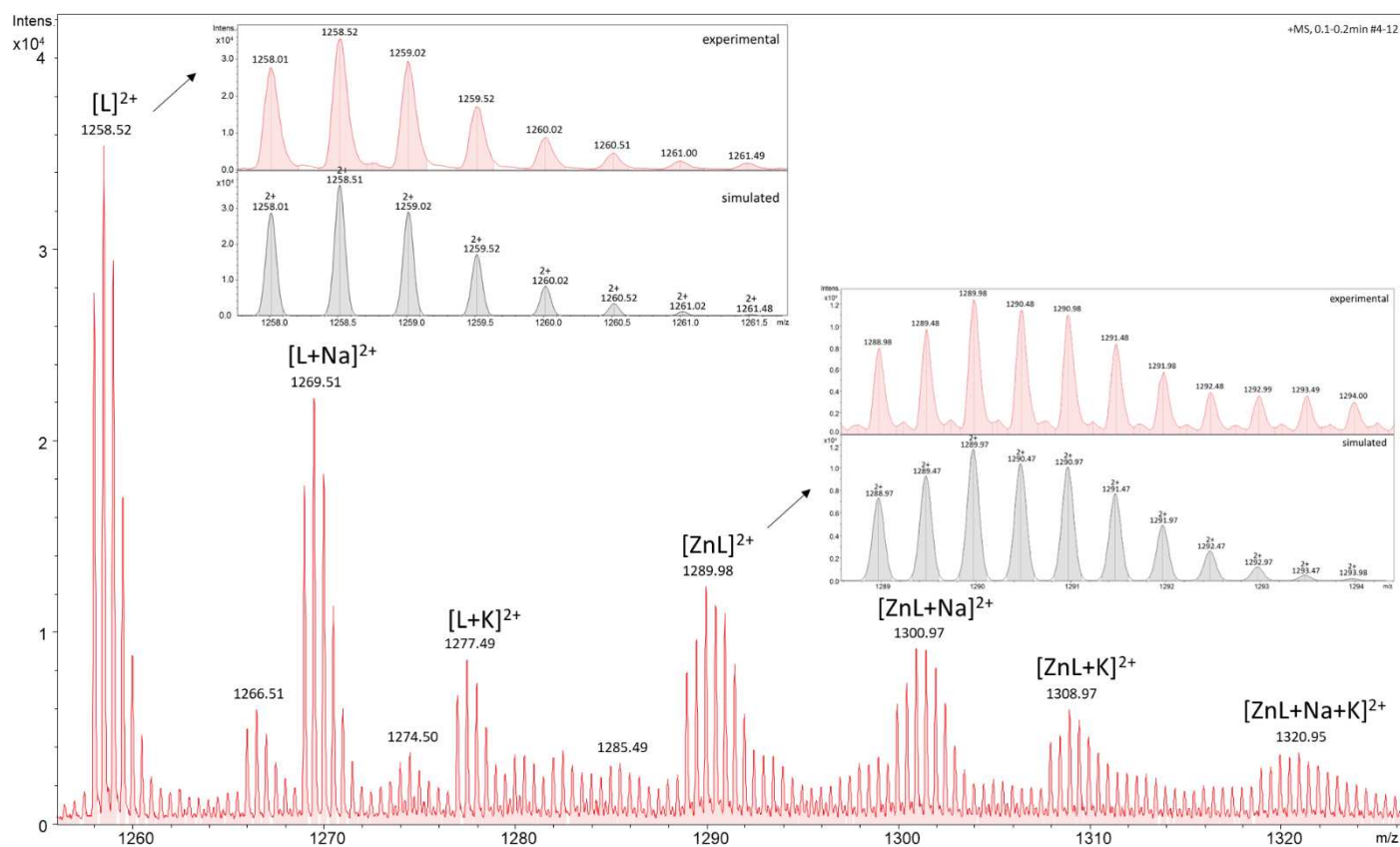


Figure 63 ESI-MS spectrum of A) Zn(II)-Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂ and B) Ni(II)-Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂

2. Methods of Zn(II) and Ni(II) coordination

a) Ac-TGCHSHGS-NH₂

The Ac-TGCHSHGS-NH₂ peptide behaves as an LH₃²⁺ acid with the protonating groups corresponding to the histidine imidazole side chain groups (pK_a values of 5.94 and 6.78, respectively) and cysteine thiol side chain with pK_a value of 8.68 (Table 7).

Table 7 Potentiometric data for the proton, Zn(II) and Ni(II) complexes of the Ac-TGCHSHGS-NH₂, Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ in a water solution of 4 mM HClO₄ with *I* = 0.1 M NaClO₄, *T* = 298 K. Standard deviations are presented in parentheses

Species	Ac-TGCHSHGS-NH ₂			Ac-MNCHFAGVEHCIGAGESESGSSQ-NH ₂		
	logβ	pK _a	residue	Logβ	pK _a	residue
H ₈ L				52.06 (3)	4.16	(E)
H ₇ L				47.90 (3)	4.21	(E)
H ₆ L				43.69 (3)	5.18	(E)
H ₅ L				38.51 (3)	6.19	(H)
H ₄ L				32.32 (3)	6.65	(H)
H ₃ L	21.40 (1)	5.94	(H)	25.67 (3)	7.49	(H)
H ₂ L	15.46 (1)	6.78	(H)	18.18 (2)	8.85	(C)
HL	8.68 (1)	8.68	(C)	9.33 (2)	9.33	(C)
Zn(II) complexes						
ZnH ₇ L				51.85 (1)		
ZnH ₆ L				47.80 (1)	4.05	(E)
ZnH ₅ L				42.98 (3)	4.82	(E)
ZnH ₄ L				37.96 (4)	5.03	(H)
ZnH ₃ L				32.84 (2)	5.12	(H)
ZnH ₂ L				27.56 (1)	5.28	(H)
ZnHL	13.28 (2)			21.34 (1)	6.23	(C)
ZnL	7.64 (1)	5.64	(C)	14.15 (1)	7.19	(C)
ZnH ₋₁ L	-0.29 (2)	7.93	(H ₂ O)	4.80 (1)	9.35	(H ₂ O)
ZnH ₋₂ L	-10.67 (2)	10.38	(H ₂ O)	-5.93 (1)	10.73	(H ₂ O)
Ni(II) complexes						
NiH ₆ L				48.72 (2)		
NiH ₅ L				44.04 (3)	4.68	(E)
NiH ₄ L				39.31 (3)	4.73	(H)
NiH ₃ L				33.58 (4)	5.74	(H)
NiH ₂ L	18.82 (7)			27.65 (4)	5.93	(H)
NiHL	13.22 (2)	5.60	(H)	21.66 (2)	5.99	(C)
NiL	6.51 (2)	6.72	(C)			
NiH ₋₁ L	-1.41 (2)	7.92	(am)	6.98 (2)		
NiH ₋₂ L	-10.07 (2)	8.66	(am)	-1.61 (3)	8.59	(am)
NiH ₋₃ L	-19.45 (2)	9.38	(am)	-11.21 (3)	9.60	(am)

(1) Zn(II)-Ac-TGCHSHGS-NH₂

The first complex observed for Ac-TGCHSHGS-NH₂ is ZnHL²⁺, with a maximum at pH 5.75 and most likely involves two histidine residues in binding (Figure 64). The loss of the next proton, leads to the ZnL⁺ form, with a maximum at pH 6.90. A significant decrease of the pK_a value calculated for the free ligand (pK_a = 8.68) and the complexed one (pK_a = 5.64) suggests that this cysteine is also involved in Zn(II) binding (Table 7). The next two forms, ZnH₋₁L and ZnH₋₂L⁻, correspond to the deprotonation of two subsequent water molecules bound to the central Zn(II)

ion. The proposed binding mode for Zn(II)-Ac-TGCHSHGS-NH₂ fragment of Zr_fC protein is shown on Figure 65.

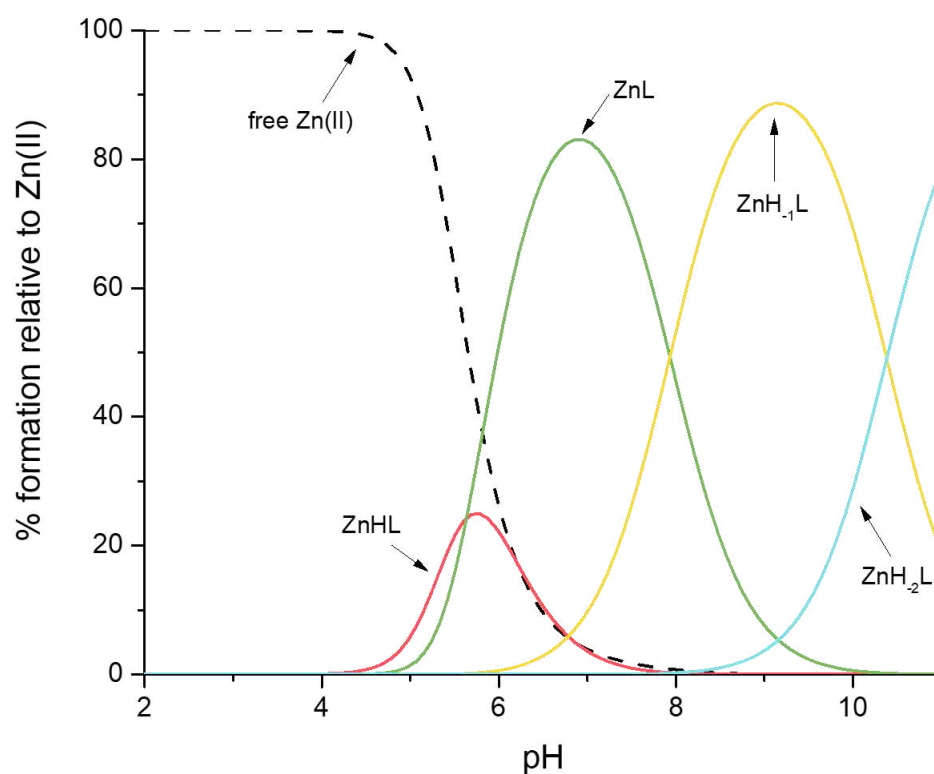


Figure 64 Species distribution diagram for the formation of Zn(II) complex with the Ac-TGCHSHGS-NH₂ Zr_fC fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

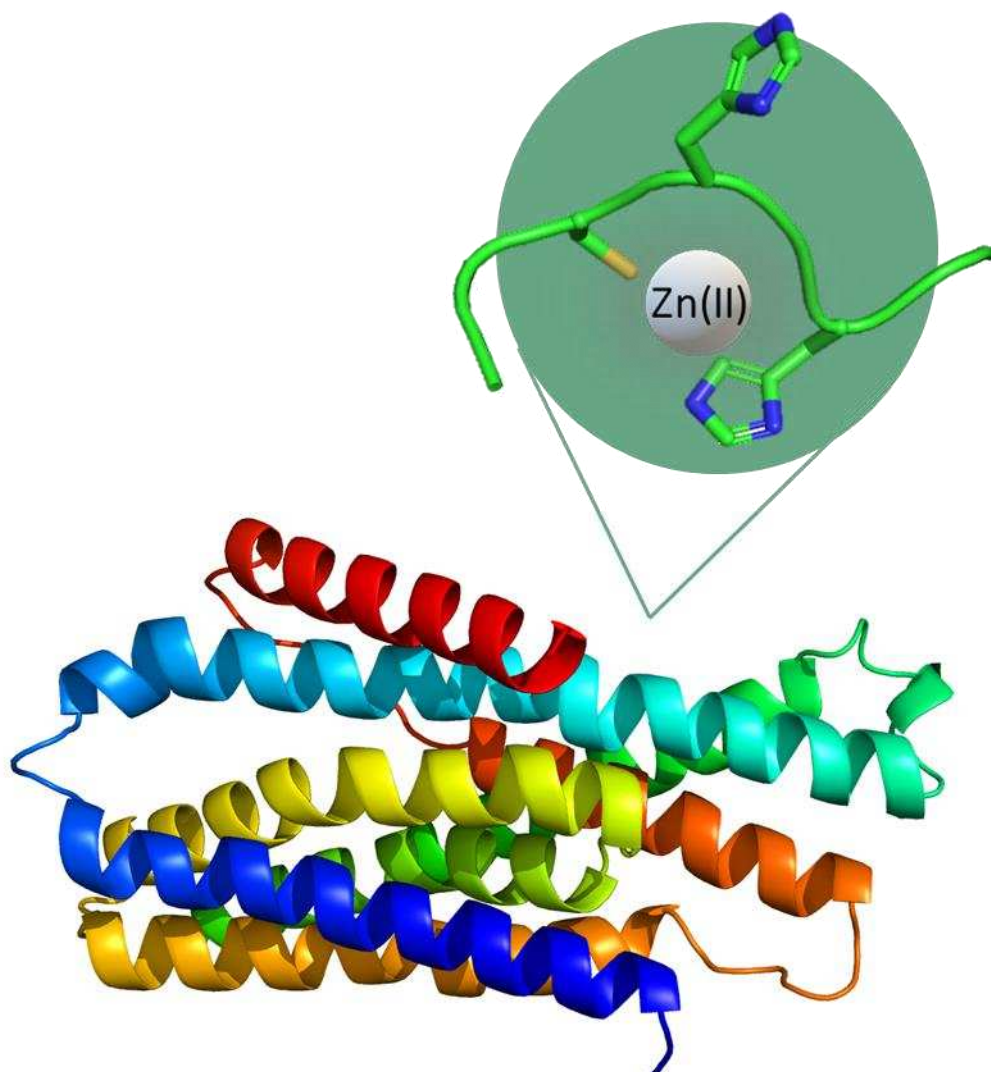


Figure 65 Proposed binding mode for Zn(II)-Ac-TGCHSHGS-NH₂ fragment of ZrfC protein at pH 7.4 (ZnL form - {2N_{im}, S⁻} binding mode). The ZrfC structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

(2) Ni(II)-Ac-TGCHSHGS-NH₂

The first Ni(II)-Ac-TGCHSHGS-NH₂ complex detected at low pH is NiH₂L³⁺, with a maximum at pH 5.50 and here, most likely, the Ni(II) ion is anchored to an imidazole of one of the histidine side chains (Figure 66). The loss of one proton leads to the NiHL²⁺ form, with a maximum at pH 6.35. The lowered histidine pK_a value in the free ligand (pK_a = 6.78) with respect to the complexed ligand (pK_a = 5.60), strongly suggest that this residue is also involved in metal binding (Table 7). The next complex, NiL⁺, dominates in solution at pH 7.35. At this pH, a pronounced CD band at 342 nm appears that is related to a S⁻ → Ni(II) transition, which together with the lowered Cys pK_a value (pK_a = 6.72) with respect to the free ligand (pK_a = 8.68) indicates thiolate binding to the Ni(II) ion (Figure 67). The next form, NiH₋₁L, dominates in solution at pH 8.30 and is associated with the coordination of an amide nitrogen. CD spectra (Figure 67) confirm this finding with pronounced bands with a negative Cotton effect at 437.5 and a positive Cotton effect at 511.2 nm, typical of amide involving square-planar nickel-peptide complex. The

increase of pH leads to the NiH_2L^- and $\text{NiH}_3\text{L}^{2-}$ forms which are associated with the coordination of two next amide nitrogens. Most likely, one of the imidazoles and one of the thiolates coordinated at acidic pH are replaced with amide nitrogens. The earlier mentioned CD band increase their intensity and most probably Ni(II) is bound to one histidine imidazole and three amide nitrogens in a $\{\text{N}_{\text{im}}, 3\text{N}^-\}$ binding mode. Also, the UV-Vis spectra indicate the formation of a square-planar nickel complex with a band around 445 nm (Figure 68).

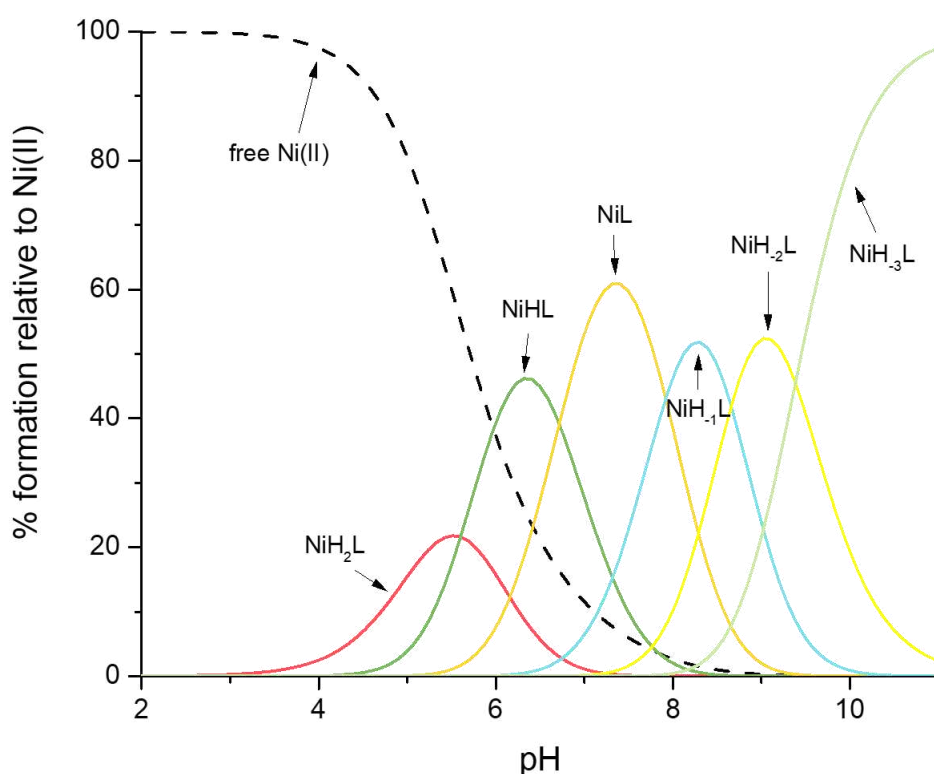


Figure 66 Species distribution diagram for the formation of Ni(II) complex with the Ac-TGCHSHGS- NH_2 ZrfC fragment, $T = 298 \text{ K}$, $I = 0.1 \text{ M NaClO}_4$, $[\text{L}] = 0.0005 \text{ M}$, $\text{Ni(II)}/\text{L}$ molar ratio = 1 : 1

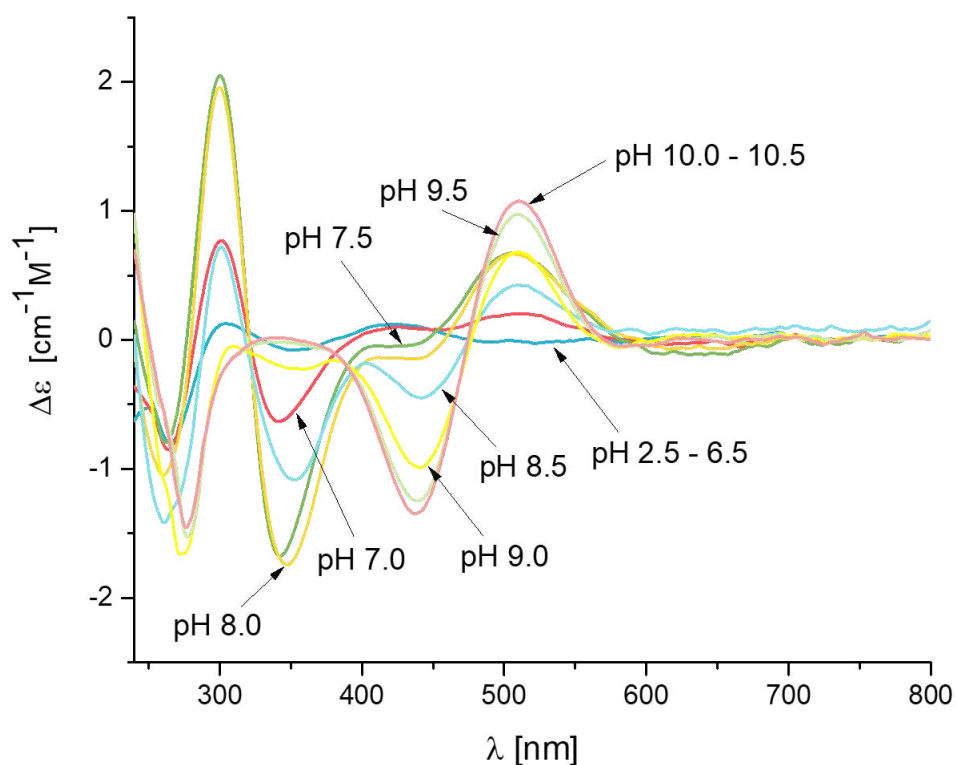


Figure 67 CD spectra of Ni(II) complex with the Ac-TGCHSHGS-NH₂ fragment from ZrfC protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

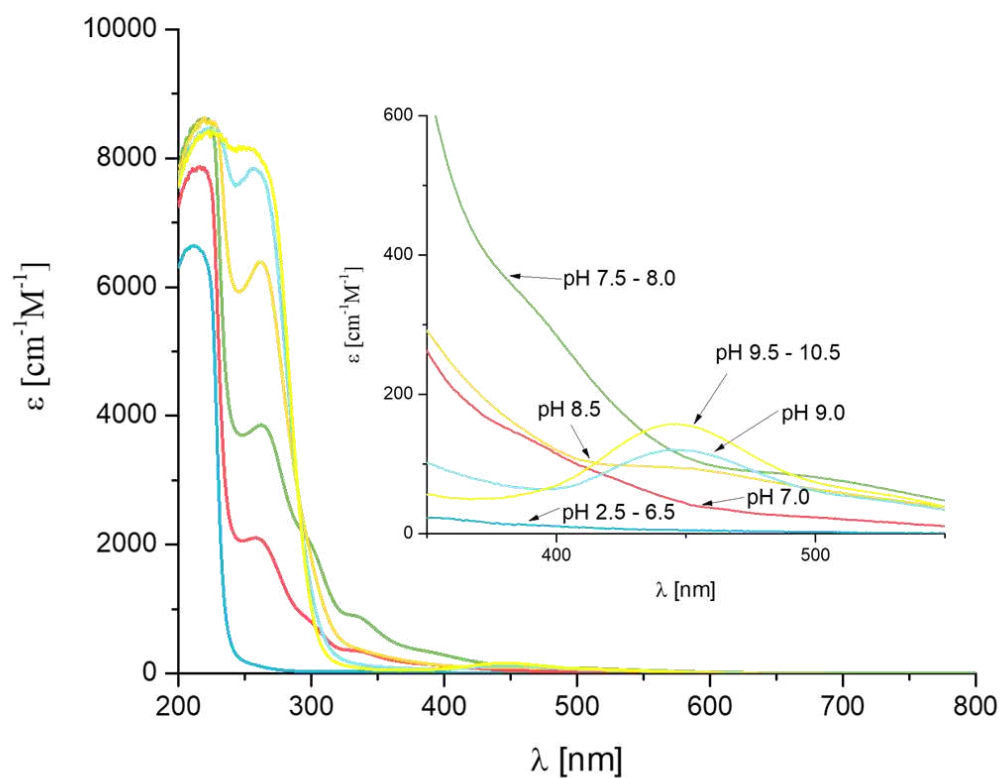


Figure 68 UV-Vis spectra of Ni(II) complex with the Ac-TGCHSHGS-NH₂ fragment from ZrfC protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

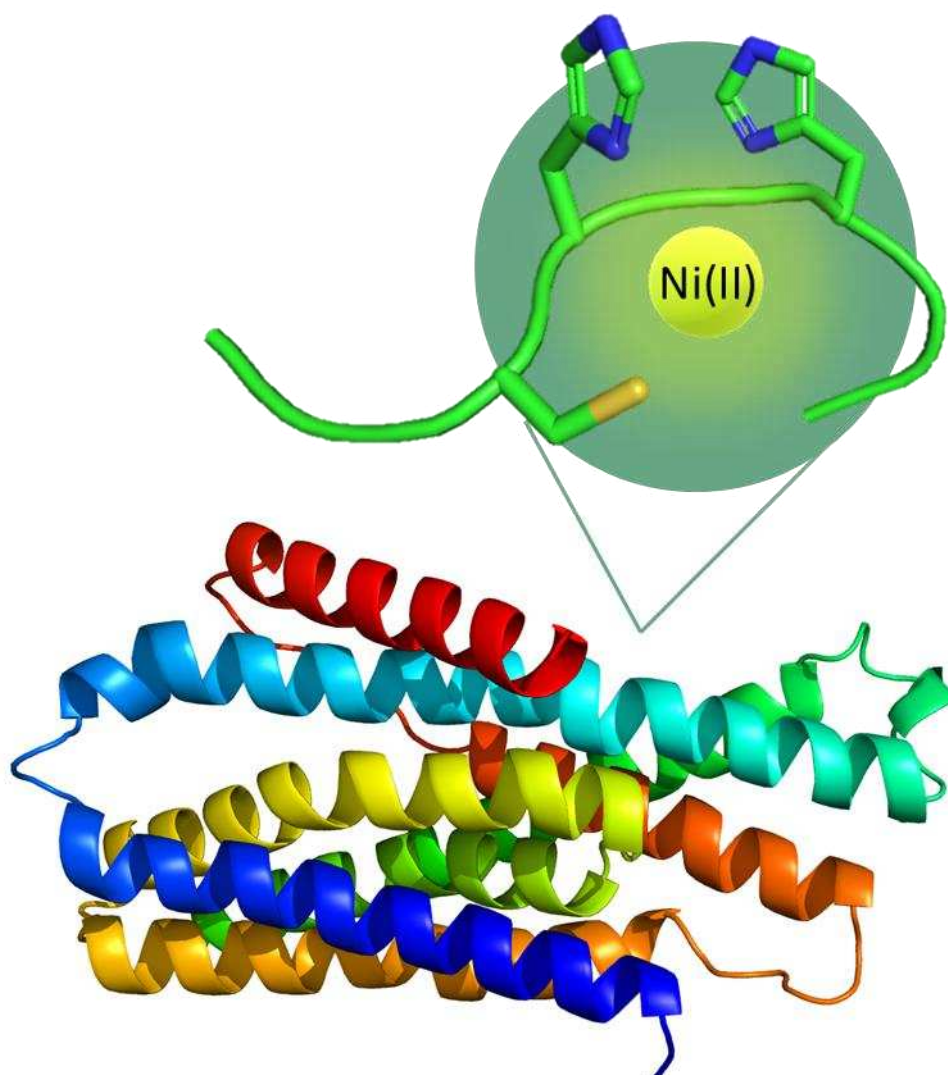


Figure 69 Proposed binding mode for Ni(II)-Ac-TGCHSHGS-NH₂ fragment of ZrfC protein at pH 7.4 (NiL form - {2N_{im}, S⁻} binding mode). The ZrfC structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

b) Ac-MNCHF^HHAGVEHCIGAGESESGSSQ-NH₂

The Ac-MNCHF^HHAGVEHCIGAGESESGSSQ-NH₂ peptide has eight protonation constants. The first three (pK_a = 4.16; 4.21 and 5.18) can be assigned to the deprotonation of three Glu carboxylic side chains. The next three constants (pK_a = 6.19; 6.65; 7.49) are related to the deprotonation of three histidine imidazole groups and the last two (pK_a = 8.85; 9.33) correspond to the deprotonation of two cysteine thiol side chains (Table 7).

(1) Zn(II)-Ac-MNCHF^HHAGVEHCIGAGESESGSSQ-NH₂

Zinc coordination to Ac-MNCHF^HHAGVEHCIGAGESESGSSQ-NH₂ starts already at quite low pH; the ZnH₇L⁴⁺ form has a maximum at pH 3.6, and here, most likely the Zn(II) ion is anchored to one of the glutamic acid side chains (Figure 70). The next two forms, ZnH₆L³⁺ and ZnH₅L²⁺, are related to the deprotonation of two other glutamic acid side chains. The loss of next three

protons, leading to ZnH_4L^+ , ZnH_3L and ZnH_2L^- forms, is most likely related to the deprotonation of three histidine side chains bound to the central Zn(II) ion. At higher pH values, the coordination mode changes. The loss of the next proton leads to the ZnHL^{2-} form, with a maximum at pH 6.7. A significant decrease of pK_a calculated for the complex ($\text{pK}_a = 6.23$) and the free ligand ($\text{pK}_a = 8.85$) suggests that this thiolate is involved in Zn(II) binding (Table 7). Analogously, in the next form, ZnL^{3-} , the lowered pK_a value for the second cysteine residue in the complexed ligand compared to the free one suggests that this residue is also bound to Zn(II) , most likely displacing one of the previously bound histidine imidazoles, and leaving the complex with the $\{2\text{N}_{\text{im}}, 2\text{S}^-\}$ coordination mode. Most probably, the most basic two forms, $\text{ZnH}_{-1}\text{L}^{4-}$ and $\text{ZnH}_{-2}\text{L}^{5-}$, correspond to the deprotonation of two water molecules bound to the metal ion. The proposed binding mode for Zn(II) to the Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂- fragment of ZrFC is show in the Figure 71.

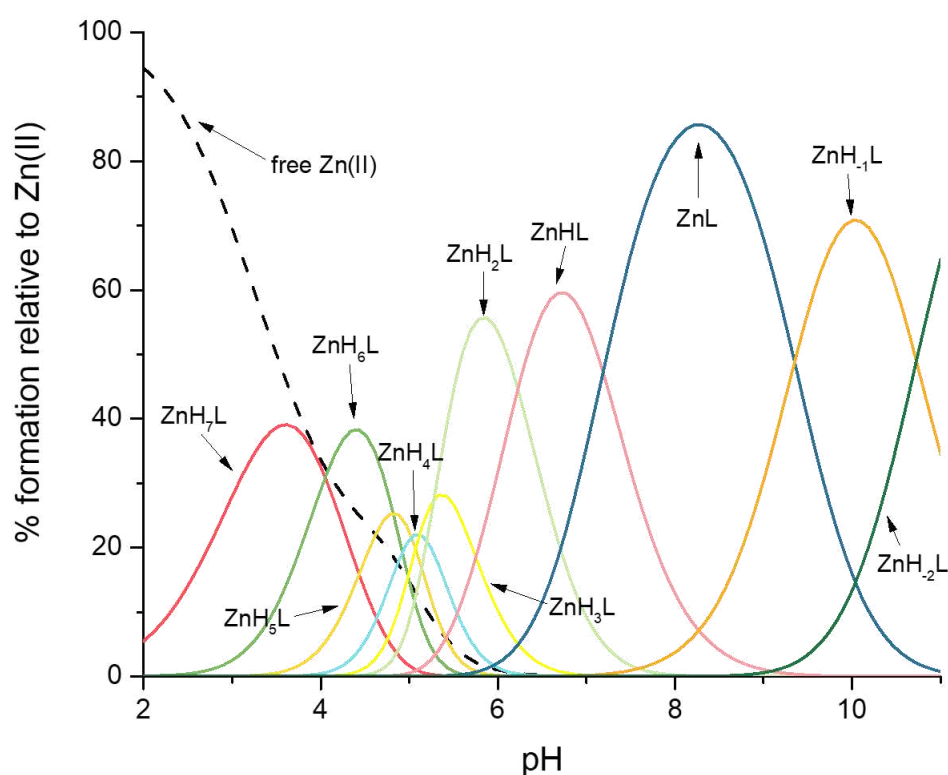


Figure 70 Species distribution diagram for the formation of Zn(II) complex with the Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ ZrFC fragment, $T = 298 \text{ K}$, $I = 0.1 \text{ M NaClO}_4$, $[\text{L}] = 0.0005 \text{ M}$, $\text{Zn(II)}/\text{L}$ molar ratio = 1 : 1

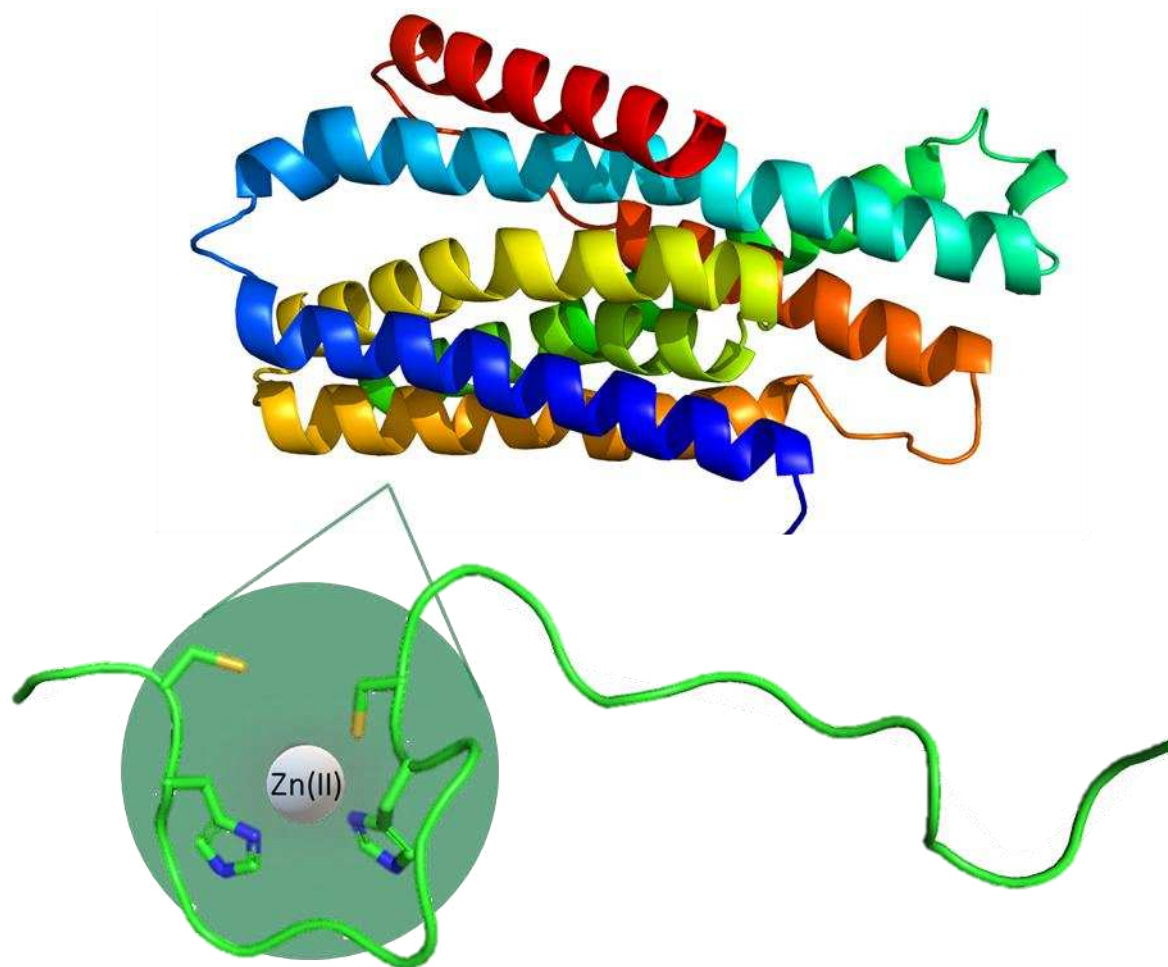


Figure 71 Proposed binding mode for Zn(II)-Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂ fragment of ZrFC protein at pH 7.4 (ZnL form - {2N_{im}, 2S⁻} binding mode). The ZrFC structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

(2) Ni(II)-Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂

The first acidic Ni(II)-Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂ complex observed at low pH is NiH₆L³⁺, with a maximum at pH 3.9 (Figure 72). Most likely, it involves two carboxylic groups of glutamic acids in coordination. The loss of one proton leads to the NiH₅L²⁺ form, where most probably the glutamic acids side chain is engaged in metal coordination. The remaining three deprotonations (NiH₄L⁺, NiH₃L, NiH₂L⁻) are related to the deprotonation and coordination of three histidine imidazoles. The lowered pK_a values for these histidine residues in the complexed ligand (4.73; 5.74 and 5.93) compared to the free one (6.19; 6.65 and 7.49) imply the participation of these groups in binding (Table 7). Moreover, the CD bands near 272.0 nm (Figure 73) that can be related to N_{im} → Ni(II) charge transfer are observed [587]. The next form which dominates at pH 6.8 is NiHL²⁺, which corresponds to the binding of a cysteine residue (its pK_a in the complexed ligand = 5.99 and in the free ligand = 8.85). The two deprotonations that occur in parallel are most likely related to the binding of the second Cys thiolate and of an amide nitrogen (Figure 72) and leads to the formation of the NiH₋₁L⁴⁻ form, as indicated by characteristic CD bands with a negative Cotton effect at 429.2 nm and positive Cotton effect at

494.5 nm (Figure 73). A typical square-planar nickel-peptide complex with amides confirms also the UV-Vis with a band at 451 nm (Figure 74). Thus, at pH 7.4 we observe a mixture of two complex forms: NiHL^{2-} and $\text{NiH}_{-1}\text{L}^{4-}$. The increase of the pH leads to the $\text{NiH}_{-2}\text{L}^{5-}$ and $\text{NiH}_{-3}\text{L}^{6-}$ forms, in which the Ni(II) coordination sphere includes two cysteine thiolates, two amide nitrogens with the $\{2\text{S}^-, 2\text{N}^-\}$ mode, and one cysteine thiolate and three amide nitrogens, with the $\{\text{S}^-, 3\text{N}^-\}$ mode, respectively.

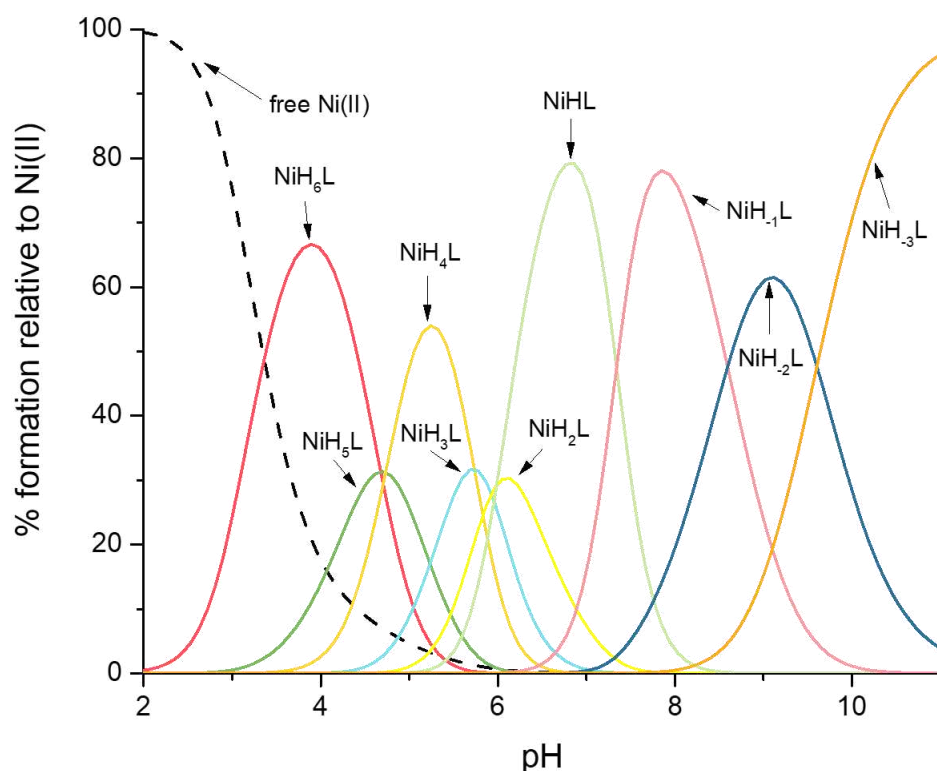


Figure 72 Species distribution diagram for the formation of Ni(II) complex with the Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂ ZrFC fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

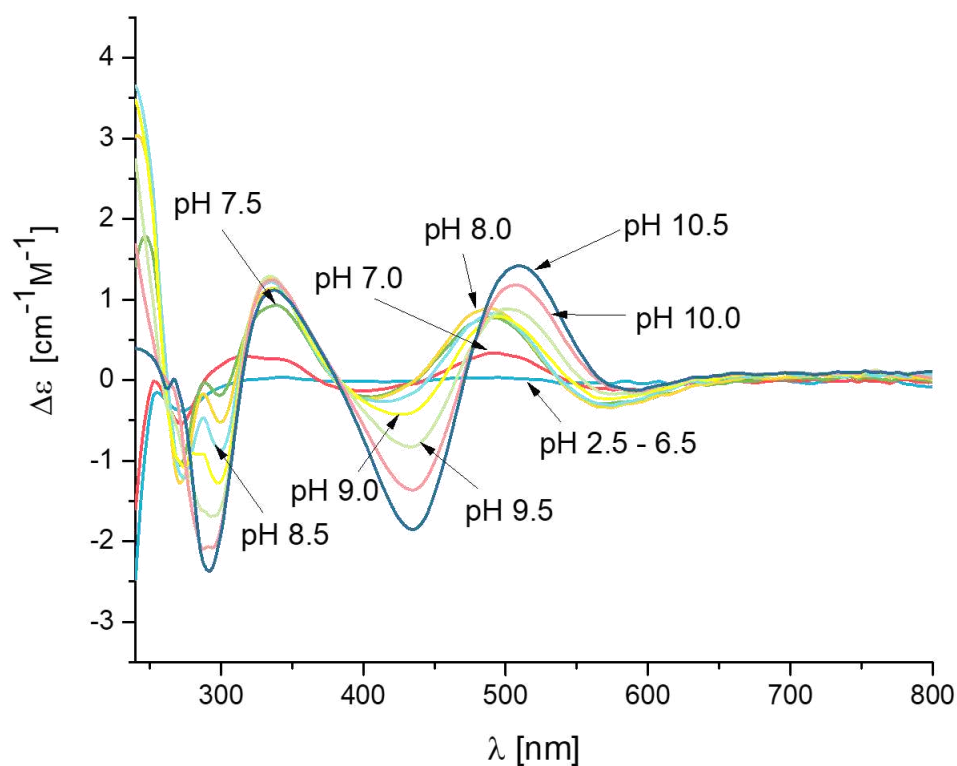


Figure 73 CD spectra of Ni(II) complex with the Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ fragment from ZrFC protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

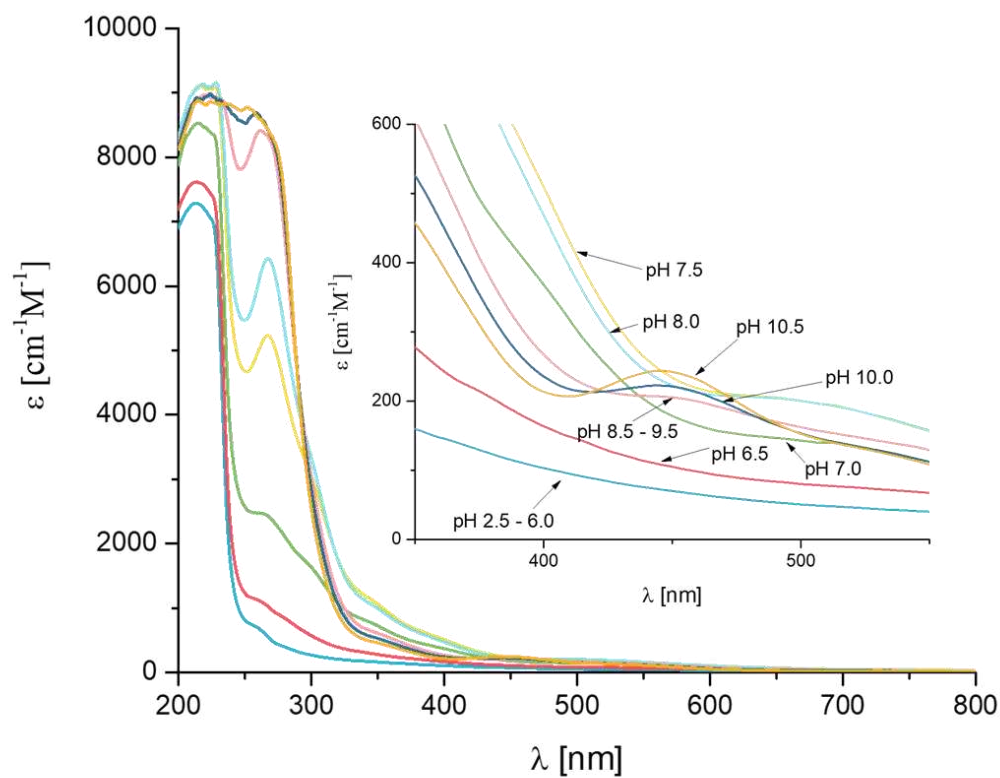


Figure 74 UV-Vis spectra of Ni(II) complex with the Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ fragment from ZrFC

protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

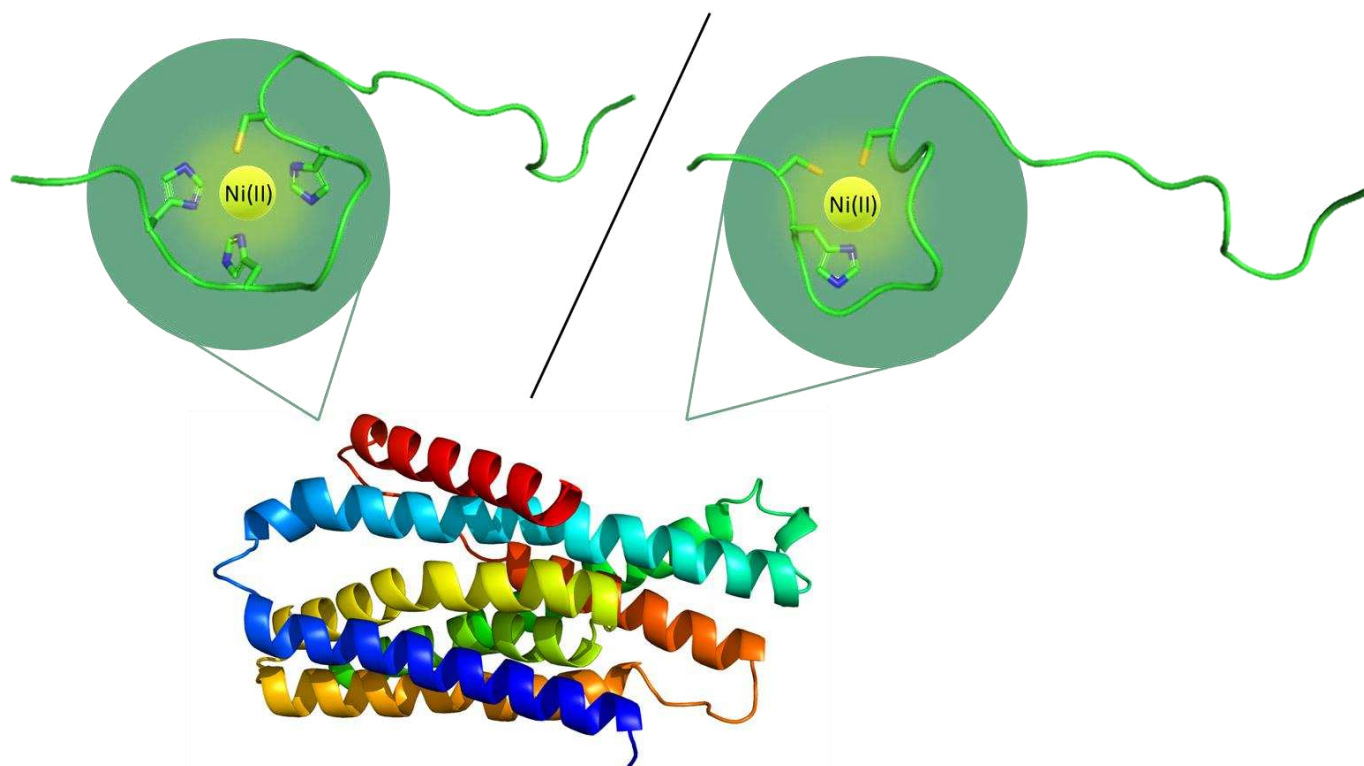


Figure 75 Proposed binding mode for Ni(II)-Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂ fragment of ZrfC protein at pH 7.4 (from the left: NiHL form – {3N_{im}, S⁻} binding mode and NiH₋₁L form – {1N_{im}, 2S⁻, N⁻} binding mode). The ZrfC structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

3. Secondary structure analysis

The comparison of the secondary structure of the Ac-TGCHSHGS-NH₂ fragment (Figure 76A) and its Ni(II) complex indicates that the addition of Ni(II) influences an undefined conformational change of Ac-TGCHSHGS-NH₂ (Figure 76C), (unlike Zn(II), which have no effect on it (Figure 76B). Both Zn(II), and Ni(II) do not influence the overall secondary structure of the Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂ ZrfC region (Figure 77).

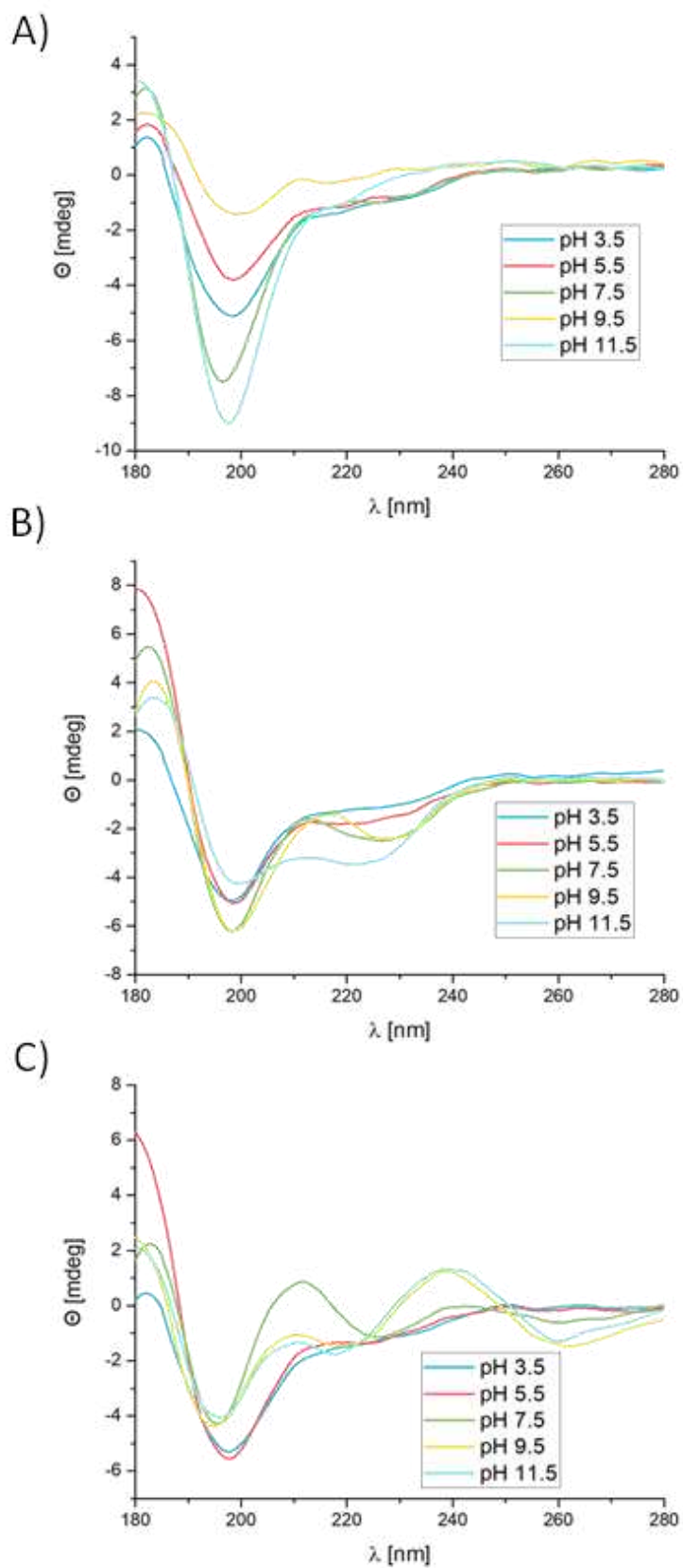


Figure 76 CD spectra of A) Ac-TGCHSHGS-NH₂ and its complexes with b) Zn(II) and C) Ni(II); optical path 0.1 mm, Ni(II)/L molar ratio = 1 : 1

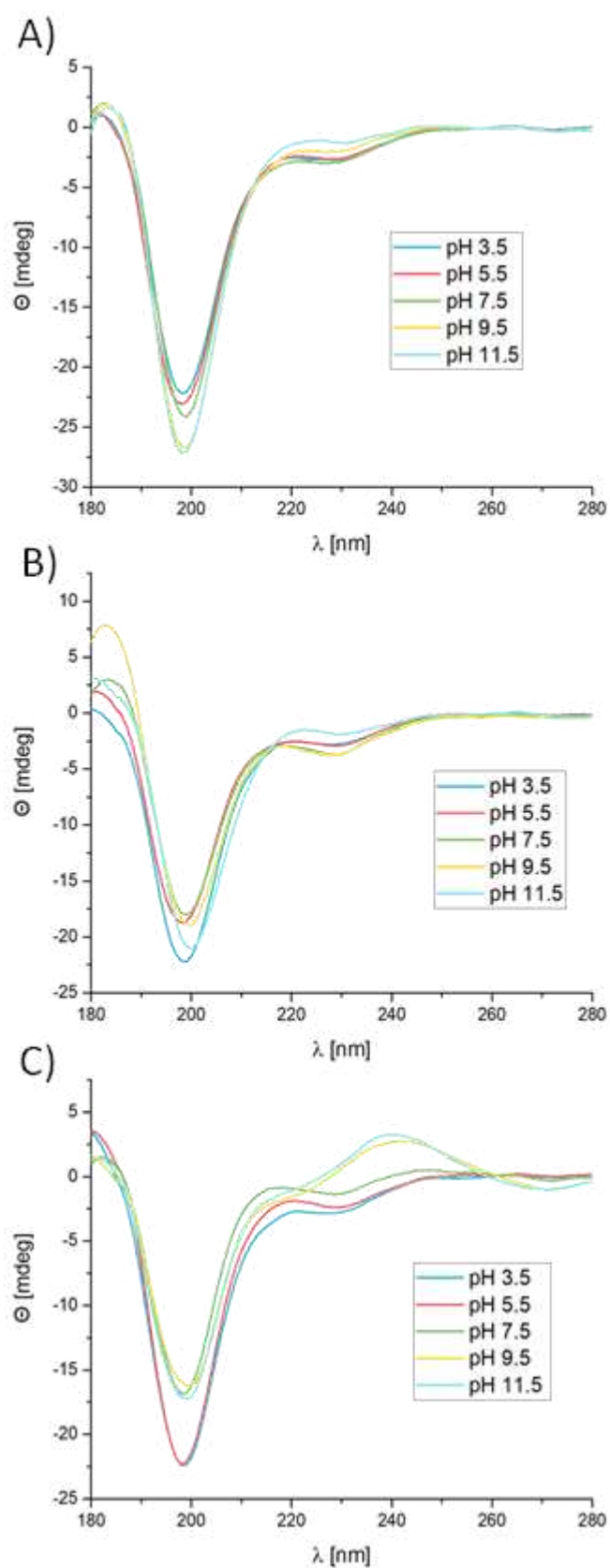


Figure 77 CD spectra of A) Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ and its complexes with b) Zn(II) and C) Ni(II); optical path 0.1 mm, Ni(II)/L molar ratio = 1 : 1

4. Comparison of the stability of Zn(II) and Ni(II) complexes

My recent studies on unstructured fragments of Asp2 zincophore from *Aspergillus fumigatus* (showed in the point A of the chapter IV and in the [493]) allowed me to determine the most probable Zn(II) binding site. In next steps of my work, I looked for the most probable Zn(II) binding site in the ZrfC transporter to understand the mechanism of Zn(II) transport in this pathogen. In order to check which ZrfC region binds Zn(II) with the highest affinity, I simulated a hypothetical situation in which equimolar amounts of Zn(II) and the two fragments of the ZrfC transporter are mixed (Figure 78). The results clearly indicate that Ac-MNCHFhAGVEHCIGAGESEGSSQ-NH₂ has a much higher affinity for Zn(II) in comparison with Ac-TGCHSHGS-NH₂ in the whole pH range. At pH 7.4, more than 95% of the metal is bound to Ac-MNCHFhAGVEHCIGAGESEGSSQ-NH₂. It suggests that most probably this region is involved in Zn(II) transport in the Asp2–ZrfC Zn(II) transfer system.

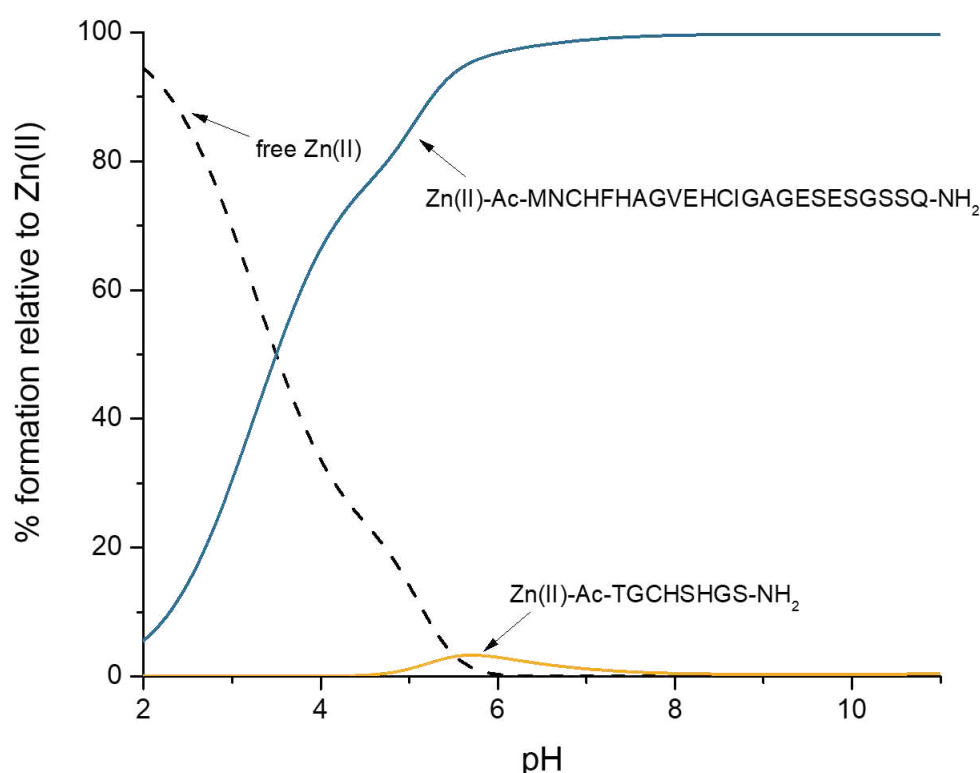


Figure 78 A competition plot between ZrfC fragments: Ac-TGCHSHGS-NH₂, Ac-MNCHFhAGVEHCIGAGESEGSSQ-NH₂ and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 7. Conditions: T=298 K, I=0.1 M NaClO₄, [Ni(II)] = [Ac-TGCHSHGS-NH₂] = [Ac-MNCHFhAGVEHCIGAGESEGSSQ-NH₂] = 0.001 M

With the same approach, I compared the affinities of Zn(II) to the most effective Zn(II) binding region of the Asp2 zincophore (Ac-PNCHTHEGGQLHCT) and of the ZrfC Zn(II) transporter (Ac-MNCHFhAGVEHCIGAGESEGSSQ-NH₂) (Figure 79). As predicted, the ZrfC transporter fragment has a much higher affinity for Zn(II). In the whole pH range, Ac-MNCHFhAGVEHCIGAGESEGSSQ-NH₂ binds Zn(II) with higher affinity than Ac-

PNCHTHEGGQLHCT, and at pH 7.4, more than 85% of Zn(II) is bound to the ZrfC transporter fragment. It suggests that from the thermodynamic point of view, Zn(II) can easily be transferred from the Aspf2 zincophore to the ZrfC transporter, making the Zn(ii) uptake from the host possible and efficient. This result is also interesting from the chemical point of view – in both cases, most probably, at physiological pH, Zn(II) is bound to the same set of donor atoms {2N_{im}, 2S⁻}. This motif is quite common Zn(II) binding motif (most commonly a structural one), present *e.g.* in zinc fingers [600]. The Zn(II)-Aspf2 binding is significantly weaker than the Zn(II)-ZrfC one, and this may be due to the proximity of the phenylalanine aromatic ring to the Zn(II) binding site in Ac-MNCHFHAGVEHCIGAGESEGSSQ-NH₂; there may be an interaction between the d-electrons of the metal ion and the π -ring system of the aromatic side chain, which provides additional stability [601]. Moreover, the number of amino acids between residues involved in binding is different in both cases. In the case of Ac-PNCHTHEGGQLHCT from the Aspf2 zincophore there are 7 amino acids between the CH and HC motif, and in the case of Ac-MNCHFHAGVEHCIGAGESEGSSQ-NH₂ from ZrfC transporter there are 6 amino acids in between. This difference may result in a different spatial arrangement, affecting bond stability [602]. The potential role of glutamic acid side chains in complex stabilization also cannot be excluded. The thermodynamical description of Zn(II)-binding to these biomolecules in perfect agreement with their role in biology- a higher affinity for this metal in the ZrfC transporter means that the transport between the zincophore and transporter is thermodynamically favourable.

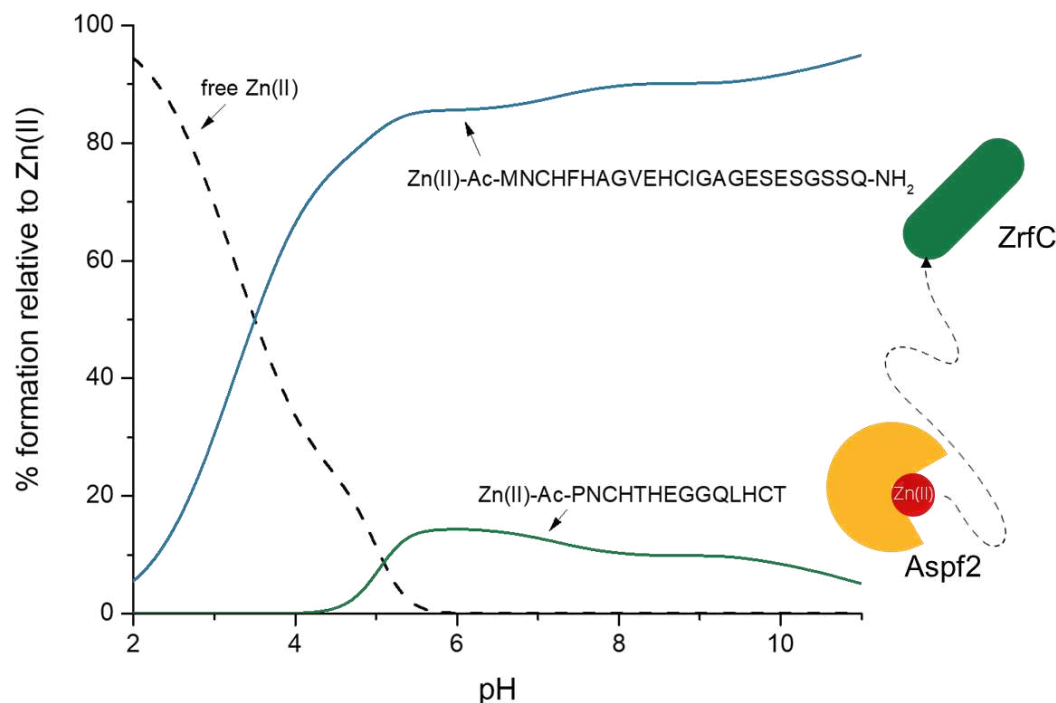


Figure 79 A competition plot between two most efficient Zn(II) binding sites in the ZrfC transporter (Ac-MNCHFHAGVEHCIGAGESEGSSQ-NH₂), in the Aspf2 zincophore (Ac-PNCHTHEGGQLHCT) and Zn(II); it describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents

are mixed. Calculations are based on binding constants from Table 7 and [493]. Conditions: $T=298\text{ K}$, $I=0.1\text{ M}$ NaClO_4 , $[\text{Zn(II)}] = [\text{Ac-MNCHFAGVEHCIGAGESESGSSQ-NH}_2] = [\text{Ac-PNCHTHEGGQLHCT}] = 0.001\text{ M}$

The analyzed ZrFC transporter fragments also bind Ni(II) , another nutrient essential for all microbes, that can theoretically compete with Zn(II) for the same binding sites. In this case, Ni(II) also chooses $\text{Ac-MNCHFAGVEHCIGAGESESGSSQ-NH}_2$ over Ac-TGCHSHGS-NH_2 and the difference in the stability of both complexes in the whole pH range is similar as in the case of Zn(II) complexes (Figure 80). Thus, the ZrFC $\text{Ac-MNCHFAGVEHCIGAGESESGSSQ-NH}_2$ fragment is also a more tempting Ni(II) binding site than the Ac-TGCHSHGS-NH_2 one. In the case of $\text{Ni(II)-Ac-MNCHFAGVEHCIGAGESESGSSQ-NH}_2$, at pH 7.4, we observe a mixture of NiHL^{2-} and $\text{NiH}_1\text{L}^{4-}$ forms, where Ni(II) is bound to two histidine and one cysteine residues or one histidine, two cysteine residues and one amide nitrogen, respectively. Under these conditions, for $\text{Ni(II)-Ac-TGCHSHGS-NH}_2$, Ni(II) occupies the same binding sites as Zn(II) , but does this mean that it can compete with Zn(II) ? In the case of Ac-TGCHSHGS-NH_2 , we notice that at pH 7.4, this fragment has a higher affinity towards Zn(II) than towards Ni(II) (Figure 81) – in our hypothetical situation, more than 70% of the sequence would bind Zn(II) . At higher pH values, the situation changes drastically, and the coordination of amide nitrogens in Ni(II) complexes gives them greater stability.

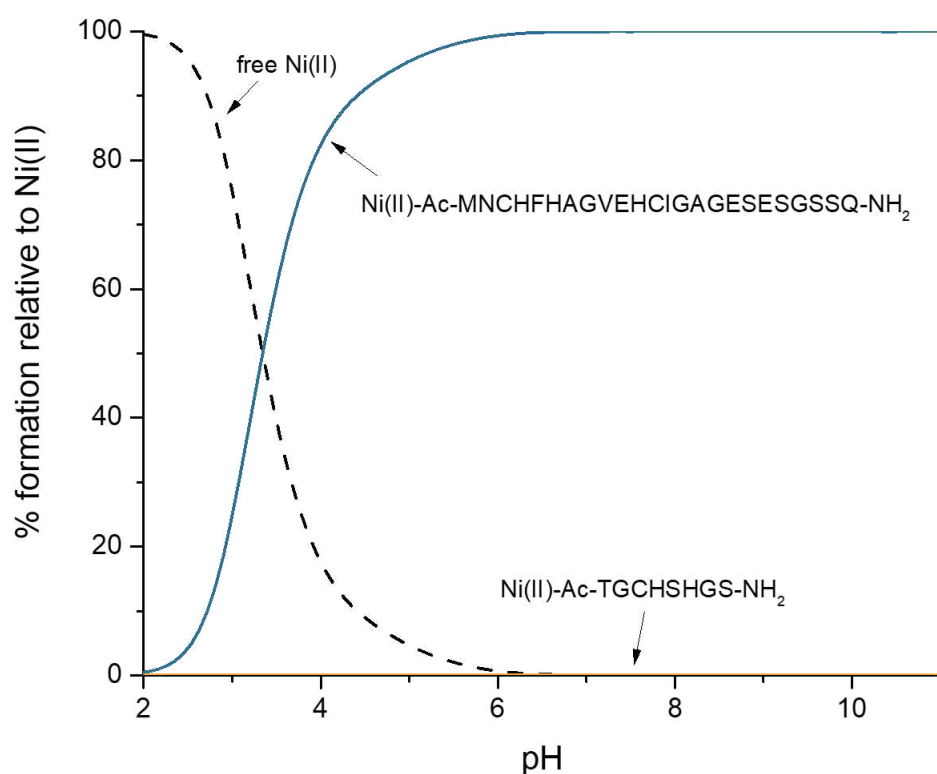


Figure 80 A competition plot between ZrFC fragments: Ac-TGCHSHGS-NH_2 , $\text{Ac-MNCHFAGVEHCIGAGESESGSSQ-NH}_2$ and Ni(II) , describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 7. Conditions: $T=298\text{ K}$, $I=0.1\text{ M}$ NaClO_4 , $[\text{Ni(II)}] = [\text{Ac-TGCHSHGS-NH}_2] = [\text{Ac-MNCHFAGVEHCIGAGESESGSSQ-NH}_2] = 0.001\text{ M}$

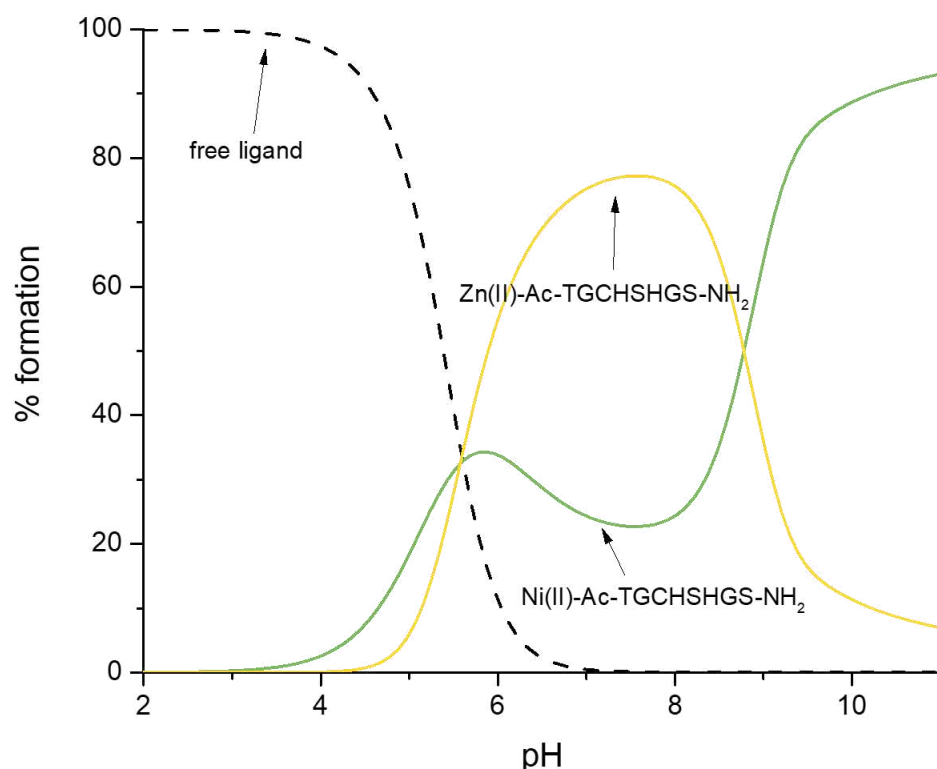


Figure 81 A competition plot between ZrFC fragment: Ac-TGCHSHGS-NH₂, Zn(II) and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 7. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ni(II)] = [Ac-TGCHSHGS-NH₂] = 0.001 M

How about the interactions of Ni(II) with Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂, the preferred Zn(II) binding site in the ZrFC transporter? That fragment is rich in histidine and cysteine residues, so it is expected to be a tempting binding site for Ni(II), which is confirmed in Figure 80. Almost in the whole pH range, Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ prefers to bind Ni(II) rather than Zn(II) (Figure 82). The difference between the stability of both complexes is the biggest at alkaline pH, at which the coordination of Ni(II) involves amide nitrogens, but at physiological pH, the stabilities of both Zn(II) and Ni(II) complexes are fairly comparable. Does this mean that Ni(II) can competitively inhibit the functioning of the zincophore based transport system present in *Aspergillus fumigatus*? This hypothesis may be too far-reaching, but it definitely may suggest a lack of ZrFC specificity and the possibility of its additional Ni(II) transporting function, but most probably, this might have little meaning for Zn(II) transport. The free Ni(II) concentration is most likely several orders of magnitude lower than that of free Zn(II) and also the binding of Ni(II) to the ZrFC transporter is not equal to its transmembrane transfer [428]. Moreover, as we have shown in our previous work [493], in a theoretical situation in which equimolar amounts of Zn(II), Ni(II) and Ac-PNCHTHEGGQLHCT would be available- at pH 7.4, more than 85% of the zincophore would bind Zn(II). That result clearly indicates, that the AspF2 zincophore strongly prefers Zn(II) to Ni(II), which additionally guarantees the Zn(II) specificity of AspF2, a crucial part of the fungal AspF2-ZrFC Zn(II) transfer system.

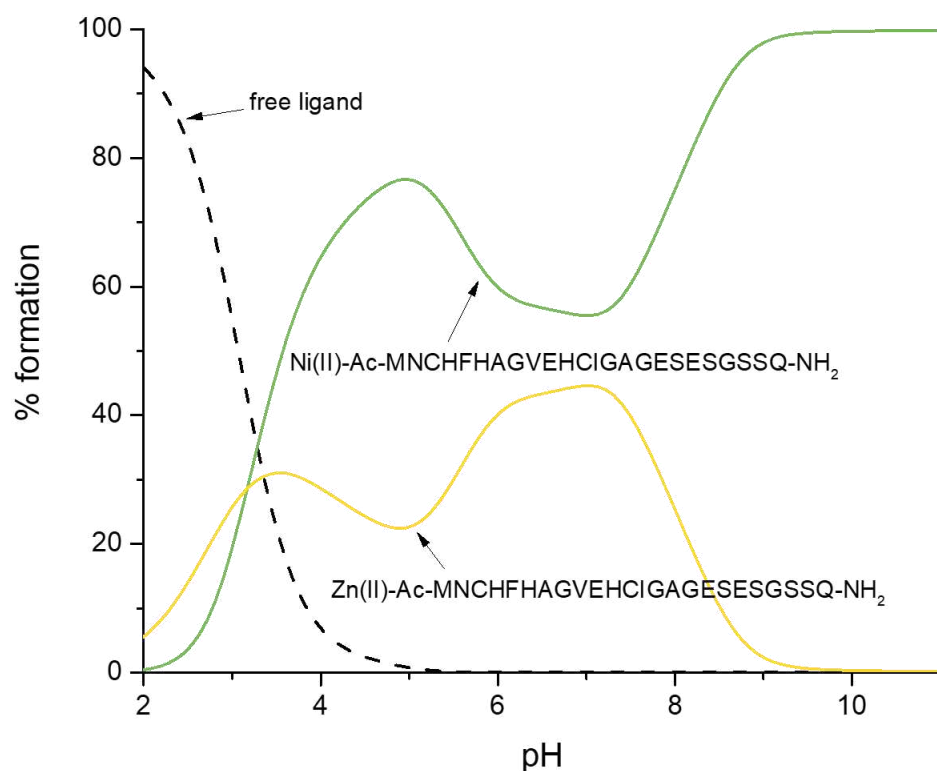


Figure 82 A competition plot between ZrFC fragment: MNCHFAGVEHCIGAGESEGSSQ-NH₂, Zn(II) and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 7. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ni(II)] = [Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂] = 0.001 M

To confirm the results based on the calculated stability constants, we carried out a CD-spectroscopic titration of the Ni(II)-Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂ species with Zn(II) at pH 7.4, showing that the fragment binds Ni(II) with higher affinity. Zn(II) is spectroscopically silent [603] and for this reason, if Zn(II) was bound, the characteristic Ni(II) band should disappear after Zn(II) would displace Ni(II) from its binding site. The observed bands decrease their intensity, but do not disappear completely, which confirms the conclusion from Figure 83 - the fact that Ni(II) binds with higher (but still comparable) affinity than Zn(II) to the Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂ region of ZrFC.

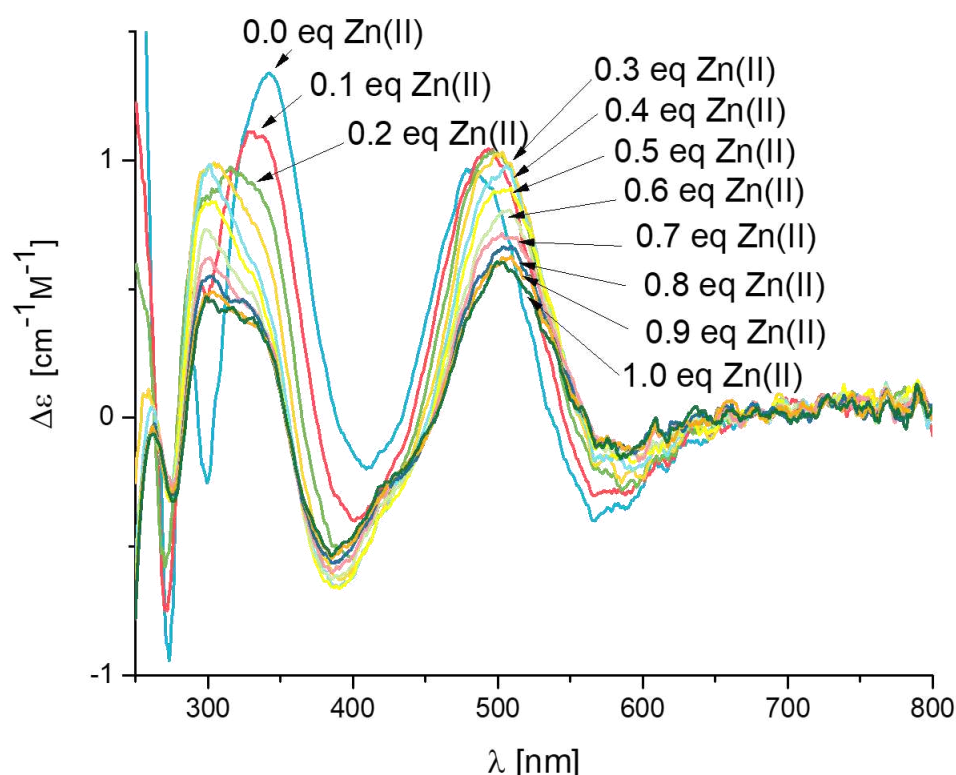


Figure 83 CD spectra of Ni(II) complexes of the ZrfC transporter fragment (Ac-MNCHFhAGVEHCIGAGESEGSSQ-NH₂) at the pH 7.4, titrated with Zn(II) ions at a step of 0.1 molar equivalents. Final conditions: [Zn(II)] : [Ni(II)] : [Ac-MNCHFhAGVEHCIGAGESEGSSQ-NH₂] = 1 : 1 : 1. Spectra were recorded at 298 K, in the range 250-800 nm and with the optical path 1 cm

We expected to deal with an analogous situation in the case of titration of the Zn(II)-Ac-MNCHFhAGVEHCIGAGESEGSSQ-NH₂ complex with Ni(II). After adding Ni(II) ions to the solution, we anticipated to observe the appearance of bands characteristic for the Ni(II)-complex, but the conducted studies clearly show that at pH 7.4, along with the addition of Ni(II) to the solution, bands characteristic for Ni(II)-complexes do not appear (Figure 84). Does this mean that Ni(II) does not displace Zn(II) from this binding site? The {2N_{im}, 2S⁻} Zn(II) binding mode ((typical for *e.g.* zinc fingers [600]) is thermodynamically very stable). That is why it is not surprising that under our experimental conditions Ni(II) is not able to displace Zn(II) from that binding site. Due to the presence of the band at a wavelength of 270 nm, we assume that Ni(II) anchors on the last available imidazole in the sequence, which is not involved in Zn(II) binding—most probably, to the His6 of our studied region (His168 in full length ZrfC numbering). The increase of pH in our [Zn(II)] : [Ni(II)] : [Ac-MNCHFhAGVEHCIGAGESEGSSQ-NH₂] = 1 : 1 : 1 system leads to the deprotonation of three amide nitrogens, which then bind Ni(II) with the {N_{im}, 3N⁻} binding mode. The observed CD bands with a negative Cotton effect at 436.5 nm and a positive Cotton effect at 520.5 nm at pH 8.0, which increase their intensity with increasing pH, clearly confirm this finding (Figure 84).

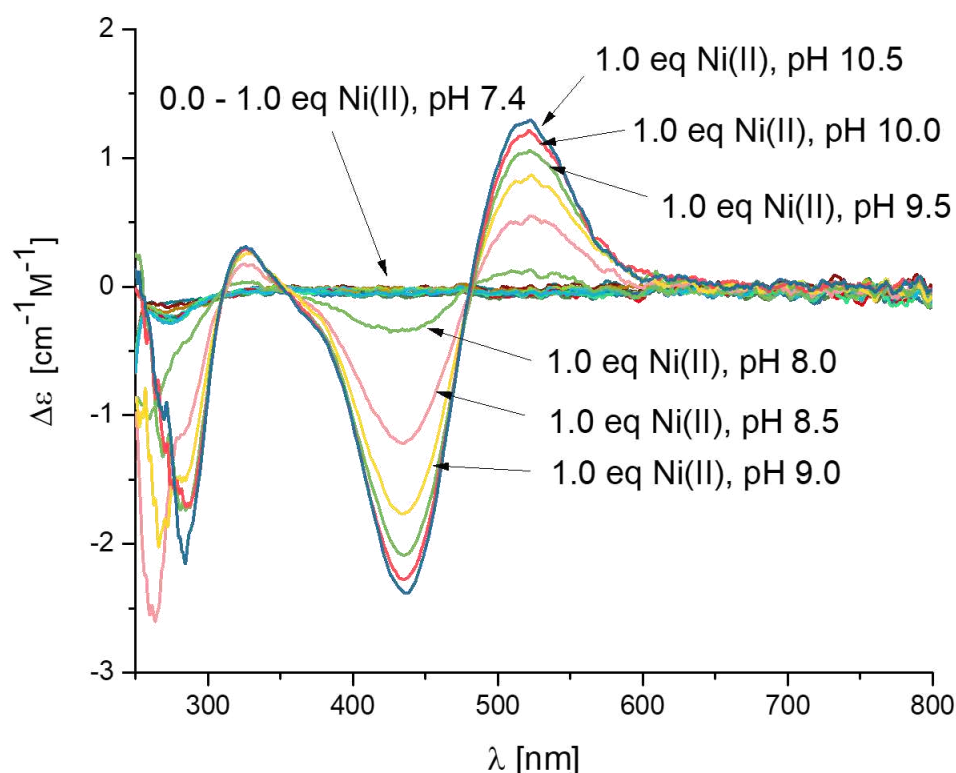


Figure 84 CD spectra of Zn(II) complexes of the ZrfC transporter fragment (Ac-MNCHF_{HAGVEHCIGAGESESGSSQ}-NH₂) at the pH 7.4, titrated with Ni(II) ions at a step of 0.1 molar equivalents. Final conditions: [Ni(II)] : [Zn(II)] : [Ac-MNCHF_{HAGVEHCIGAGESESGSSQ}-NH₂] = 1 : 1 : 1. Spectra were recorded at 298 K, in the range 250-800 nm and with the optical path 1 cm. Finally, the pH value was raised to pH 10.5 at a step of 0.5

5. Conclusions

As described above, the most probable Zn(II) binding site on the Aspf2 zincophore from *Aspergillus fumigatus* is its C-terminal region, PNCHTHEGGQLHCT, and on the ZrfC transporter – the MNCHF_{HAGVEHCIGAGESESGSSQ} fragment. The Zn(II) binding site on ZrfC coordinates this metal with higher affinity than the Zn(II) binding site on Aspf2, allowing Zn(II) transfer from the Aspf2 zincophore to the ZrfC transporter.

The binding preferences of Ni(II), a metal that could potentially compete with Zn(II) binding on ZrfC, are similar to those of Zn(II), and it is also the Ac-MNCHF_{HAGVEHCIGAGESESGSSQ}-NH₂ region that binds Ni(II) with the highest affinity among the studied ligands. At physiological pH, the Aspf2 zincophore prefers to bind Zn(II) rather than Ni(II), while for the transporter, the MNCHF_{HAGVEHCIGAGESESGSSQ} fragment binds the two metals with comparable affinity, thus, making transporter selectivity much more complicated.

These findings are an input to the basic chemistry of Zn(II) and Ni(II), but also an input to understanding these metals' transport; they elevate our understanding of the mechanism of fungal metal transport.

C. Thermodynamic and structural properties of Zn(II) and Ni(II) complexes with Ac-Ac-ACHA-NH₂ and Ac-AHCA-NH₂ ligands

The model sequences are presented in the Figure 85 and article [604]. Histidine and cysteine residues, with their imidazole and thiol moieties, respectively are primary binding sites for Zn(II) and Ni(II) ions and are thus ubiquitous both in peptidic metallophores and in antimicrobial peptides that may use nutritional immunity as a way to limit pathogenicity during infection. Cys-Hys and His-Cys motifs are present in 411 of antimicrobial compounds (168 Cys-His and 243 His-Cys motifs, respectively). For the sake of comparison – 348 Cys-Cys motifs and only 94 His-His motifs can be found in the AMP database [605]. Usually, the repeat of one single amino acid has a biological reason. Proteins rich in Cys residues often protect against heavy metals and oxidant damage [606]. Gln-rich proteins mediate protein-protein interactions [607], and Gly and Ala-rich stretches are considered structural elements of proteins [534]. Most probably, Asp residues compensate for the positive charge of His residues and provides an additional metal-binding site. Whereas Gly residues might increase the flexibility of His-rich domains [534]. Quite often, His-rich motifs are interrupted by alanine, serine or glutamic acid [608]. According to the Person's classification, His-rich sequences are potential binding sites for several so-called 'intermediate' metal ions. Their imidazoles, with a pK_a value of about 6.5, are partially deprotonated at physiological pH and are available for metal ion binding, wanting to give the lone pair of electrons from nitrogen to the metal ion [534]. Due to this fact, sequences with numerous histidines have been chosen not only by nature, but also by molecular biologists as effective metal binders. Cysteine is another amino acid residue, which is tempting for metal ions. Cys residues constitute one-third of all amino acid residues in metallothioneins, which are responsible for toxic heavy metal ions and its homeostatic regulation. They come in various motifs: CXC, CXXC and CXCC [609], where X may stand for any amino acid. The CC binding motif is present in several chaperones, like mentioned Hpn and HspA, where Ni(II) is bound to two sulfurs of neighboring cysteine residues and to the amide between them [187]. In the CXC motif Ni(II) also binds to two cysteines and two amides between them with a {2S⁻, 2N⁻}. Examples of a CXXC type sequences can be found for example in HypA protein and those motifs usually have a structural role and bind Zn(II). They can also bind Ni(II), which is anchored to the *N*-terminal MHE motif [534]. It is worth mentioning that if in Ni(II) binding no amides are involved, Zn(II) may also displace it from their binding sites [187].

Due to this, I also determined the thermodynamic properties for two model sequences encompassing Cys–His and His–Cys motifs. The results are presented in the article [604]. The aim of the study was to check whether: i) His or Cys—does it matter for the stability of their complexes which one goes first? (ii) Do His–Cys and Cys–His ligands have a metal preference? (iii) Do non-binding residues affect complex stability?



Figure 85 Amino acid sequences of model ligands

1. Zn(II) and Ni(II) coordination modes

a) Ac-AHCA-NH₂

The first protonation constant for Ac-AHCA-NH₂ corresponds to the imidazole nitrogen atom of histidine residue (6.42) and the second one, with the value 8.72, to the thiolate of the cysteine residue (Table 8).

Table 8 Potentiometric data for the proton, Zn(II) and Ni(II) complexes of the Ac-AHCA-NH₂ and Ac-ACHA-NH₂ in a water solution of 4 mM HClO₄ with *I* = 0.1 M NaClO₄, *T* = 298 K. Standard deviations are presented in parentheses

Species	Ac-AHCA-NH ₂			Ac-ACHA-NH ₂		
	logβ	pK _a	residue	logβ	pK _a	residue
H ₂ L	15.14 (2)	6.42	(H)	15.18 (2)	6.42	(H)
HL	8.72 (1)	8.72	(C)	8.76 (1)	8.76	(C)
Zn(II)-complexes						
ZnL	6.22 (1)	7.84	(H ₂ O)	6.43 (1)	8.05	(H ₂ O)
ZnH ₋₁ L	-1.62 (1)	9.98	(H ₂ O)	-1.62 (1)	9.82	(H ₂ O)
ZnH ₋₂ L	-11.60 (2)			-11.44 (2)		
Ni(II)-complexes						
NiL	3.86 (1)	7.39	(am)			
NiH ₋₁ L	-3.53 (2)	8.05	(am)	-3.41 (5)	8.45	(am)
NiH ₋₂ L	-11.58 (2)	8.56	(H ₂ O)	-11.86 (2)	9.63	(H ₂ O)
NiH ₋₃ L	-20.14 (1)			-21.49 (3)		
NiL ₂				9.85 (4)		

(1) Zn(II)-Ac-AHCA-NH₂

In the case of the Zn(II)-Ac-AHCA-NH₂ complex, the first detected species, with a maximum at pH 7, is ZnL²⁺ (Table 8 and Figure 86). Most probably, in this form, the Zn(II) is coordinated to one imidazole nitrogen and one Cys thiol. The remaining coordination positions are likely occupied by water molecules. The next observed species, ZnH₋₁L (maximum at pH 8.90), has a {N_{im}, S⁻, OH⁻} binding mode, and it forms with pK_a = 7.84, corresponding to the deprotonation of a coordinated water molecule. Above pH 10.0, ZnH₋₂L⁻ dominates in solution. This case is most likely related to the deprotonation of the second water molecule (pK_a = 9.98),

and in this form, the metal ion coordination sphere includes the imidazole, the sulfur and two oxygen atoms from the water molecules (Figure 87).

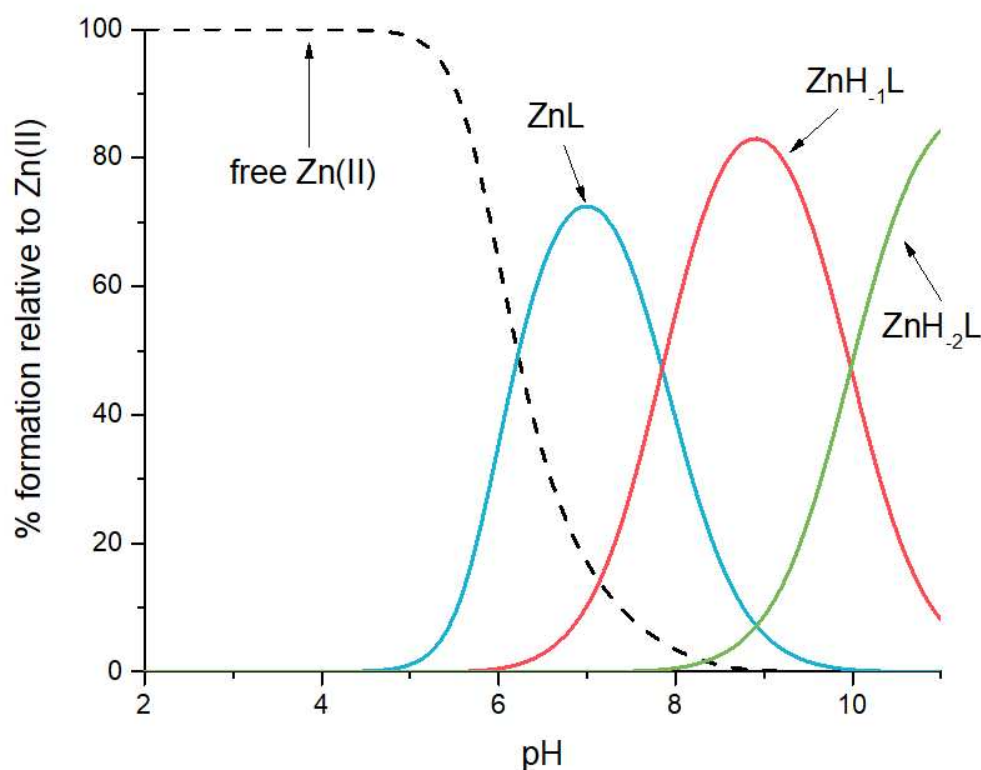


Figure 86 Species distribution diagram for the formation of Zn(II) complex with the Ac-AHCA-NH₂, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

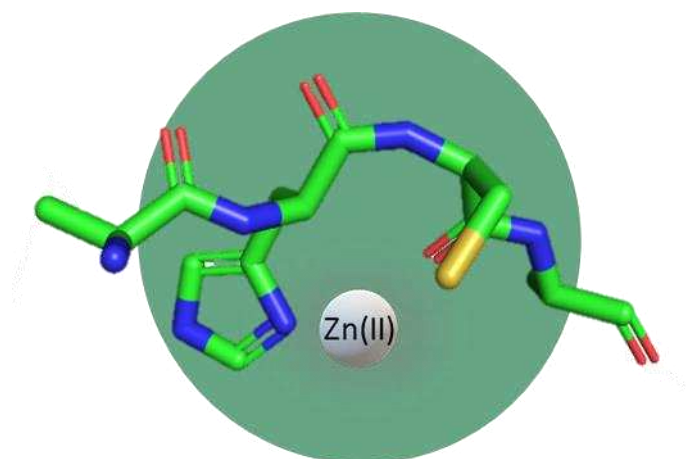


Figure 87 Proposed binding mode for Zn(II)-Ac-AHCA-NH₂

(2) Ni(II)-Ac-AHCA-NH₂

The first Ni(II) complex observed at low pH, NiL²⁺ reaches its maximum at pH 7.5 (Table 8 and Figure 88). Most probably, in this complex, Ni(II) is bound to the His imidazole and Cys

sulfur. CD spectra confirm this finding with pronounced bands around 270 nm and 350 nm, typical for $N_{im} \rightarrow Ni(II)$ [587] and $S^- \rightarrow Ni(II)$ [596] charge transfer transitions, respectively (Figure 89). The next deprotonation leads to the formation of $NiH_{-1}L^+$ species, in which an amide nitrogen binds to $Ni(II)$. The loss of the next proton leads to the $NiH_{-2}L$ form and is most likely related to the deprotonation and binding of a second amide. The last observed form, $NiH_{-3}L^-$, most likely corresponds to the deprotonation of a water molecule bound to the central $Ni(II)$ ion. In this form, one cysteine thiolate, one histidine imidazole and two amide nitrogens already participate in binding, forming a square planar nickel complex (Figure 90). The hypothesis is supported also by the UV-Vis spectra (Figure 91), where no changes in the wavelength of maximum absorption are observed above pH 8.5.

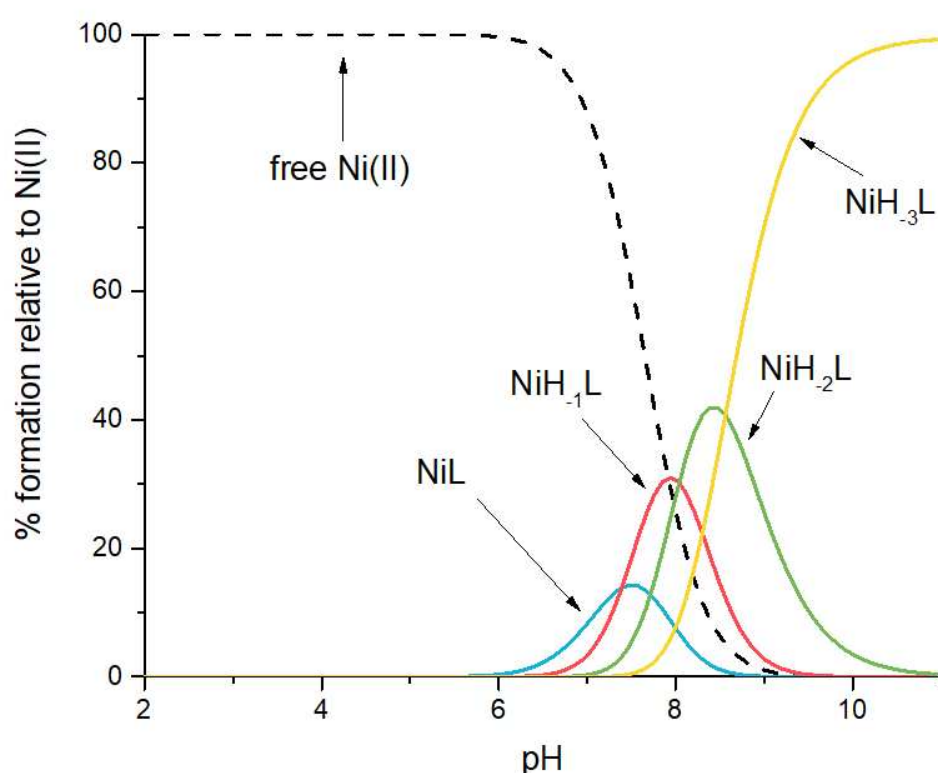


Figure 88 Species distribution diagram for the formation of $Ni(II)$ complex with the $Ac-AHCA-NH_2$, $T = 298\text{ K}$, $I = 0.1\text{ M NaClO}_4$, $[L] = 0.0005\text{ M}$, $Ni(II)/L$ molar ratio = 1 : 1

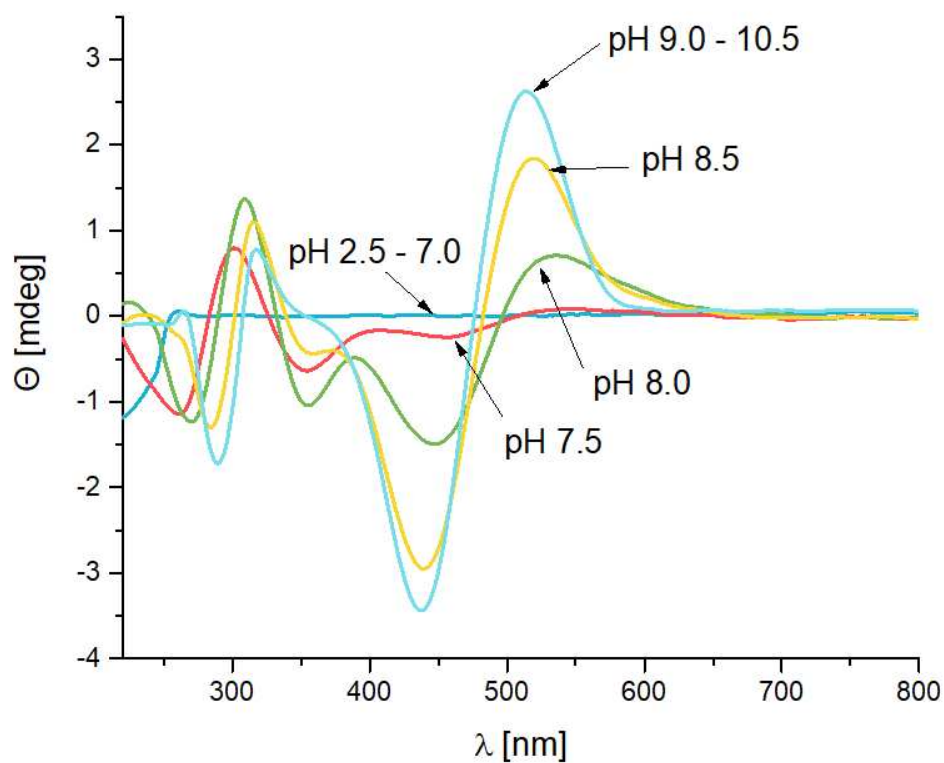


Figure 89 CD spectra of Ni(II) complex with the Ac-AHCA-NH₂ in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

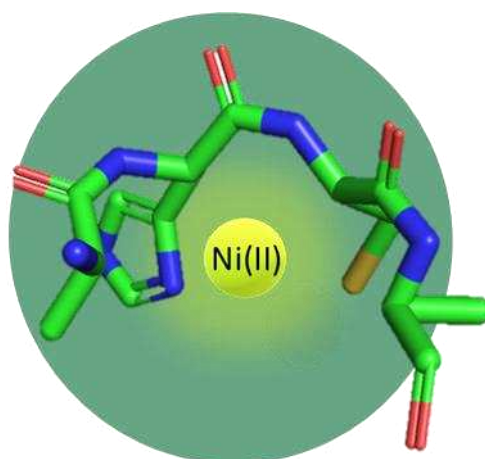


Figure 90 Proposed binding mode for Ni(II)-Ac-AHCA-NH₂

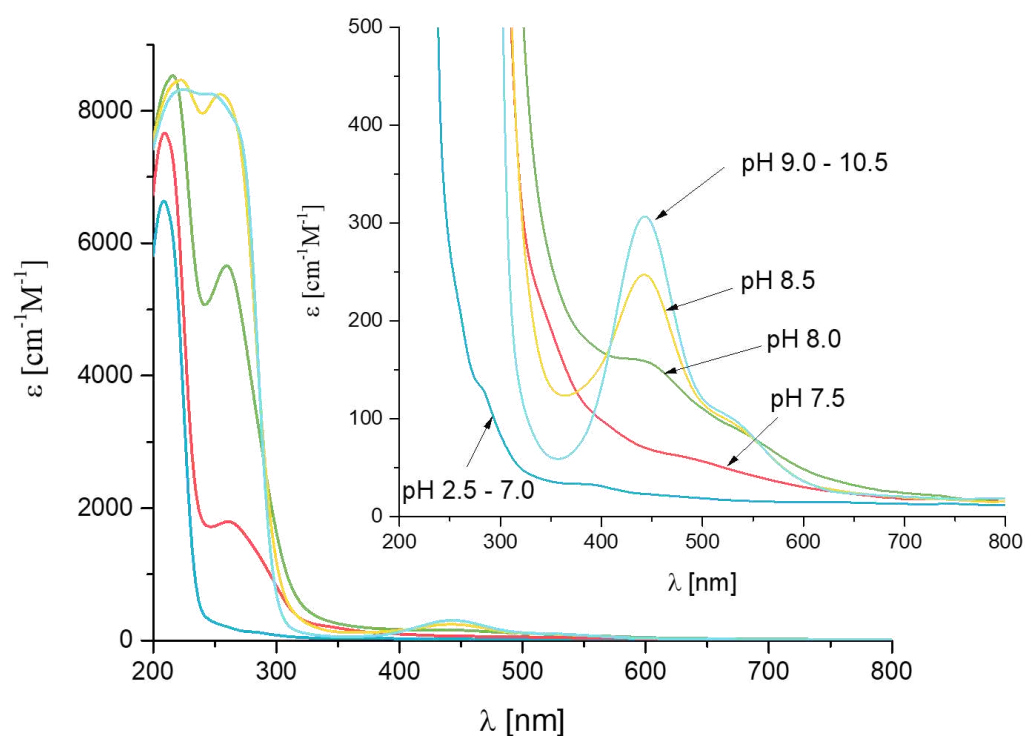


Figure 91 UV-Vis spectra of Ni(II) complex with the Ac-AHCA-NH₂ in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

b) Ac-ACHA-NH₂

The Ac-ACHA-NH₂ peptide also behaves as an H₂L acid with the deprotonating groups corresponding to the His and Cys residues with pK_a values: 6.42 and 8.76, respectively (Table 8).

(1) Zn(II)-Ac-ACHA-NH₂

Ac-ACHA-NH₂ coordination to Zn(II) results in three different species in the studied pH range: ZnL²⁺, ZnH₋₁L⁺ and ZnH₋₂L. ZnL²⁺ is the first detected species, where most probably histidine and cysteine residues are already bound to Zn(II). This form has a maximum of around pH 7.0 (Table 8 and Figure 92). The second one, ZnH₋₁L⁺, which dominates in solution in the pH range 8.0 - 10.0, is most likely related to the deprotonation of the first water molecule bound to the central Zn(II). Most probably, in the ZnH₋₂L complex, a subsequent coordinated water molecule deprotonates (Figure 93).

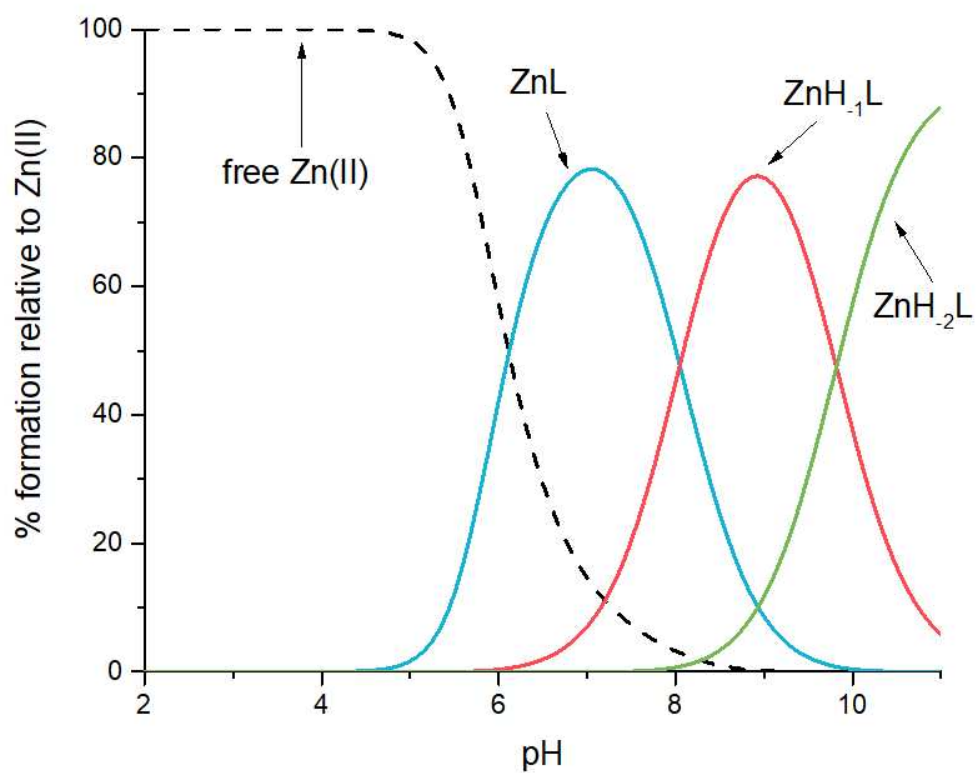


Figure 92 Species distribution diagram for the formation of Zn(II) complex with the Ac-ACHA-NH₂, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

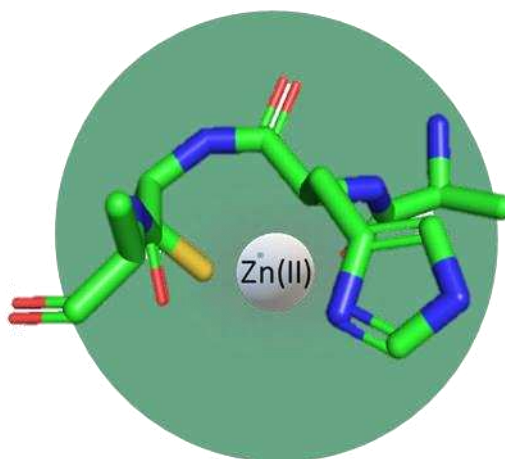


Figure 93 Proposed binding mode for Zn(II)-Ac-ACHA-NH₂

(2) Ni(II)-Ac-ACHA-NH₂

In the studied pH range (2.5- 10.5) and at a metal:ligand molar ratio of 1:1, we observed four different species of Ni(II) complex (Table 8 and Figure 94). The first species is a bis-complex with two ligand molecules bound to the metal (NiL_2). This species occurs at pH 6.0 and achieves maximum concentration at around pH 8.0. Spectroscopic data obtained in this pH range, in particular the shoulders in the spectra at around 500 and 410 nm, suggest that in NiL_2 , Ac-ACHA- NH_2 binds Ni(II) via four donor atoms in a $\{2\text{S}^-, 2\text{N}_{\text{im}}\}$ binding mode (Figure 95). This is consistent with previously studied Ni(II) complexes with Cys- and His-containing peptides. CD spectra also confirm this finding with pronounced d-d bands at 305 nm, which can be assigned to $\text{S}^- \rightarrow \text{Ni(II)}$ charge transfer transitions [596]. In the NiH_{-1}L form, apart from Cys and His side chains, an amide also takes part in metal binding, as confirmed by the presence of negative and positive bands at 550 and 480 nm in the CD spectra (Figure 96) [538]. Above pH 8.5, the square planar nickel complex reorganizes; the next two forms, $\text{NiH}_{-2}\text{L}^-$ and $\text{NiH}_{-3}\text{L}^{2-}$, most likely involve additional amide nitrogen atoms and a water molecule in binding, which can be confirmed via d-d transitions in the UV-Vis spectra at around 430 nm (Figure 97).

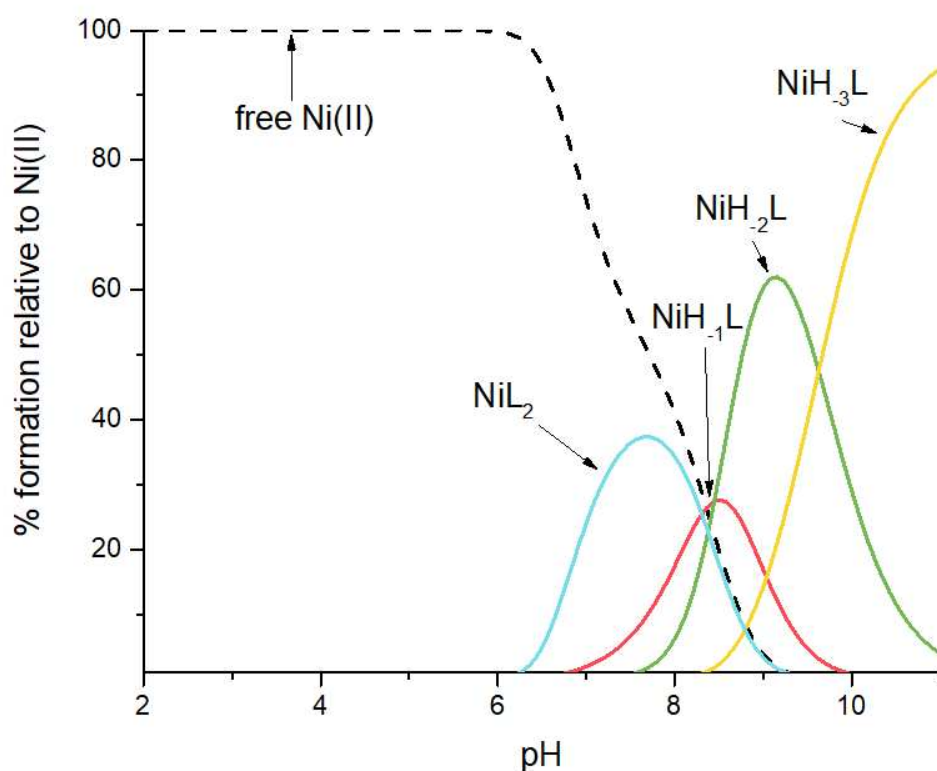


Figure 94 Species distribution diagram for the formation of Ni(II) complex with the Ac-ACHA- NH_2 , $T = 298\text{ K}$, $I = 0.1\text{ M NaClO}_4$, $[\text{L}] = 0.0005\text{ M}$, $\text{Ni(II)}/\text{L}$ molar ratio = 1 : 1

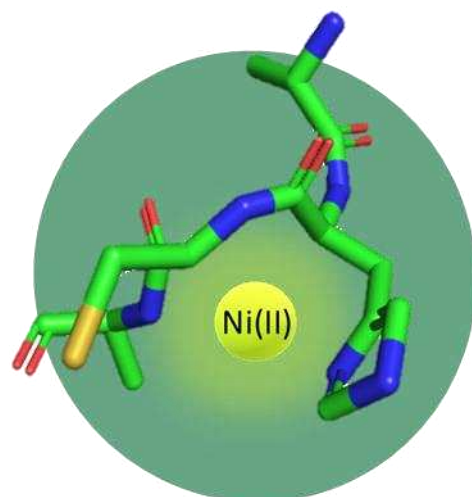


Figure 95 Proposed binding mode for Ni(II)-Ac-ACHA-NH₂

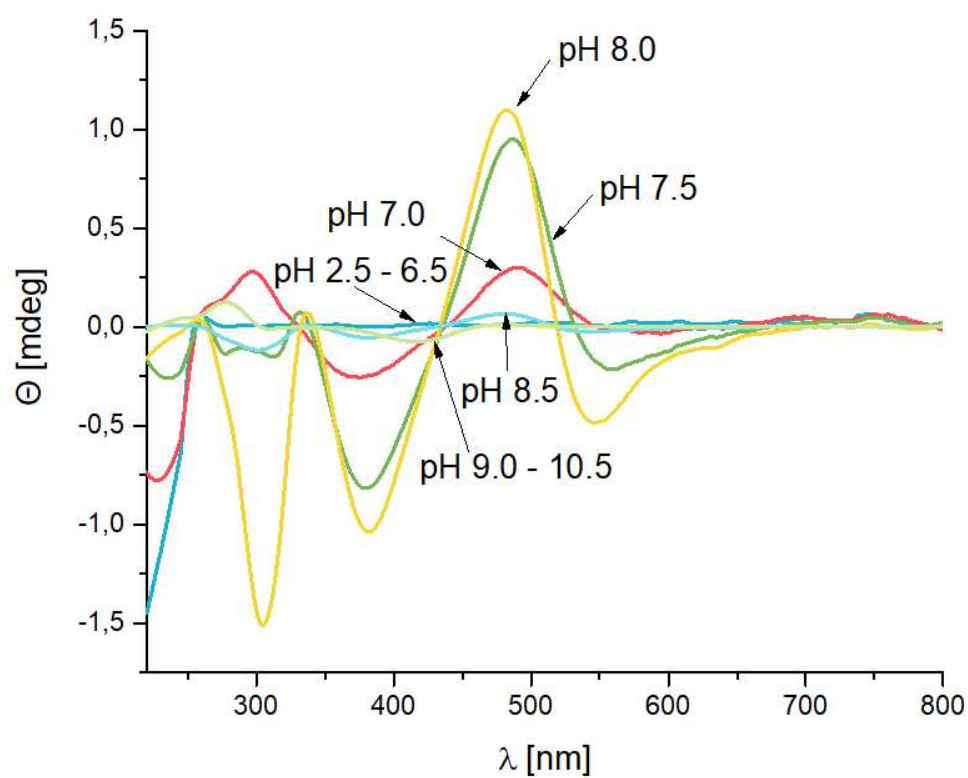


Figure 96 CD spectra of Ni(II) complex with the Ac-ACHA-NH₂ in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

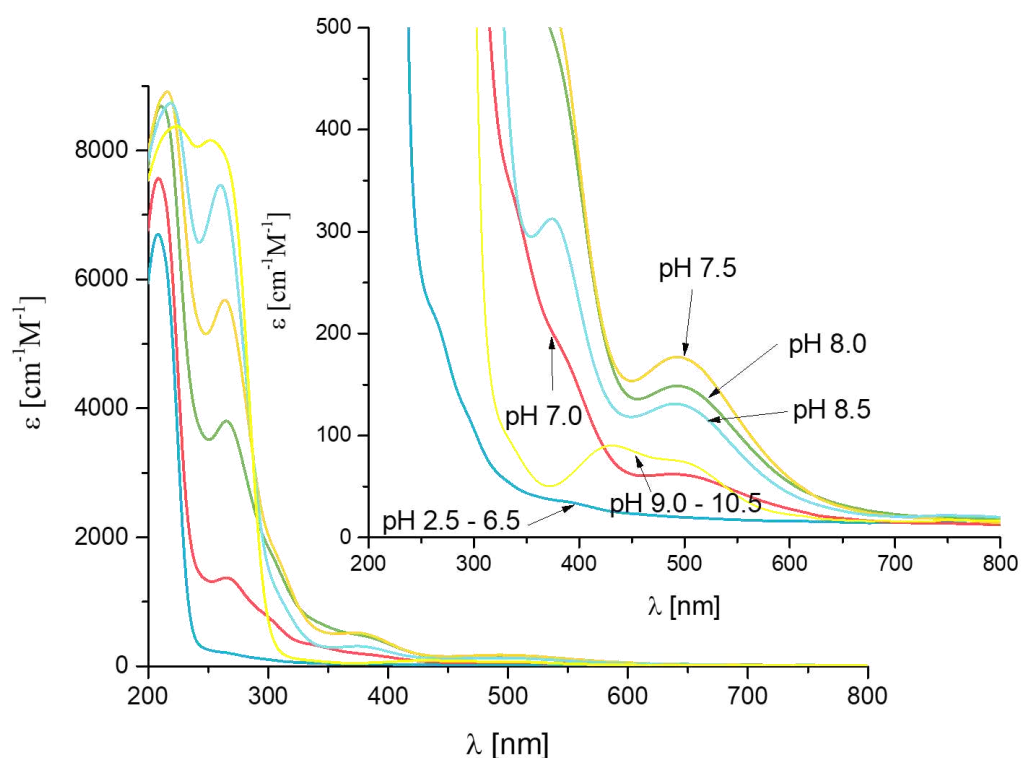


Figure 97 UV-Vis spectra of Ni(II) complex with the Ac-ACHA-NH₂ in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

2. Comparison of the stability of Zn(II) and Ni(II) complexes

To compare the affinity of Zn(II) and Ni(II) binding to Ac-AHCA-NH₂, Ac-ACHA-NH₂ ligands and other peptides described in the literature, I used their calculated stability constants to prepare so-called competition plots, which illustrate the comparison of the complexes' stability. Such plots show a theoretical situation in which equimolar amounts of all reagents, metals and peptides are mixed.

In the whole pH range, the Ac-ACHA-NH₂ and Ac-AHCA-NH₂ bind Zn(II) with comparable affinity (Figure 98). In both cases, the coordination of Zn(II) involves one histidine imidazole and one cysteine thiolate, and the coordination sphere is filled with two water molecules. The results suggest that the location of the histidine and cysteine residues, which are next to each other, does not have a significant effect on the stability of the Zn(II) complexes- Zn(II) is equally ready to bind to the HC motif as it is to bind to the CH one.

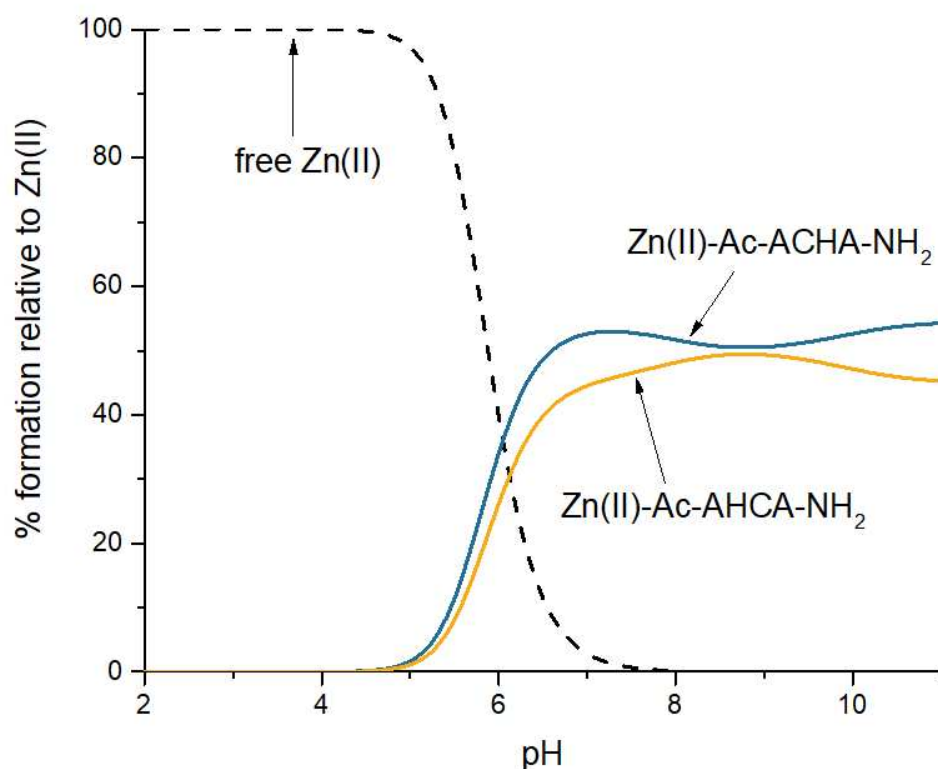


Figure 98 A competition plot between ligands: Ac-AHCA-NH₂, Ac-ACHA-NH₂ and Zn(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 8. Conditions: 298 K, I = 0.1 M NaClO₄, [Zn(II)] = [Ac-AHCA-NH₂] = [Ac-ACHA-NH₂] = 0.001 M

Nickel's preferences in binding Ac-ACHA-NH₂ and Ac-AHCA-NH₂ are substantially different (Figure 99). Ni(II) binds to both ligands with comparable affinity up to pH 8, while at a higher pH, at which amides take part in binding, Ni(II) coordination to Ac-AHCA-NH₂ is strongly preferred, suggesting that the {S⁻, 2N⁻} binding mode is more stable than the {N_{im}, 2N⁻} one.

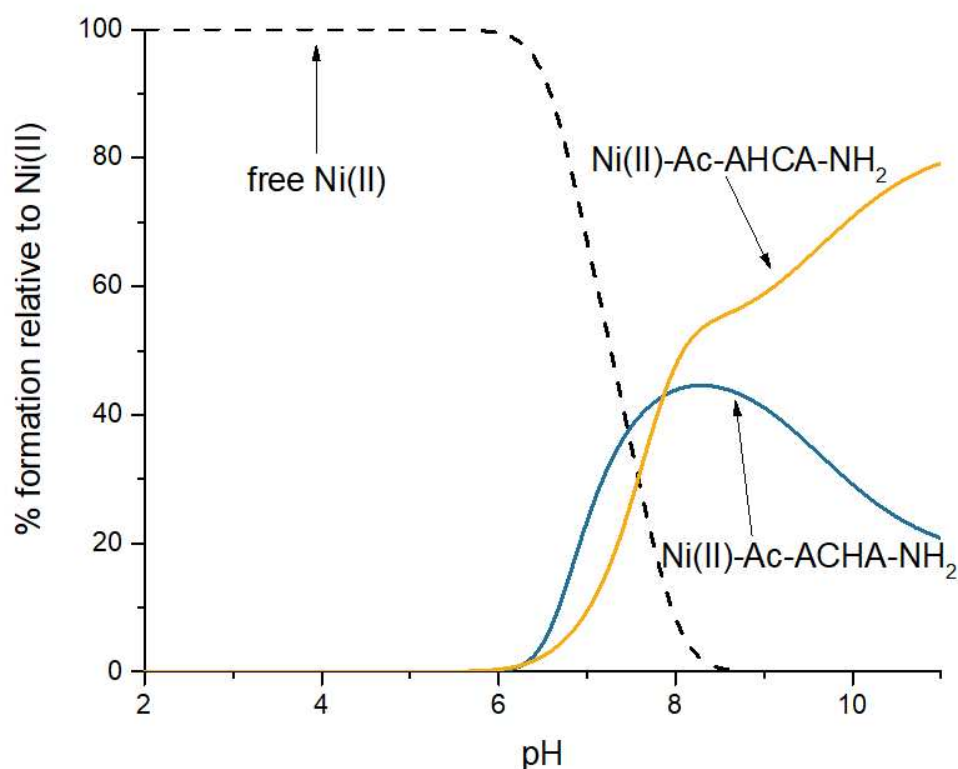


Figure 99 A competition plot between ligands: Ac-AHCA-NH₂, Ac-ACHA-NH₂ and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 8. Conditions: 298 K, I = 0.1 M NaClO₄, [Ni(II)] = [Ac-AHCA-NH₂] = [Ac-ACHA-NH₂] = 0.001 M

Which is the preferred Zn(II) binding site—CH/HC or CC? Figure 100 gives us a straightforward answer—the 'CC' motif clearly binds Zn(II) with higher affinity, at least in the compared sequences: Ac-CC-NH₂, Ac-GSCCHTGNHD-NH₂, Ac-EEGCCHGHHE-NH₂ and Ac-CCSTSDSHHQ-NH₂ [187].

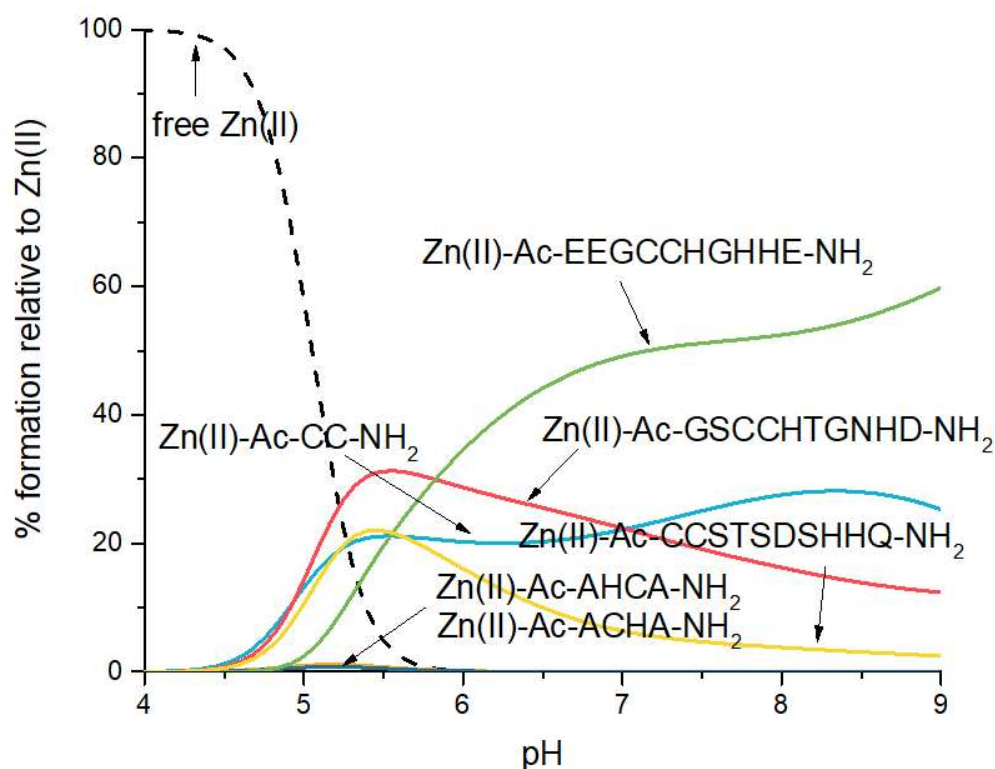


Figure 100 A competition plot between ligands: Ac-AHCA-NH₂, Ac-ACHA-NH₂, Ac-CC-NH₂, Ac-GSCCHTGNHD-NH₂, Ac-EEGCCHGHHE-NH₂, Ac-CCSDSHHQ-NH₂ and Zn(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 8 and [187]. Conditions: 298 K, I = 0.1 M NaClO₄, [Zn(II)] = [Ac-AHCA-NH₂] = [Ac-ACHA-NH₂] = [Ac-CC-NH₂] = [Ac-GSCCHTGNHD-NH₂] = [Ac-CCSDSHHQ-NH₂] = 0.001 M

Thiolates are clearly the most likely binding sites for Zn(II)—our HC/CH ligands (Ac-AHCA-NH₂ and Ac-ACHA-NH₂) bind this metal much more effectively than peptides which contain two His imidazoles (Ac-GHEITHG-NH₂, Ac-GHTIEHG-NH₂, Figure 101), showing that the Zn(II)-N_{im}, S⁻ complex turns out to be thermodynamically stronger than Zn(II)-2N_{im}- in our hypothetical situation, at physiological pH (pH 7.4), nearly 50% of Zn(II) is bound to Ac-ACHA-NH₂, nearly 40% of Zn(II) is bound to Ac-AHCA-NH₂ and only around 15% is bound to Ac-GHEITHG-NH₂ and Ac-GHTIEHG-NH₂ [591].

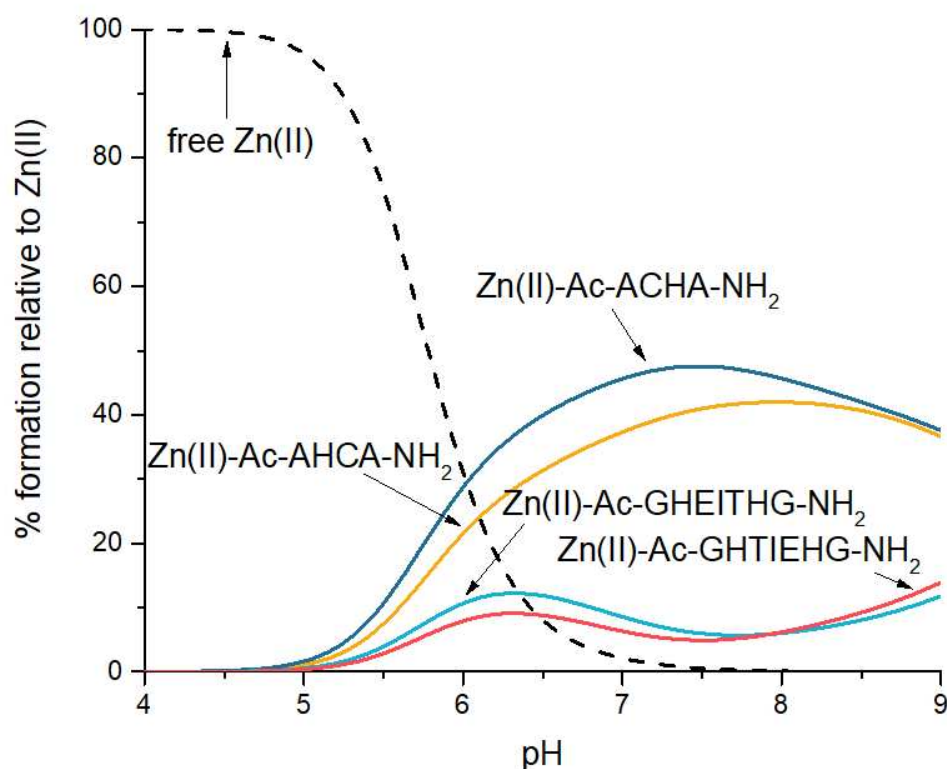


Figure 101 A competition plot between ligands: Ac-AHCA-NH₂, Ac-ACHA-NH₂, Ac-GHEITHG-NH₂, Ac-GHTIEHG-NH₂ and Zn(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 8 and [591]. Conditions: 298 K, I = 0.1 M NaClO₄, [Zn(II)] = [Ac-AHCA-NH₂] = [Ac-ACHA-NH₂] = [Ac-GHEITHG-NH₂] = [Ac-GHTIEHG-NH₂] = 0.001 M

Nickel's preferences for CH- and HC-type ligands differ somewhat from those of Zn(II). Ni(II) forms very stable square-planar complexes with the CC motif, binding to two thiolates and the amide in between. Ni(II)-CC complexes are, as Zn(II) complexes, more stable than CH and HC ones, but for different reasons- in the case of Zn(II), the affinity for sulfur seems to be the reason for the high affinity, while in the case of Ni(II), the planar geometry and additional stabilization by the amide seem to play the major role (the competition plot between Ni(II), Ac-AHCA-NH₂, Ac-ACHA-NH₂, Ac-CC-NH₂, Ac-GSCCHTGNHD-NH₂, Ac-EEGCCHGHHE-NH₂ and Ac-CCSTSDSHHQ-NH₂ [187] is shown on Figure 102).

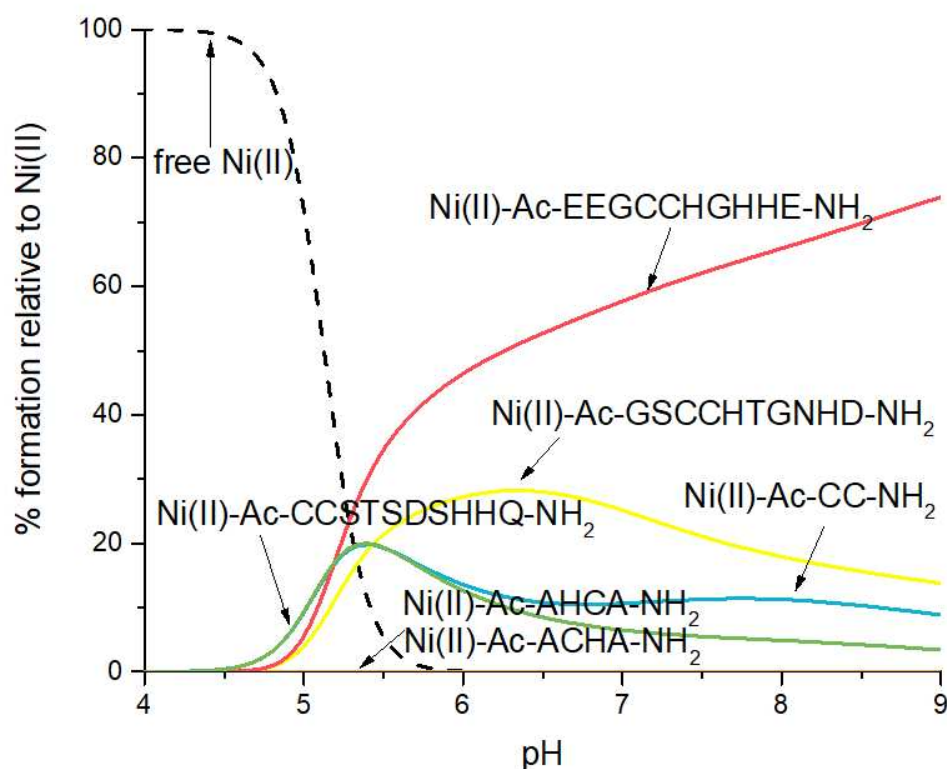


Figure 102 A competition plot between ligands: Ac-AHCA-NH₂, Ac-ACHA-NH₂, Ac-CC-NH₂, Ac-GSCCHTGNHD-NH₂, Ac-EEGCCHGHHE-NH₂, Ac-CCSTSDSHHQ-NH₂ and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 8 and [187]. Conditions: 298 K, I = 0.1 M NaClO₄, [Ni(II)] = [Ac-AHCA-NH₂] = [Ac-ACHA-NH₂] = [Ac-CC-NH₂] = [Ac-GSCCHTGNHD-NH₂] = [Ac-CCSTSDSHHQ-NH₂] = 0.001 M

What is the impact of the non-binding residues on the Ni-CH complex stabilities with a {N_{im}, N⁻, S⁻} binding mode? As hypothesized, the non-binding residues are able to protect the complex from hydrolysis, most likely forming a network of hydrogen bonds that protects the central Ni(II) ion. In our hypothetical situation in Figure 103, Ni(II) complexes with Ac-AHCA-NH₂ and Ac-ACHA-NH₂ are substantially weaker than those with Ac-GGKPDLRPCHP-NH₂ and Ac-PCHYIPRPKPR-NH₂ [610].

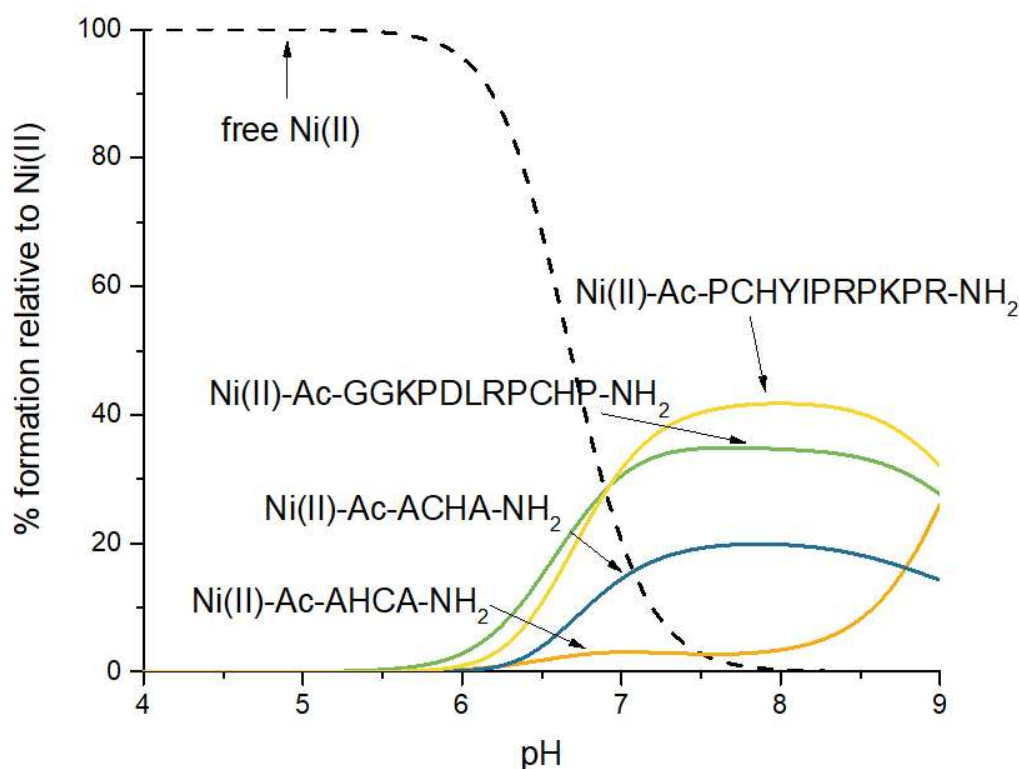


Figure 103 A competition plot between ligands: Ac-AHCA-NH₂, Ac-ACHA-NH₂, Ac-GGKPDLRPCHP-NH₂, Ac-PCHYIPRPKPR-NH₂, Ac-PCHPPCHYIPR-NH₂ and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 8 and [610]. Conditions: 298 K, I = 0.1 M NaClO₄, [Ni(II)] = [Ac-AHCA-NH₂] = [Ac-ACHA-NH₂] = [Ac-GGKPDLRPCHP-NH₂] = [Ac-PCHYIPRPKPR-NH₂] = [Ac-PCHPPCHYIPR-NH₂] = 0.001 M

The position of His and Cys residues affects the stability of the complexes with Ni(II), but not those with Zn(II). Ni(II) will more likely bind to Ac-AHCA-NH₂ than to Ac-ACHA-NH₂ above pH 8.0, when amide nitrogen atoms begin to take part in coordination (Figure 104 and Figure 105). Moreover, His-Cys and Cys-His ligands do have a strong metal preference; up to pH 8.5, Zn(II) complexes are clearly the strongest ones. At alkaline pH (above 9.0), in which amides start to bind Ni(II), the square planar Ni(II) species become strongest (Figure 104 and Figure 105). Non-binding residues have a clear enhancing effect on the stability of Ni(II) complexes, most likely through shielding the central Ni(II) ion from surrounding solvent molecules.

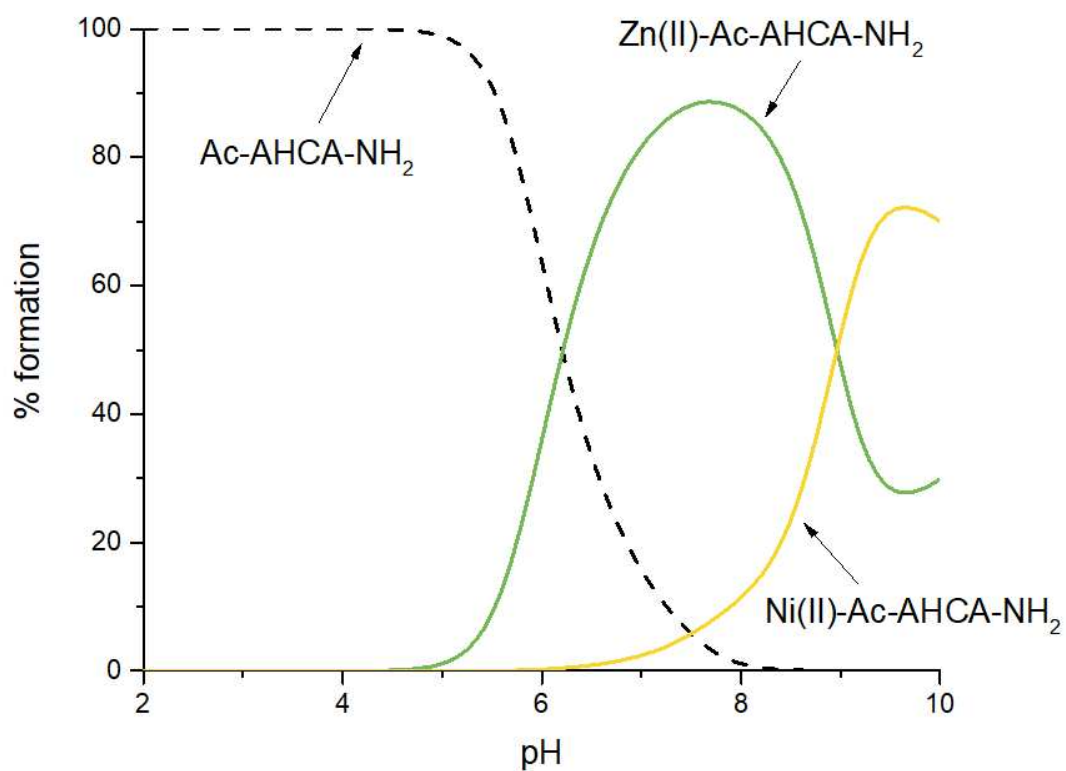


Figure 104 A competition plot between the Ac-AHCA-NH₂ peptide, Zn(II) and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 8. Conditions: 298 K, I = 0.1 M NaClO₄, [Zn(II)] = [Ni(II)] = [Ac-AHCA-NH₂] = 0.001 M

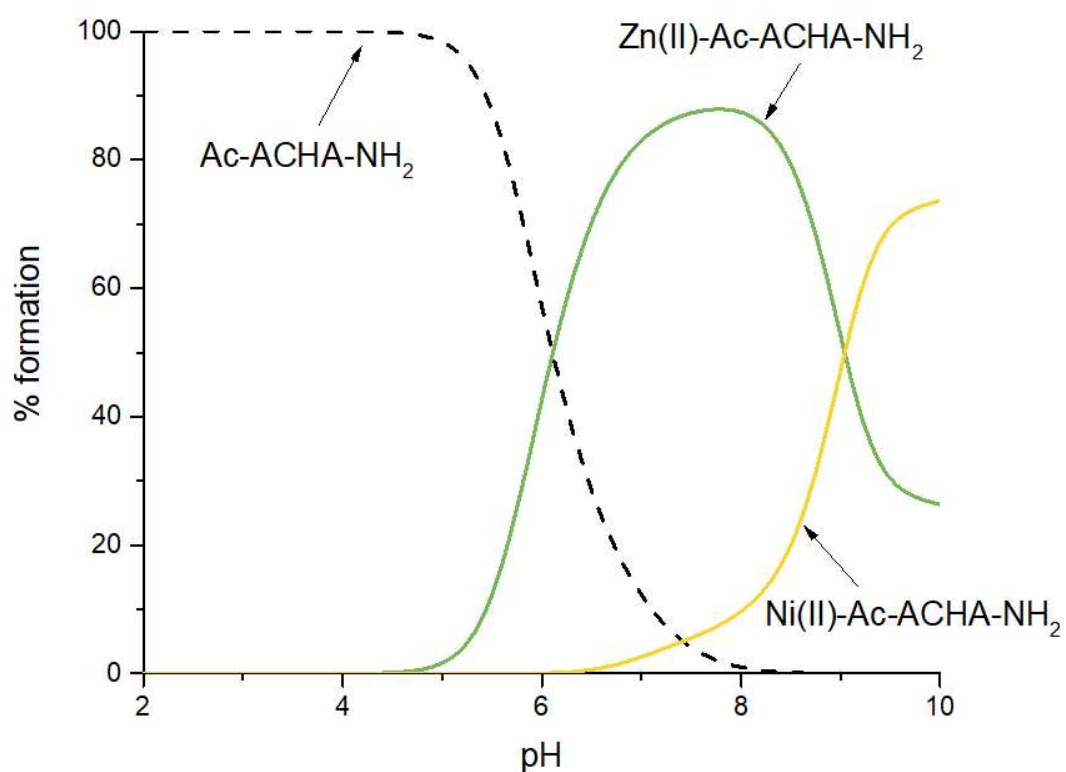


Figure 104 A competition plot between the Ac-ACHA-NH₂ peptide, Zn(II) and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations

are based on binding constants from Table 8. Conditions: 298 K, I = 0.1 M NaClO₄, [Zn(II)] = [Ni(II)] = [Ac-ACHA-NH₂] = 0.001 M

3. Conclusions

The position of His and Cys residues affects the stability of the complexes with Ni(II), but not those with Zn(II). Moreover, Ni(II) will more likely bind to Ac-AHCA-NH₂ than to Ac-ACHA-NH₂ above pH 8.0, when amide nitrogen atoms begin to take part in coordination. The measurements indicate that the His–Cys and Cys–His ligands do have a strong metal preference; up to pH 8.5, Zn(II) complexes are clearly the strongest ones. At alkaline pH (above 9.0), in which amides start to bind Ni(II), the square planar Ni(II) species become strongest. Furthermore, non-binding residues have a clear enhancing effect on the stability of Ni(II) complexes, most likely through shielding the central Ni(II) ion from surrounding solvent molecules.

D. Thermodynamic and structural properties of Zn(II) and Ni(II) complexes with unstructured fragments of ZnuA and AztC transporters

A Gram-negative, non-motile and denitrifying bacteria, *Paracoccus denitrificans*, and the mechanism of its Zn(II) transport are presented in point B.2. of the Introduction (Zn(II) transport in *Paracoccus denitrificans*) and in the article [492]. To understand the transport of Zn(II) in *Paracoccus denitrificans*, I focus on Zn(II) and Ni(II) complexes of two ZnuA regions (Ac-HEHEHEHEHEHEHEHEH-NH₂ and Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂) and one AztC regions (Ac-GGGHYHYIDGKAVFHAG-NH₂). The amino acid sequences of the ZnuA and AztC transporters are presented in Figure 105 and Figure 106, respectively. The probable Zn(II)-binding sites are highlighted in different colors. I explain their structural and thermodynamic properties, using a variety of complementary methods: mass spectrometry, potentiometry, UV-Vis spectroscopy and CD spectroscopy. As usual, the ZnuA and AztC regions are chosen on the basis of three criteria:

- (i) having probable Zn(II)-binding residues,
- (ii) being unstructured,
- (iii) being evolutionally conserved sites,

and therefore, they are appropriate for our peptide-based approach. The structure was predicted by Phyre2, a remote homology recognition technique, capable of generating reliable protein models [593]. I also check the thermodynamic parameters of Ni(II) binding sites, keeping in mind that Ni(II) is a biologically relevant metal ion, essential for pathogens, and could theoretically compete with Zn(II) for the binding sites in its transporter. It is important to keep in mind that in the full-length protein, the metal binding sites might also occur in locally structured regions.

10	20	30	40	50
MIRPSSLVLA	AALGTAALPA	RAEVPRVVD	IPVVGAMVQQ	VMGDLGQPEI
60	70	80	90	100
LLQAGGDPHS	YQLRPSQARS	LQDADLLIWV	GPELTPWLER	AATSLSAQSE
110	120	130	140	150
MLALLDLPAT	HRRDYAGGEH	EHEHEHEHEH	EHEHEHDGHG	HAEEQAHHDH
160	170	180	190	200
DHSGTDPHAW	LDPANGQAWL	AGIAETLSRH	DPDNAGVYAA	NAAKAADEIA
210	220	230	240	250
ALDEELKSAL	TPTQGKRFVV	FHDAYGYFTE	HYGLEPAIAV	SLGDASTPSA
260	270	280	290	300
ARLRAIRDEI	AEEGAVCAFP	EANHDPKLI	AVIEGSEIRQ	GAALDPEGTG
310	320			
ATPGAGLYAE	LLRGMGQALA	DCLGAD		

Figure 105 Amino acid sequence of ZnuA transporter; probable Zn(II) binding sites are highlighted in different colors

10	20	30	40	50
MKDWLFRIAT	CSIMTFSSLA	AAQAEPLDVV	ATFSIIGDFA	AKVGGDRIRL
60	70	80	90	100
NVLVGPDSDT	HVYEPRPADA	IALAGADVVL	TNGLEFEGFL	TRLIAASGTD
110	120	130	140	150
AAVATLTLDGV	ETMEEPGGGH	YHYIDGKAVF	HAGAHDPHAW	QAVPNAKVYV
160	170	180	190	200
QNIAAAFCAA	DAEGCAAYQA	NAARYIGELD	ALDTEIRAAI	AALPQDRRTV
210	220	230	240	250
VVAHNAFRYF	EAAYGVHFLS	PQGVSTESAE	AAADVAGLIR	EIRARNASAI
260	270	280	290	300
FAENISDTRL	LEQIAREAGL	PLAGTLYSDA	LSGPDGPASN	YIAMMRHNAG
310				
AIAAALAAR				

Figure 106 Amino acid sequence of AztC transporter; probable Zn(II) binding sites are highlighted in different colors

1. Stoichiometry of Zn(II) and Ni(II) complexes with ZnuA and AztC fragments

Mass spectrometry confirmed peptide purity and showed that all ligands form mononuclear complexes with Zn(II) and Ni(II).

a) Ac-HEHEHEHEHEHEHEHEH-NH₂

The signal: $m/z = 776.30$, $z = 3+$ correspond to the free ligand for the Ac-HEHEHEHEHEHEHEHEH-NH₂ (fragment of the ZnuA protein). The assignment peaks were compared to simulated isotopic patterns, which fit perfectly to experimental data. In all measured mass spectra, the signals corresponding to the sodium and potassium adducts of free ligands were observed. In the mass spectra of Zn(II)-Ac-HEHEHEHEHEHEHEHEH-NH₂ (Figure 107A) beside the signals from the ligand and its sodium and potassium adducts, I observe a signals which comes from the Zn(II) complex ($m/z = 797.27$, $z = 3+$) and its sodium ($m/z = 804.59$, $z = 3+$), chloride ($m/z = 809.62$, $z = 3+$), sodium and one chloride ($m/z = 816.61$, $z = 3+$), potassium ($m/z = 821.98$, $z = 3+$) and potassium and sodium ($m/z = 842.60$, $z = 3+$) adducts. In the case of Ni(II)-Ac-HEHEHEHEHEHEHEHEH-NH₂ (Figure 107B) the signal ($m/z = 794.93$, $z = 3+$) correspond to the equimolar Ni(II) complex. In these spectra also the sodium ($m/z = 802.26$, $z = 3+$); ($m/z = 808.95$, $z = 3+$) adducts of the nickel peptide are present and the signals correspond to adducts with one sodium atom and two potassium atoms.

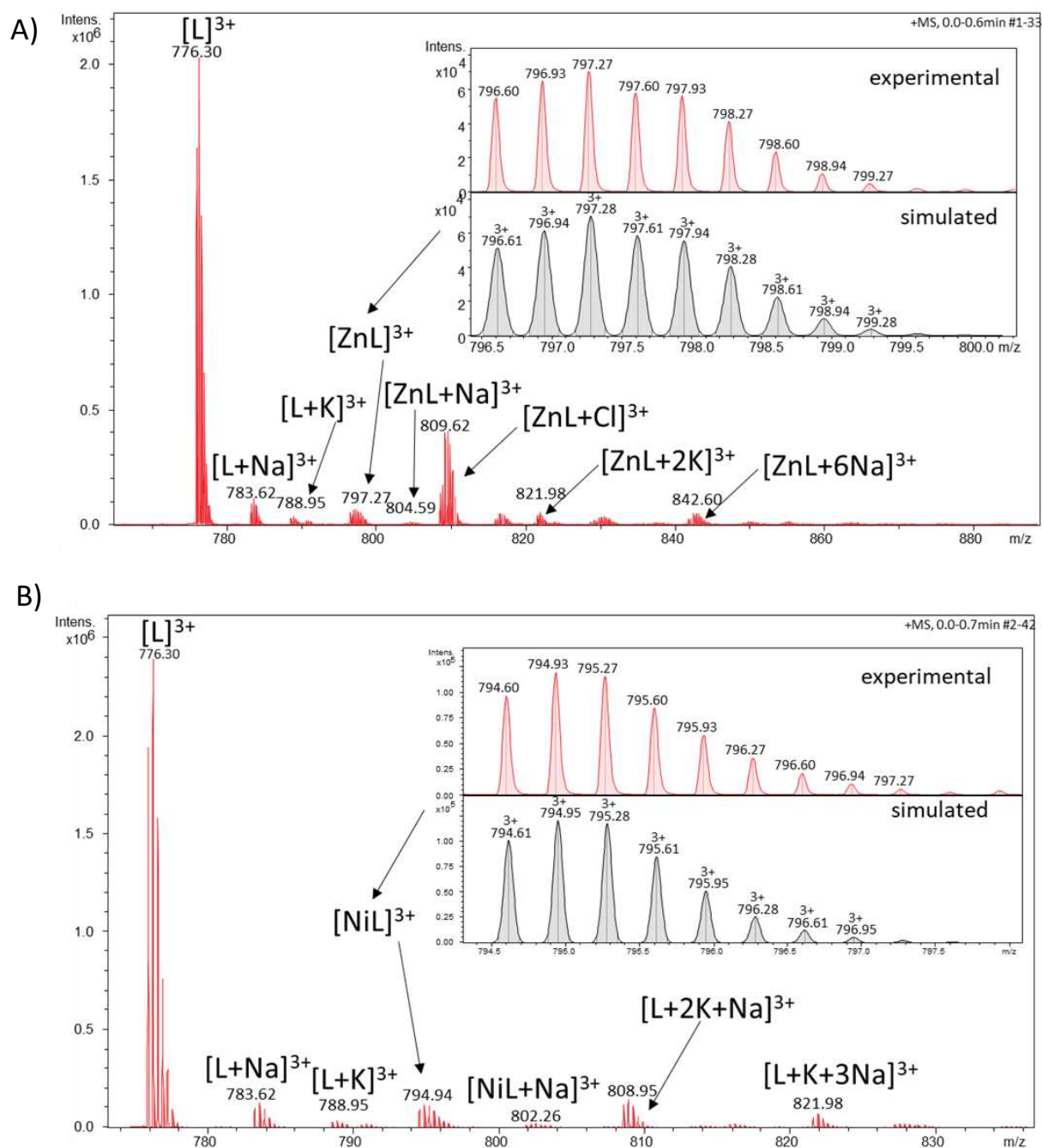


Figure 107 ESI-MS spectrum of A) Zn(II)-Ac-HEHEHEHEHEHEHEHEH-NH₂ and B) Ni(II)-Ac-HEHEHEHEHEHEHEHEH-NH₂

b) Ac-GGEHEHEHEHEHEHEHEHEHDGHHGAEEQAHHDDHDSG-NH₂

The signal: m/z = 1131.92, z = 4+ and m/z = 914.44, z = 2+ correspond to the free ligand for the Ac-GGEHEHEHEHEHEHEHEHEHDGHHGAEEQAHHDDHDSG-NH₂ (fragment of the ZnuA protein). In the Zn(II)-Ac-GGEHEHEHEHEHEHEHEHEHDGHHGAEEQAHHDDHDSG-NH₂ spectra, aside from the signals which comes from the free ligand and its adducts, a equimolar Zn(II) complex (m/z = 1147.90, z = 4+), its different adducts can be observed (Figure 108A). In the spectra of the same ligand with Ni(II) (Figure 108B), the signals can be assigned to the nickel

complex ($m/z = 1146.16$, $z = 4+$) and a nickel complex with a sodium adduct ($m/z = 1151.91$, $z = 4+$), potassium adduct ($m/z = 1156.41$, $z = 4+$) and sodium chloride adduct ($m/z = 1166.18$, $z = 4+$).

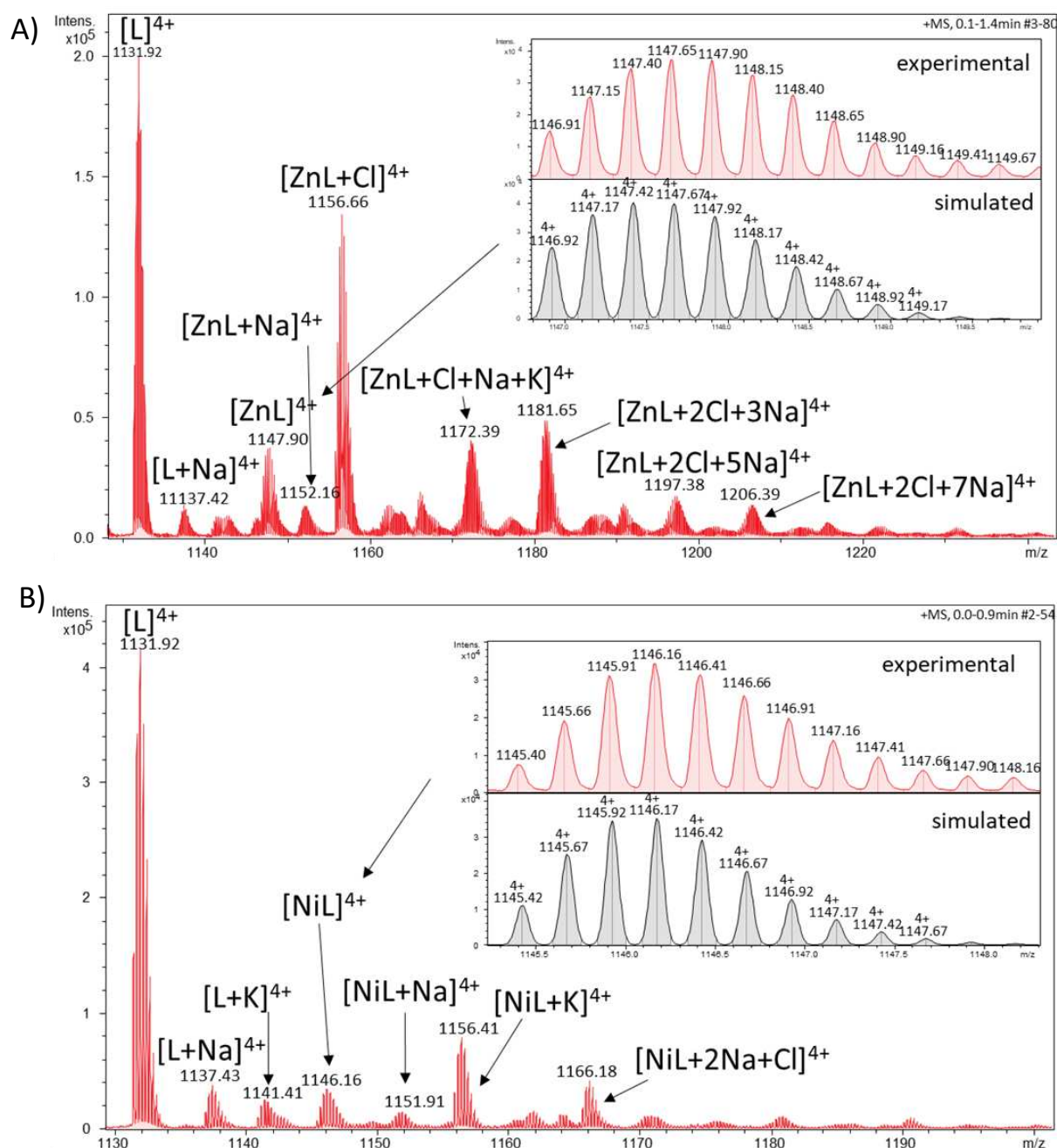


Figure 108 ESI-MS spectrum of A) Zn(II)-Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDS-NH₂ and B) Ni(II)-Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDS-NH₂

c) Ac-GGGHYHYIDGKAVFHAG-NH₂

The signal: $m/z = 914.44$, $z = 2+$ correspond to the free ligand for the Ac-GGGHYHYIDGKAVFHAG-NH₂ (fragment of the AztC protein). In the case of Zn(II)-Ac-GGGHYHYIDGKAVFHAG-NH₂ mass spectra (Figure 109A) the prevailing signals correspond to

the Zn(II) complex ($m/z = 945.90$, $z = 2+$), its sodium adducts ($m/z = 955.89$, $z = 2+$) and its potassium adducts ($m/z = 964.88$, $z = 2+$). In the Ni(II)-Ac-GGGHYHYIDGKAVFHAG-NH₂ spectra (Figure 109B) the signals which come from Ni(II) complex ($m/z = 942.40$, $z = 2+$), its sodium adducts ($m/z = 953.39$, $z = 2+$) and potassium adducts ($m/z = 961.38$, $z = 2+$) are observed.

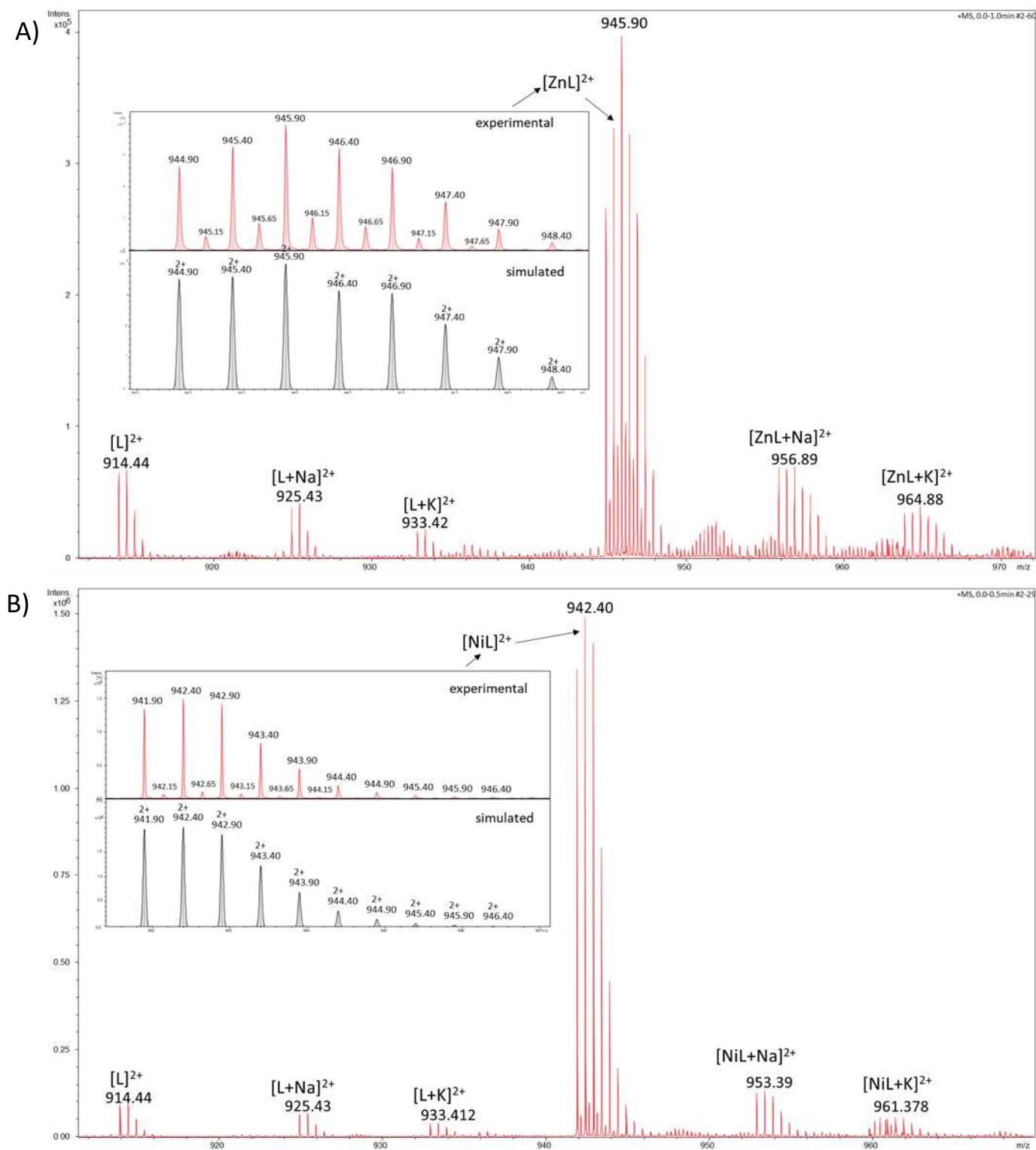


Figure 109 ESI-MS spectrum of A) Zn(II)-Ac-GGGHYHYIDGKAVFHAG-NH₂ and B) Ni(II)-Ac-GGGHYHYIDGKAVFHAG-NH₂

2. Zn(II) and Ni(II) coordination modes

a) Ac-HEHEHEHEHEHEHEHEH-NH₂

The Ac-HEHEHEHEHEHEHEHEH-NH₂ fragment behaves as an [H₁₇L]⁹⁺ acid with the protonating groups corresponding to the glutamic acid side chain groups with pK_a values of 2.62, 3.35, 3.42, 3.82, 4.15, 4.22, 4.72, and 4.94 and histidine imidazole side chain groups with pK_a values of 5.74, 5.99, 6.45, 6.54, 6.96, 7.01, 7.48, 7.62 and 8.30, respectively (Table 9).

Table 9 Potentiometric data for the proton, Zn(II) and Ni(II) complexes of the Ac-HEHEHEHEHEHEHEHEH-NH₂, Ac-GGEHEHEHEHEHEHEHEHDGHGHAEEQAHHDDHDSG-NH₂ and Ac-GGGHYHYIDGKAVFHAG-NH₂ in a water solution of 4 mM HClO₄ with *I* = 0.1 M NaClO₄, *T* = 298 K. Standard deviations are presented in parentheses

Species	Ac-HEHEHEHEHEHEHEHEH-NH ₂			Ac-GGEHEHEHEHEHEHEHEH DGHGHAEEQAHHDDHDSG-NH ₂			Ac-GGGHYHYIDGKAVFHAG-NH ₂		
	logβ	pK _a	residue	logβ	pK _a	residue	logβ	pK _a	residue
H ₂₆ L				144.67 (2)		(2E)			
H ₂₄ L				138.17 (3)	3.00	(E)			
H ₂₃ L				135.17 (2)		(2E)			
H ₂₁ L				127.61 (4)	3.70	(E)			
H ₂₀ L				123.91 (3)		(2E)			
H ₁₈ L				115.47 (3)	4.43	(E)			
H ₁₇ L	93.34 (4)	2.62	(E)	111.04 (3)		(2E)			
H ₁₆ L	90.72 (3)	3.35	(E)						
H ₁₅ L	87.37 (4)	3.42	(E)	101.04 (3)		(2H)			
H ₁₄ L	83.95 (4)	3.82	(E)						
H ₁₃ L	80.13 (3)	4.15	(E)	90.77 (2)		(2H)			
H ₁₂ L	75.98 (3)	4.22	(E)						
H ₁₁ L	71.76 (2)	4.72	(E)	78.79 (2)		(2H)			
H ₁₀ L	67.04 (2)	4.94	(E)						
H ₉ L	62.10 (1)	5.74	(H)	66.02 (2)		(2H)			
H ₈ L	56.36 (1)	5.99	(H)						
H ₇ L	50.37 (2)	6.45	(H)	52.63 (2)		(2H)	54.37 (1)	3.58	(D)
H ₆ L	43.92 (2)	6.54	(H)				50.79 (1)	5.97	(H)
H ₅ L	37.38 (2)	6.96	(H)	38.57 (2)		(2H)	44.82 (1)	6.24	(H)
H ₄ L	30.42 (2)	7.02	(H)				38.58 (1)	7.71	(H)
H ₃ L	23.40 (1)	7.48	(H)	23.97 (1)		(2H)	30.87 (1)	9.69	(Y)
H ₂ L	15.92 (1)	7.62	(H)				21.18 (1)	10.14	(Y)
HL	8.30 (1)	8.30	(H)	8.31 (2)	8.31	(H)	11.04 (1)	11.04	(K)
Zn(II) complexes									
ZnH ₁₃ L				95.19 (1)					
ZnH ₁₁ L				84.57 (1)					
ZnH ₉ L				72.72 (1)					
ZnH ₇ L	55.19 (2)			59.99 (1)					
ZnH ₆ L	49.88 (1)	5.30	(H)						
ZnH ₅ L				46.27 (1)					
ZnH ₄ L	37.64 (1)			39.28 (1)	6.99	(H)			
ZnH ₃ L	30.77 (2)	6.87	(H)				38.75 (1)		
ZnH ₂ L	23.83 (2)	6.94	(H)	24.55 (1)			30.93 (1)	7.82	(H ₂ O)
ZnHL	16.82 (1)	7.01	(H)	16.42 (1)	8.13	(H)	21.49 (1)	9.44	(Y)
ZnL	8.51 (2)	8.31	(H _{non-binding})	8.22 (1)	8.20	(H)	11.65 (1)	9.84	(Y)
ZnH ₋₁ L	-0.95 (2)	9.46	(H ₂ O)	-0.59 (1)	8.81	(H ₂ O)	0.60 (1)	11.05	(K)
Ni(II) complexes									
NiH ₂₀ L				127.79 (1)					

NiH ₁₇ L				115.68 (1)				
NiH ₁₅ L				106.67 (1)				
NiH ₁₃ L				97.11 (1)				
NiH ₁₁ L				86.65 (1)				
NiH ₉ L				75.81 (2)				
NiH ₈ L	60.96 (2)							
NiH ₇ L				63.99 (3)				
NiH ₆ L	49.83 (2)			57.98 (5)	6.01 (H)			
NiH ₅ L	44.37 (3)	5.46 (H)						
NiH ₄ L				45.38 (4)		42.70 (1)		
NiH ₃ L	32.05 (1)			38.67 (5)	6.71 (H)	37.10 (1)	5.60 (H)	
NiH ₂ L	25.32 (2)	6.73 (H)				28.21 (1)	8.89 (am)	
NiHL	18.56 (2)	6.76 (H)		25.25 (2)				
NiL	11.52 (2)	7.04 (H)				9.79 (1)		
NiH ₋₁ L	3.65 (2)	7.87 (am)		10.82 (3)		-0.10 (2)	9.89 (Y)	
NiH ₋₂ L						-10.09 (2)	9.99 (Y)	
NiH ₋₃ L	-16.62 (2)			-4.61 (3)		-20.46 (2)	10.37 (K)	

(1) Zn(II)-Ac-HEHEHEHEHEHEHEH-NH₂

The first Zn(II)-Ac-HEHEHEHEHEHEHEH-NH₂ peptide complex observed at low pH, [ZnH₇L]⁺, reaches its maximum concentration at pH 5.3 and gets involved in binding two histidine residues (Figure 110). The zinc coordination sphere may also contain carboxylic oxygens from Glu residues. The loss of one proton leads to the [ZnH₆L] complex and it is most likely related to the coordination of an additional histidine residue. The loss of more protons leads to first the [ZnH₄L]²⁻ complex and then to the [ZnH₃L]³⁻ species, in which another histidine is possibly involved in Zn(II) binding. The loss of the next three protons leads to the formation of three species: [ZnH₃L], [ZnH₂L]⁴⁻ and [ZnHL]⁵⁻, which are related to the deprotonation of further histidine residues. Most probably, in each of these species, three or four imidazoles of histidine residues participate in Zn(II) binding (Figure 111), but the donor atoms can be different from one complex molecule to another. Thus, we can hypothesize that this Zn(II)-Ac-HEHEHEHEHEHEHEH-NH₂ complex system is an example of the so-called polymorphic binding site, with the metal “moving back and forth” along the polyhistidine region [611]. The loss of the next proton leads to the [ZnL]⁶⁻ form, with its maximum concentration at pH 8.88. The pK_a values calculated for the complexed (8.31) and free (8.30) ligands suggest that this histidine is not involved in Zn(II) binding. The last observed complex, [ZnH₋₁L]⁷⁻, most likely corresponds to the formation of a hydroxylated complex.

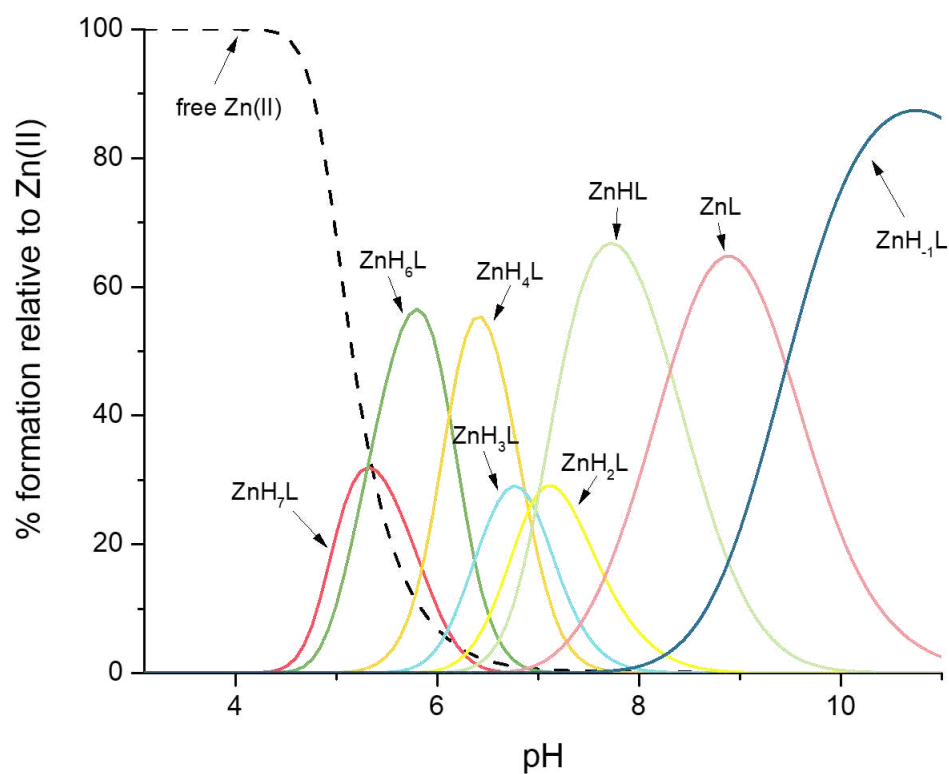


Figure 110 Species distribution diagram for the formation of Zn(II) complex with the Ac-HEHEHEHEHEHEHEH-NH₂ ZnuA fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

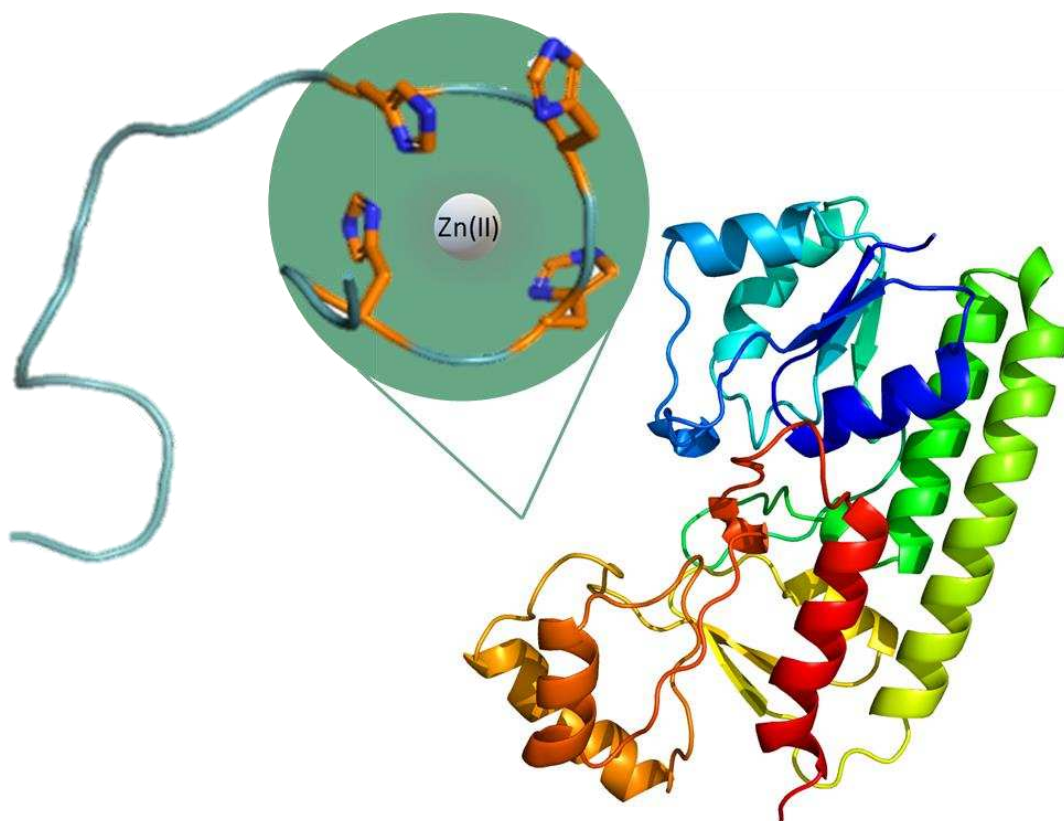


Figure 111 Proposed binding mode for Zn(II)-Ac-HEHEHEHEHEHEHEH-NH₂ fragment of ZnuA protein at pH 7.4

(ZnHL form - {4N_{im}} binding mode). The ZnuA structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

(2) Ni(II)-Ac-HEHEHEHEHEHEHEH-NH₂

The first Ni(II)-Ac-HEHEHEHEHEHEHEH-NH₂ complex, formed at acidic pH, is [NiH₈L]²⁺, with its maximum concentration at around pH 5.2 (Figure 112 and Table 9). It involves one imidazole group of histidine residue in coordination. The increase of pH leads to the [NiH₆L] form, in which the Ni(II) coordination sphere includes nitrogen atoms from three histidine residues. The next complex, [NiH₅L]⁻, dominates in the solution at around pH 5.9 and here, most probably, a fourth histidine residue is bound to the Ni(II) ion. The available data do not permit us to exactly define which histidines of the sequence are involved in complexation. The presence of numerous histidine residues allows the peptide to coordinate the metal ion via different sets of imidazoles, leading to an equilibrium between several different complex forms with the same stoichiometry. In the pH range from 7.0 to 10.0, the [NiH₃L]³⁻, [NiH₂L]⁴⁻, [NiHL]⁵⁻ and [NiL]⁶⁻ complexes dominate in solution; the suggested binding mode is {6N_{im}} (Figure 113). UV-Vis spectra (Figure 114) confirm this finding with pronounced bands at 610 nm, which can be assigned to the octahedral complex. The loss of the next proton leads to the [NiH₋₁L]⁷⁻ complex, which shows its maximum formation in solution at around pH 9.3. Spectroscopic data suggest a square-planar nickel complex with the coordination of three histidine imidazoles and one amide nitrogen {3N_{im}, N⁻}. The binding of the backbone amide is confirmed by the CD band with a negative Cotton effect at 420 nm and a positive Cotton effect at around 520 nm (Figure 115) at pH 10.0. Also, the d-d transitions at around 430 nm in the UV-Vis spectra indicate the formation of a square-planar nickel complex. The last formed complex, [NiH₋₃L]⁹⁻, appears above pH 10.0. The increase of the CD and UV-Vis band intensities at this pH suggests the replacement of one imidazole by two amide groups and the formation of a square-planar complex with the {N_{im}, 3N⁻} binding mode. The increase of CD band intensity, which appears near 265 nm, can be assigned to the N⁻ → Ni(II) transition [587].

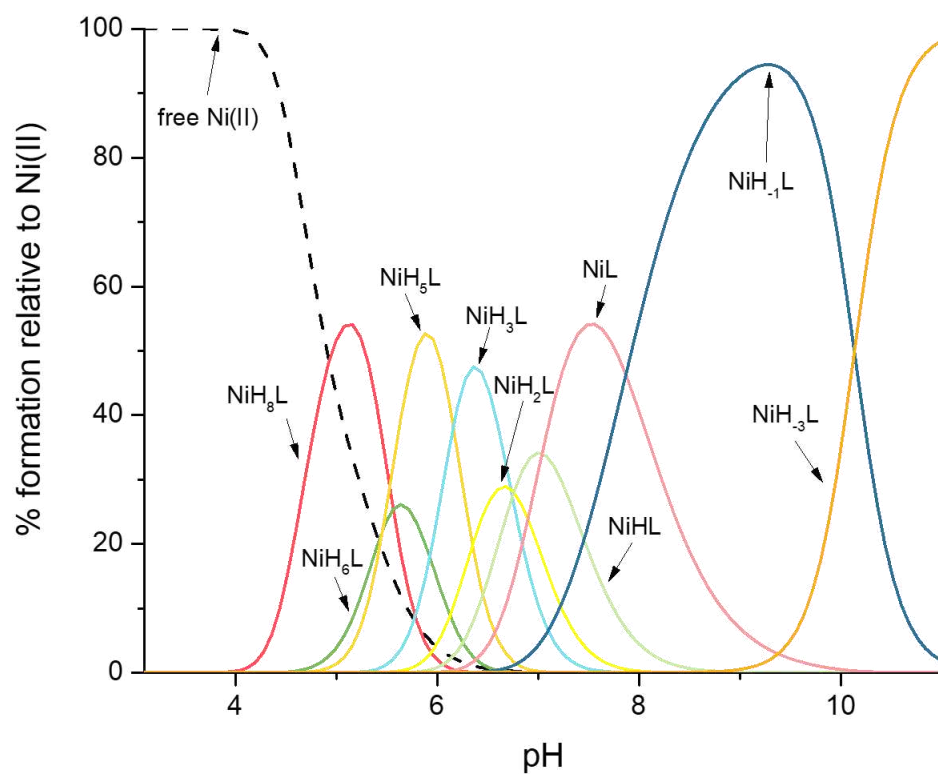


Figure 112 Species distribution diagram for the formation of Ni(II) complex with the Ac-HEHEHEHEHEHEHEHEH-NH₂ ZnA fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

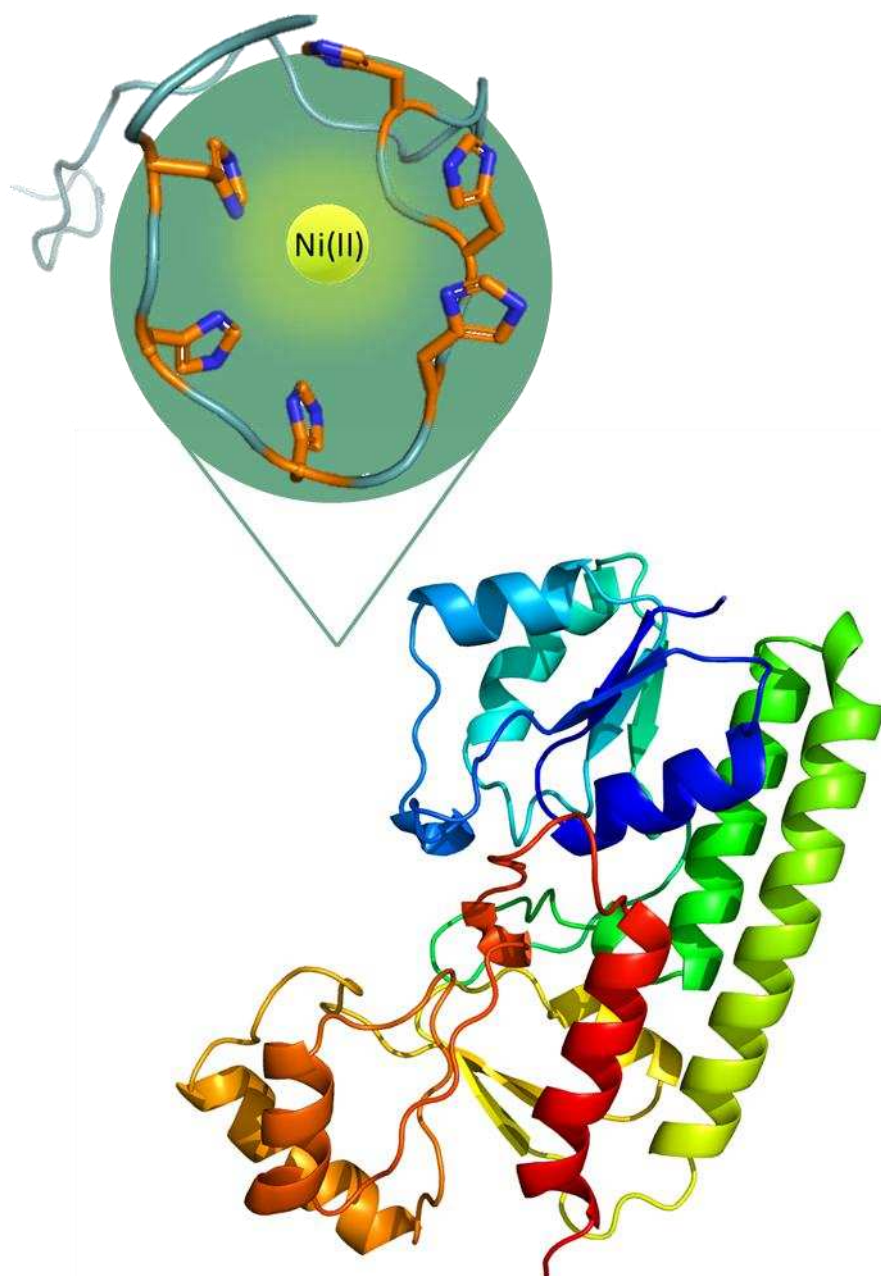


Figure 113 Proposed binding mode for Ni(II)-Ac-HEHEHEHEHEHEHEH-NH₂ fragment of ZnuA protein at pH 7.4 (NiL form - {6Ni_{im}} binding mode). The ZnuA structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

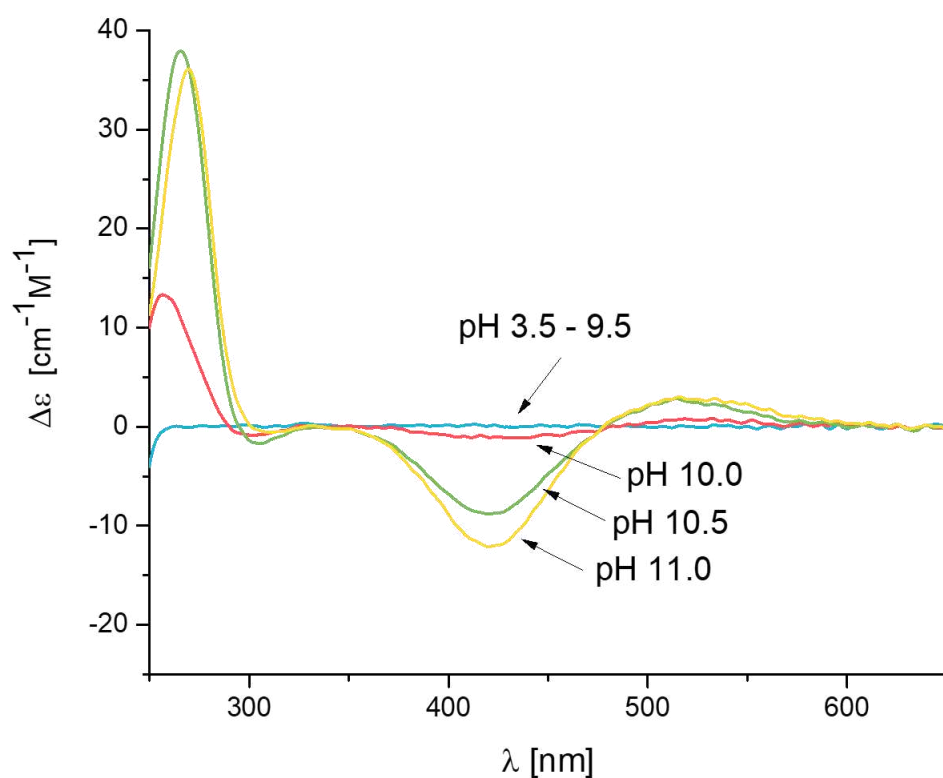


Figure 114 CD spectra of Ni(II) complex with the Ac-HEHEHEHEHEHEHEHEH-NH₂ fragment from ZnuA protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

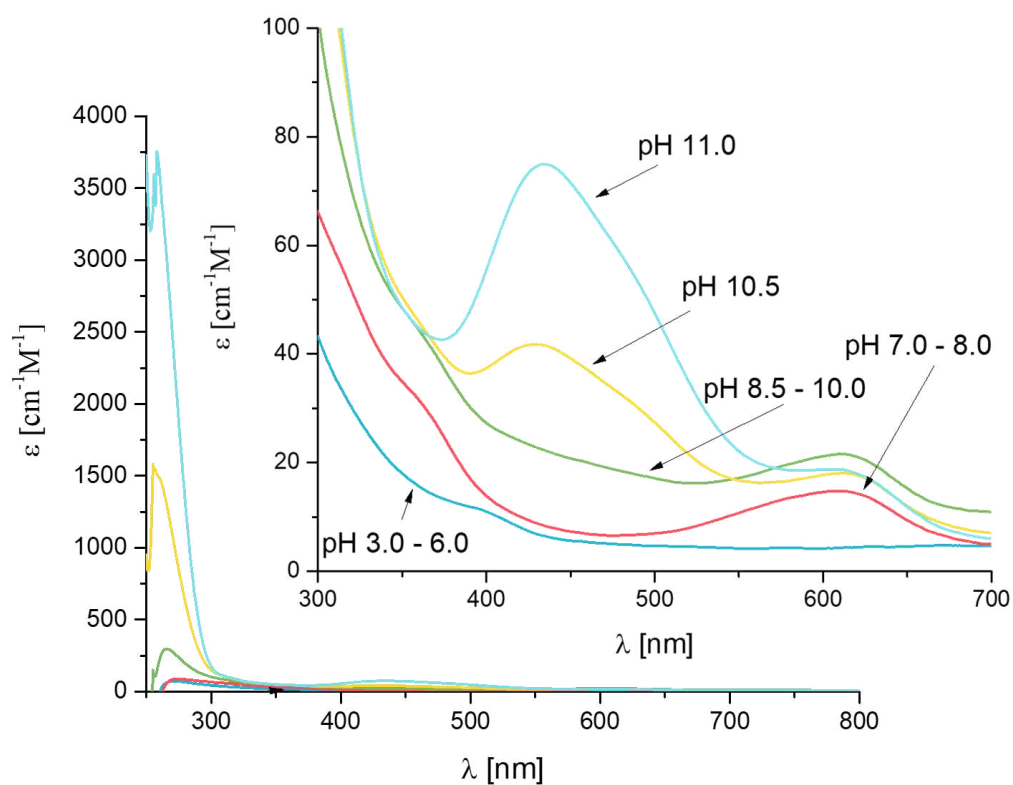


Figure 115 UV-Vis spectra of Ni(II) complex with the Ac-HEHEHEHEHEHEHEHEH-NH₂ fragment from ZnuA protein

in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

b) Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂

The Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ region consists of 29 sites involved in acid–base equilibria, which correspond to 15 His imidazole nitrogens, 10 Glu and 4 Asp carboxylates. Due to the large amount of histidyl residues deprotonating in a narrow pH range, it was not possible to determine all protonation constants for this peptide. Similar situations have previously been described in the literature [612]. The logβ values obtained from the potentiometric data are presented in Table 9.

(1) Zn(II)-Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂

The longest examined ZnuA region: Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂, contains a shorter Ac-HEHEHEHEHEHEHEHEH-NH₂ peptide (described in the point 2a of the Results and discussion) in its sequence, which may be helpful in predicting the structure and thermodynamics of the longer peptide complex. Zn(II) coordination to the Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ region starts at pH 4.0; the first detected complex is [ZnH₁₃L]⁺. In this species, 16 acid/base sites are already deprotonated (3 Asp, 11 Glu, and 2 His); thus, imidazole nitrogens of two histidines should participate in metal binding (Figure 116 and Table 9). The increase of pH leads to the [ZnH₁₁L]⁻, [ZnH₉L]³⁻, [ZnH₇L]⁵⁻, [ZnH₅L]⁷⁻ complexes, with their maximum concentrations at around pH 5.7, 6.2, 6.6 and 7.0, respectively. In these complexes, it is expected that up to four imidazole nitrogen atoms are bound to Zn(II). The loss of a further proton leads to the [ZnH₄L]⁸⁻ form, with a pK_a value of 6.99, which corresponds to the deprotonation of another histidine residue. In this form, a four-nitrogen coordination mode ({4N_{im}}) is hypothesized (Figure 117), thus suggesting that the “new” histidine residue replaces the one already coordinated. In the next formed species: [ZnH₂L]¹⁰⁻ and [ZnHL]¹¹⁻, most likely other histidine residues are bound, replacing the previous ones (which could suggest polymorphic binding sites). At this point, it was underlined that the above statements are only hypotheses since stepwise deprotonations of this peptide are not observed. The loss of the next proton leads to the [ZnL]¹²⁻ species, with its maximum concentration at around pH 8.60. A significant decrease of the pK_a values (free ligand - 8.31 and the complexed one - 8.22) suggests that this His residue is involved in Zn(II) coordination and replaces another one in the binding. The last observed form, [ZnH₋₁L]¹³, is related to the deprotonation of one water molecule.

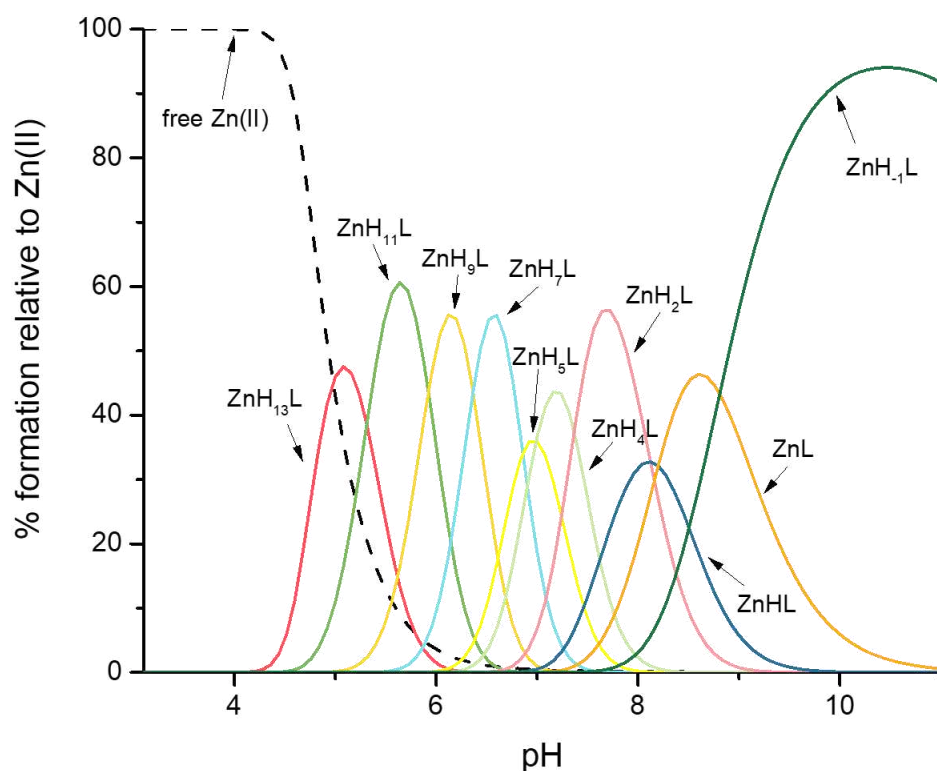


Figure 116 Species distribution diagram for the formation of Zn(II) complex with the Ac-GGEHEHEHEHEHEHEHEHDGHHGAEEQAHHDDHDSG-NH₂ ZnuA fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

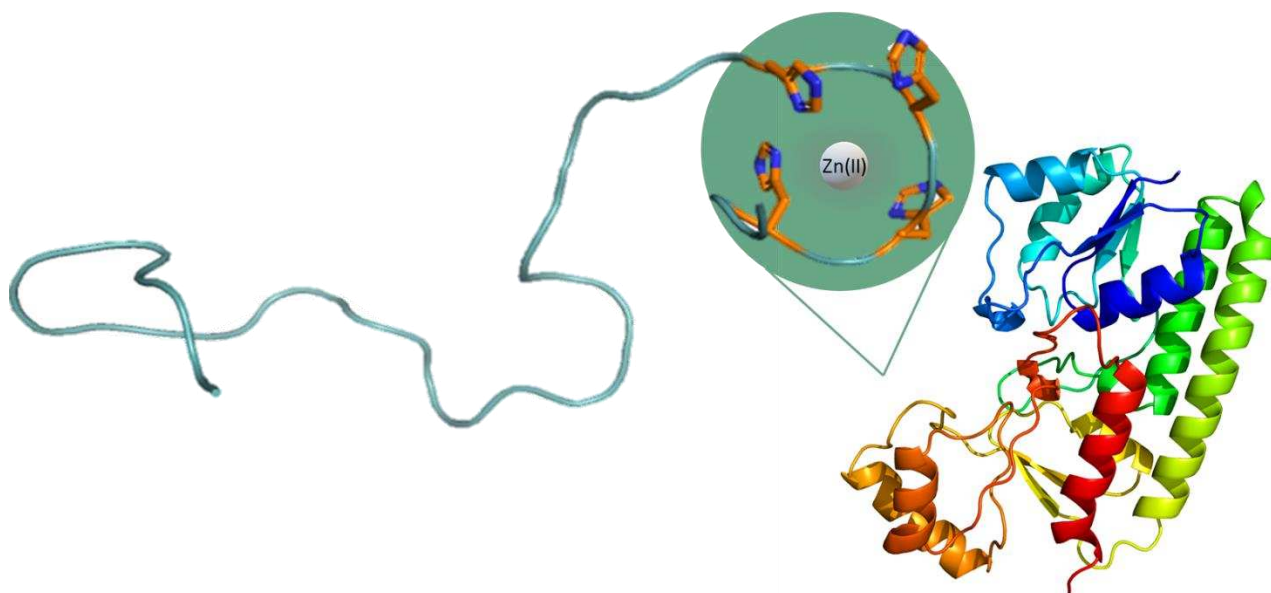


Figure 117 Proposed binding mode for Zn(II)-Ac-GGEHEHEHEHEHEHEHEHDGHHGAEEQAHHDDHDSG-NH₂ fragment of ZnuA protein at pH 7.4 (ZnH₂L form - {4N_{im}} binding mode). The ZnuA structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

(2) Ni(II)-Ac-
GGEHEHEHEHEHEHEHEHDGHHGAEEQAHHDDHDSG-NH₂

Ni(II) coordination for this sequence starts below pH 3.0 (Figure 118 and Table 9) and the first two forms, [NiH₂₀L]⁸⁺ and [NiH₁₇L]⁵⁺ (with their maximum concentrations at pH 3.8 and 4.3), are most likely connected with the deprotonation and coordination of acidic residues in this peptide. The release of further protons leads to [NiH₁₅L]³⁺, [NiH₁₃L]⁺ and [NiH₁₁L]⁻ with the possible coordination of some histidine residues. In the pH range 5.0–7.5, [NiH₉L]³⁻, [NiH₇L]⁵⁻, [NiH₆L]⁶⁻, [NiH₄L]⁸⁻, [NiH₃L]⁹⁻ and [NiHL]¹¹⁻ species co-exist in solution. In this pH region, the observed UV-Vis band near 590 nm can be attributed to an octahedral nickel complex with the {6N_{im}} binding mode (Figure 119). Above pH 7.50, the [NiH₋₁L]¹³⁻ form dominates in solution; it most likely derives from the deprotonation and coordination of a backbone amide, with a change in coordination geometry from octahedral to square planar. This is confirmed by the CD bands, showing a negative Cotton effect at 485 nm and a positive Cotton effect at 410 nm (Figure 120). The disappearance of the UV-Vis band near 590 nm and the increase of band at 370 nm also confirm the above structural suggestion (Figure 121). In the complex [NiH₋₃L]¹⁵⁻, which dominates above pH 8.0, two more amide nitrogens replace imidazoles in the equatorial plane, giving a {3N⁻, N_{im}} binding mode (Figure 118). These findings are confirmed by UV-Vis spectra (band at around 425 nm) and CD spectra: bands with a negative Cotton effect at around 485 nm and a positive Cotton effect at around 405 nm. The band near 270 nm can be assigned to a N⁻ → Ni(II) transition [587].

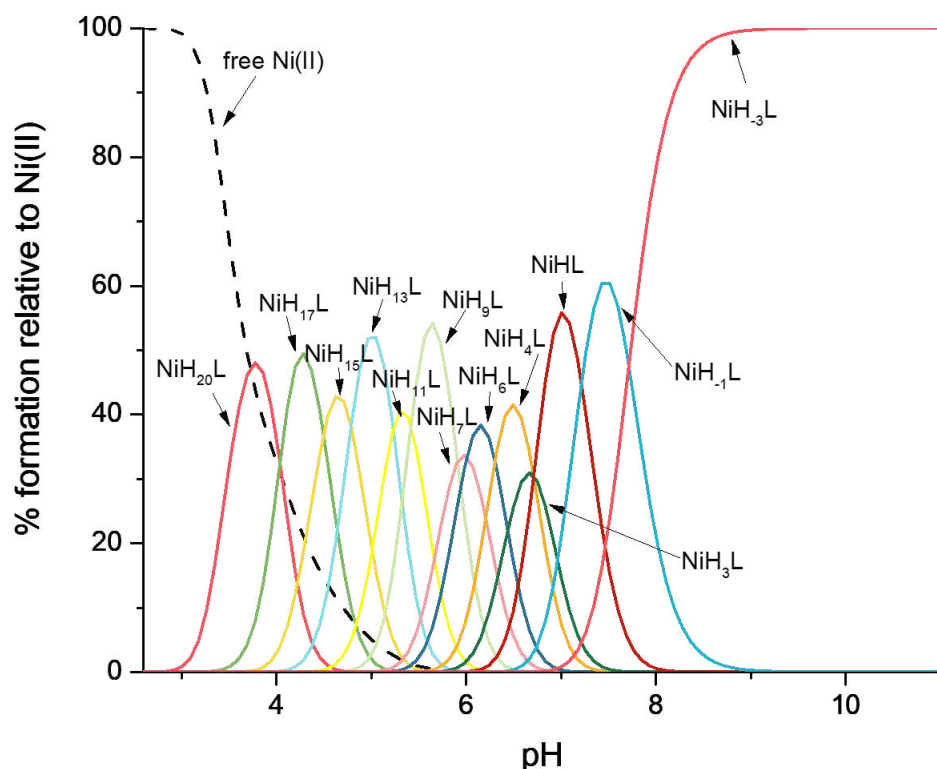


Figure 118 Species distribution diagram for the formation of Ni(II) complex with the Ac-GGEHEHEHEHEHEHEHEHDGHHGAEEQAHHDDHDSG-NH₂ ZnuA fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

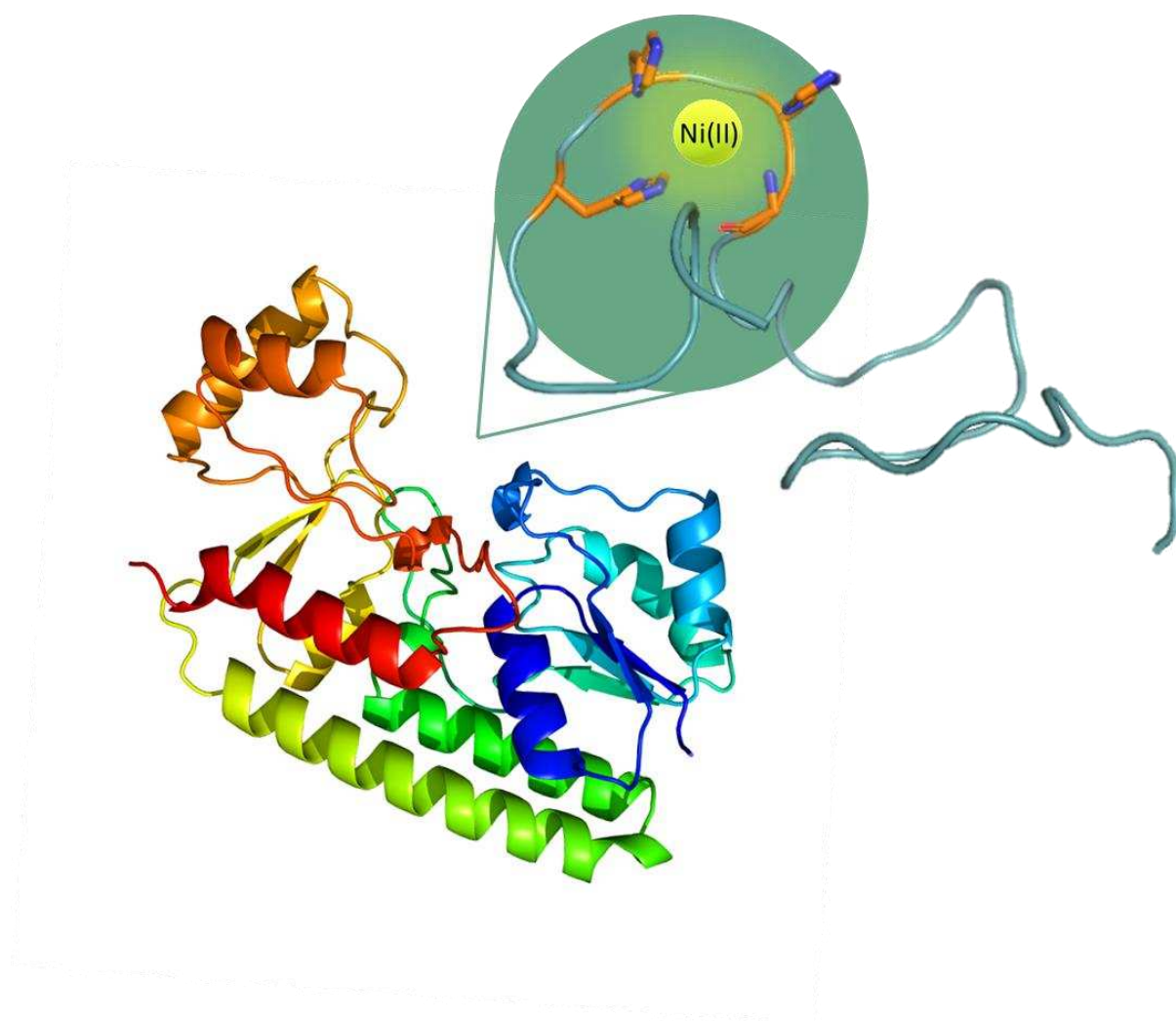


Figure 119 Proposed binding mode for Ni(II)-Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ fragment of ZnuA protein at pH 7.4 (NiH₋₁L form - {3N_{im}, N⁻} binding mode). The ZnuA structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

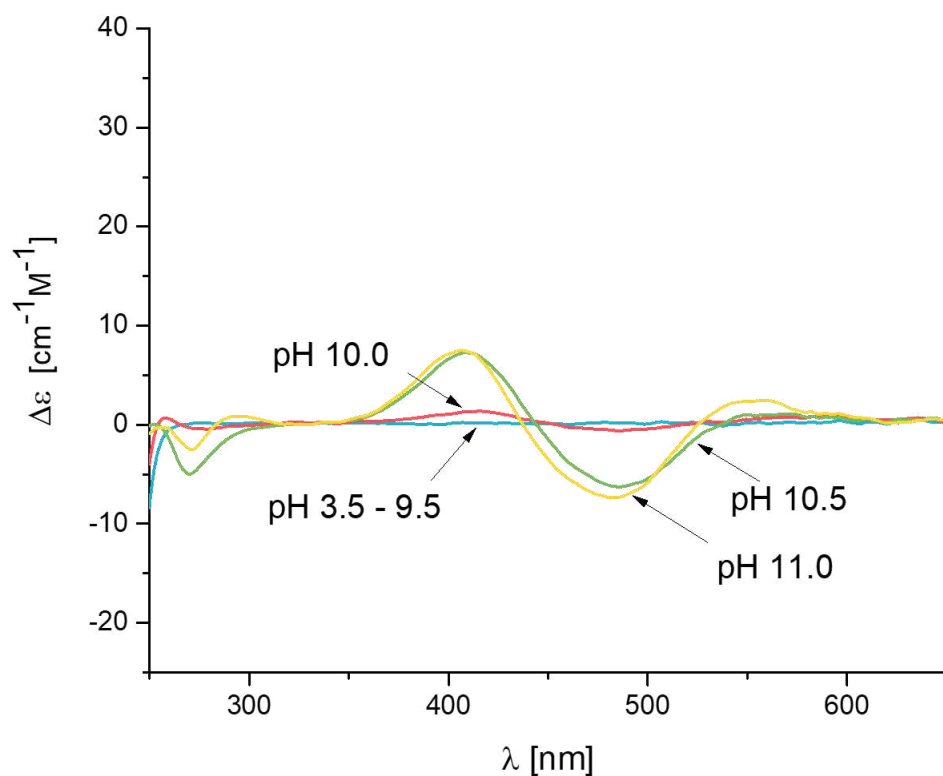


Figure 120 CD spectra of Ni(II) complex with the Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ fragment from ZnuA protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

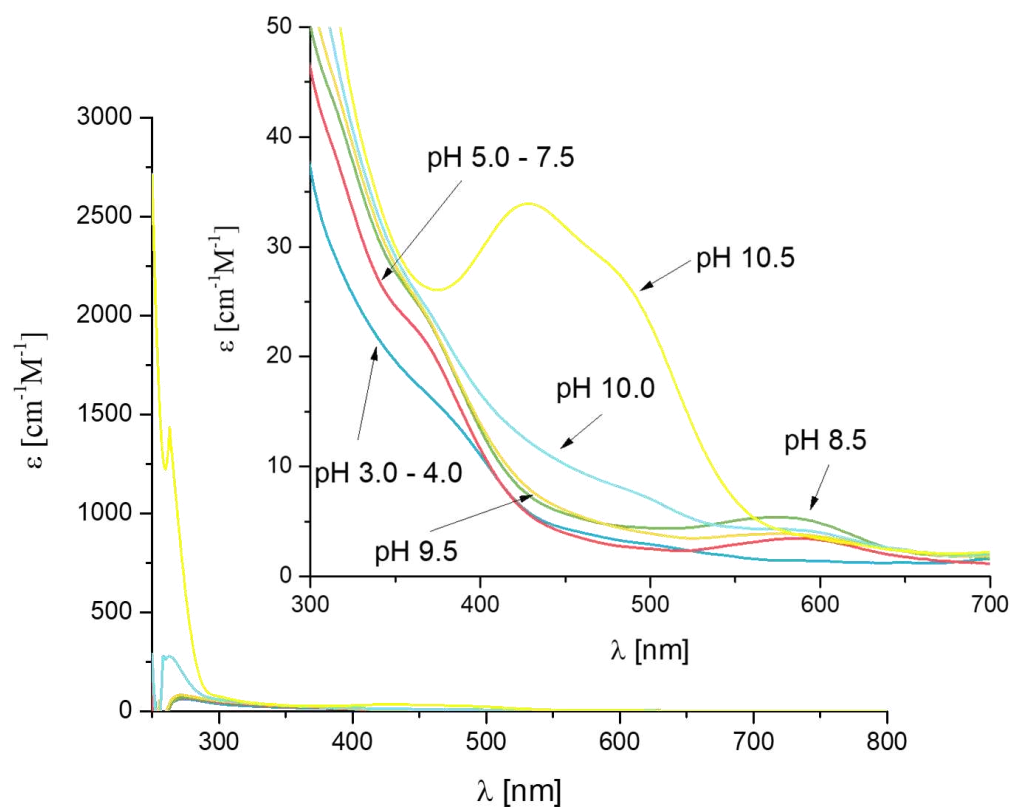


Figure 121 UV-Vis spectra of Ni(II) complex with the Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ fragment from ZnuA protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

NH₂ fragment from ZnuA protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

c) Ac-GGGHYHYIDGKAVFHAG-NH₂

The Ac-GGGHYHYIDGKAVFHAG-NH₂ peptide acts as a typical (H₇L)⁶⁺ acid. The first pK_a value (3.58) comes from the deprotonation of the carboxylic side chain of aspartic acid. The next three constants (5.97, 6.24 and 7.71) correspond to the deprotonation of three imidazole groups of the histidine residues. The next two constants (9.69 and 10.14) are related to the deprotonation of the tyrosine residues and the most basic one (11.04) corresponds to the deprotonation of the lysine side chain group (Table 9).

(1) Zn(II)-Ac-GGGHYHYIDGKAVFHAG-NH₂

In the case of the Zn(II)-Ac-GGGHYHYIDGKAVFHAG-NH₂ fragment, in the first complex observed at acidic pH, [ZnH₃L]⁴⁺, with its maximum concentration at pH 6.3, Zn(II) is coordinated to three histidine residues (Figure 122 and Table 9). The loss of the next proton leads to the [ZnH₂L]³⁺ form and it probably corresponds to the deprotonation of a coordinated water molecule, which occupies the fourth coordination position of a tetrahedral zinc complex (Figure 123). The loss of two further protons, leading to [ZnHL]²⁺ and [ZnL]⁺ complexes, is related to the deprotonation of two tyrosine side chains, which do not participate in binding. The last species, [ZnH₋₁L], is formed with a pK_a value of 11.05, which corresponds to the deprotonation of the unbound lysine side chain group.

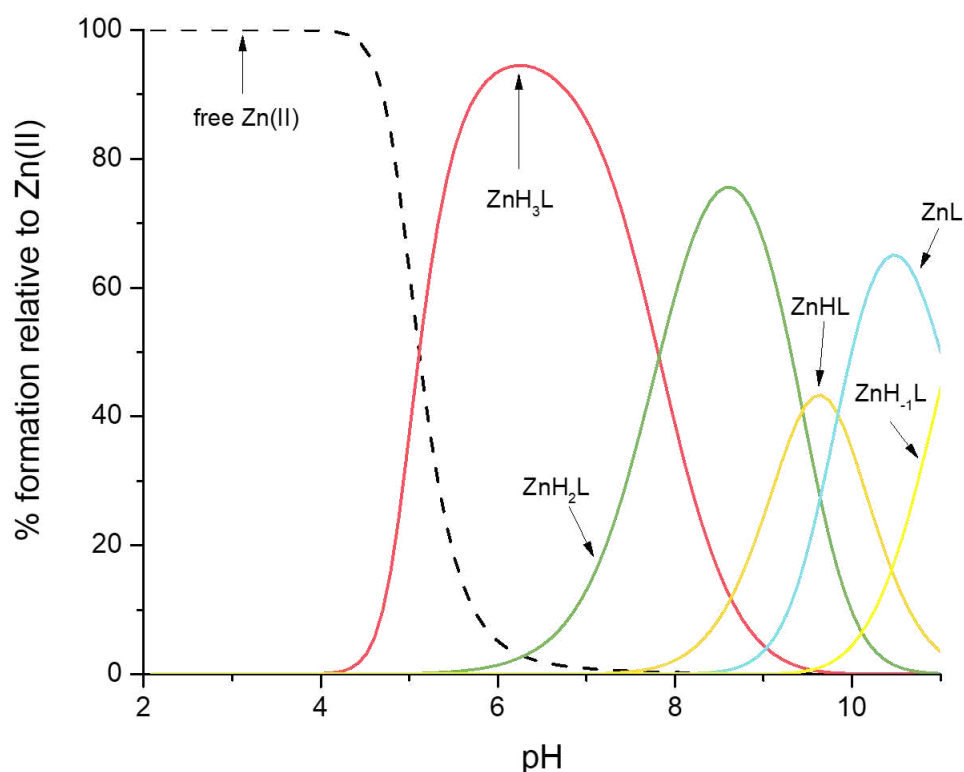


Figure 122 Species distribution diagram for the formation of Zn(II) complex with the Ac-GGGHYHYIDGKAVFHAG-NH₂ AztC fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

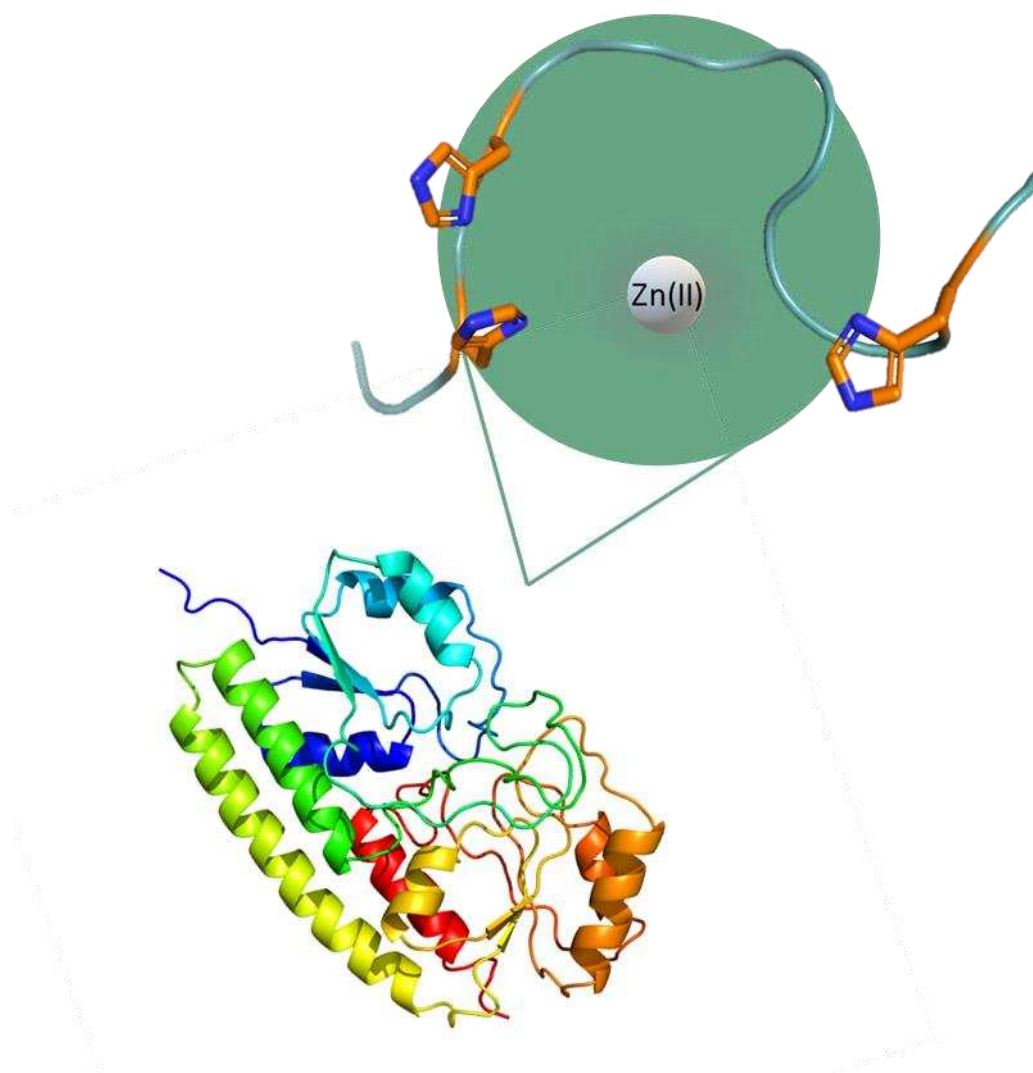


Figure 123 Proposed binding mode for Zn(II)-Ac-GGGHYHYIDGKAVFHAG-NH₂ fragment of AztC protein at pH 7.4 (ZnH₂L form - {3N_{im}, H₂O} binding mode). The AztC structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

(2) Ni(II)-Ac-GGGHYHYIDGKAVFHAG-NH₂

For the Ac-GGGHYHYIDGKAVFHAG -NH₂ region, seven complexes with Ni(II) are observed (Figure 124 and Table 9). In the [NiH₄L]⁵⁺ species, most likely two imidazoles and possibly an aspartic acid side chain are bound to the Ni(II) ion. The loss of the next proton leads to the formation of [NiH₃L]⁴⁺ with its maximum concentration at pH 7.4, which is connected to the coordination of the third histidine residue (pK_a = 5.60)- Figure 125. The increase of pH leads to the formation of the [NiH₂L]³⁺ complex, in which the Ni(II) coordination sphere includes three histidine imidazole groups and one amide nitrogen- the CD band, which appears near 415 and 485 nm, confirms the formation of a square-planar Ni(II) complex (Figure 126). The loss of the next two protons leads to the [NiL]⁺ form, which is most probably connected to the deprotonation and coordination of the second and third amide nitrogens. The CD spectra confirm this finding with a band that reduces its intensity near 251 nm and that can be assigned to the N⁻ → Ni(II) transition [587]. The further release of acidic protons from non-coordinating

Tyr and Lys residues leads to the formation of $[\text{NiH}_{-1}\text{L}]$, $[\text{NiH}_{-2}\text{L}]^-$ and $[\text{NiH}_{-3}\text{L}]^{2-}$ species, which dominate in solution at pH above 9.0. In these complexes the Ni(II) coordination sphere includes one histidine imidazole group and three amide nitrogens with the $\{\text{N}_{\text{im}}, 3\text{N}^-\}$ binding mode and a square-planar geometry, as confirmed by CD (a negative Cotton effect at around 495 nm and a positive Cotton effect at around 415 nm) and UV-Vis (shifting of a band from 365 nm at pH 3.17 to 422 nm at pH 11.0) results (Figure 127).

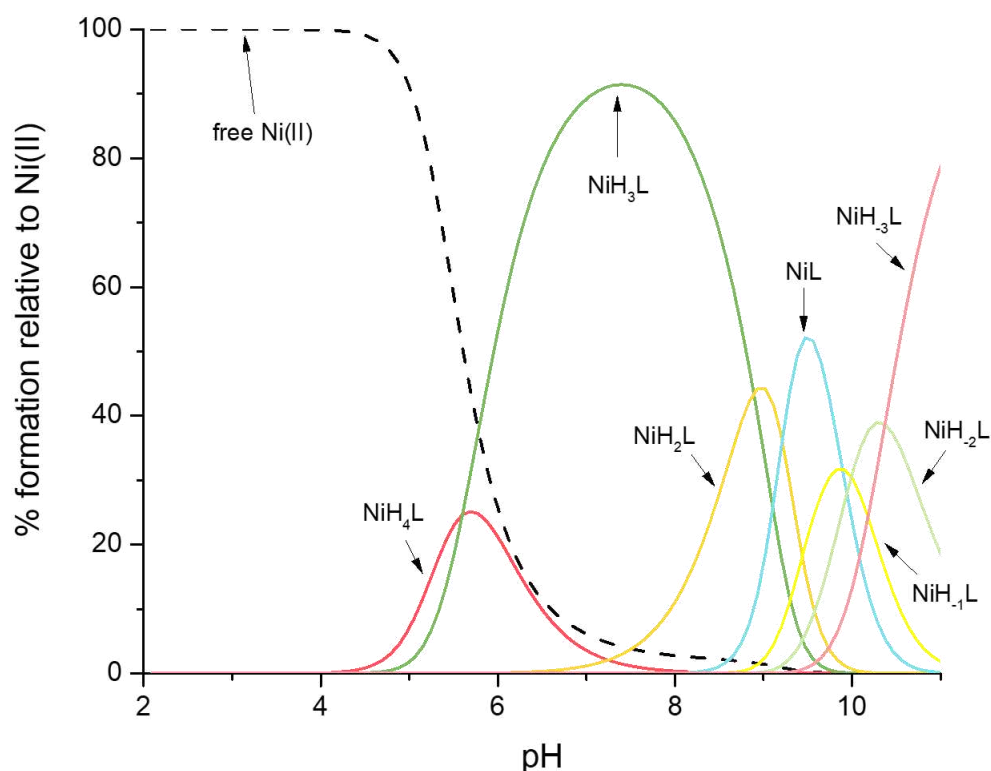


Figure 124 Species distribution diagram for the formation of Ni(II) complex with the Ac-GGGHYHYIDGKAVFHAG-NH₂ AztC fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

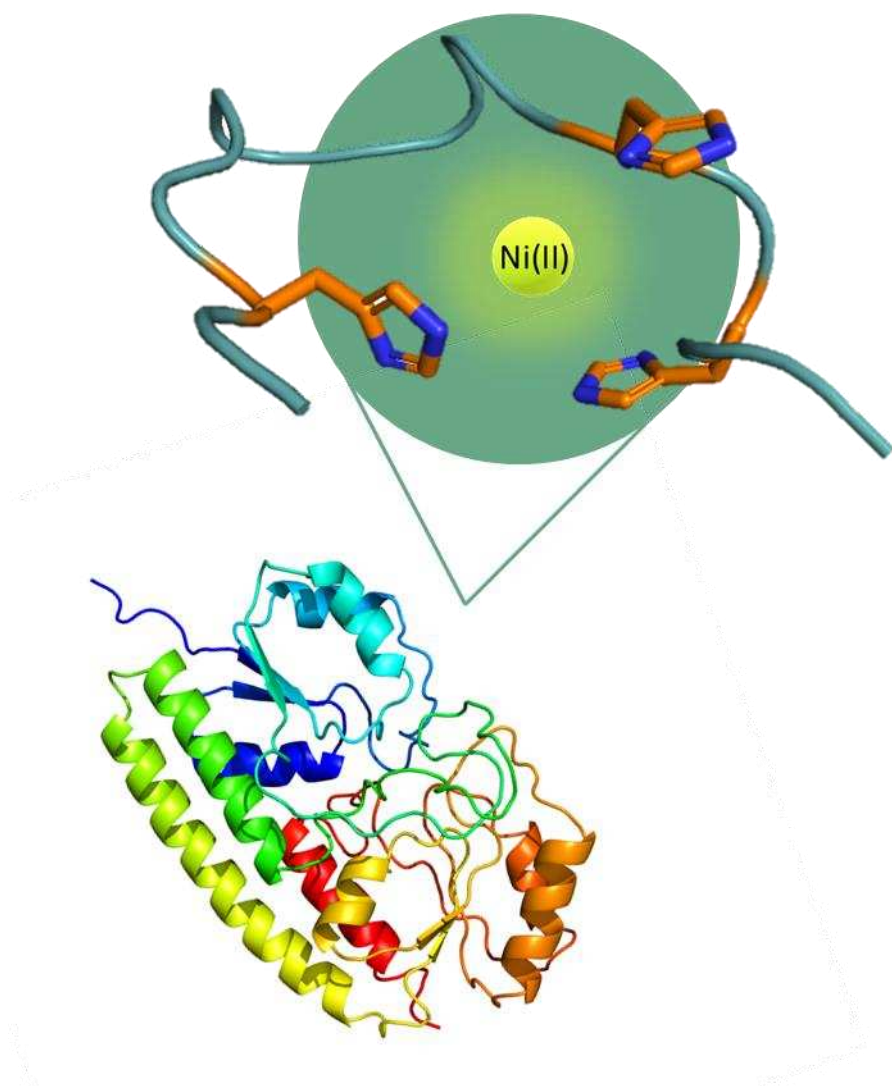


Figure 125 Proposed binding mode for Ni(II)-Ac-GGGHYHYIDGKAVFHAG-NH₂ fragment of AztC protein at pH 7.4 (NiH₃L form - {3N_{im}} binding mode). The AztC structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

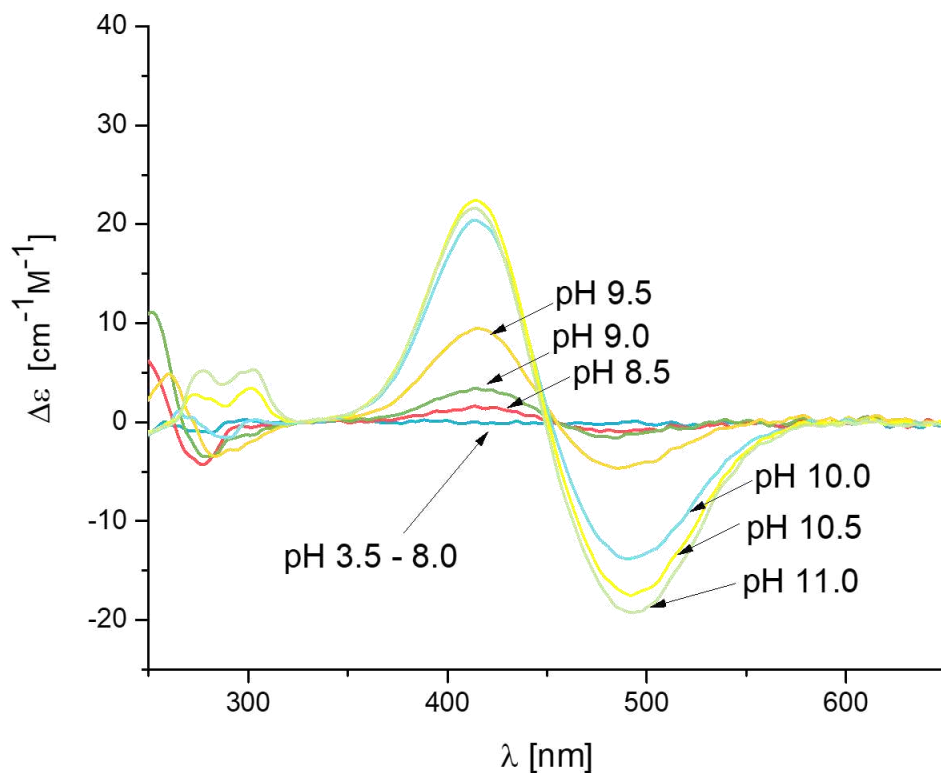


Figure 126 CD spectra of Ni(II) complex with the Ac-GGGHYHYIDGKAVFHAG-NH₂ fragment from AztC protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

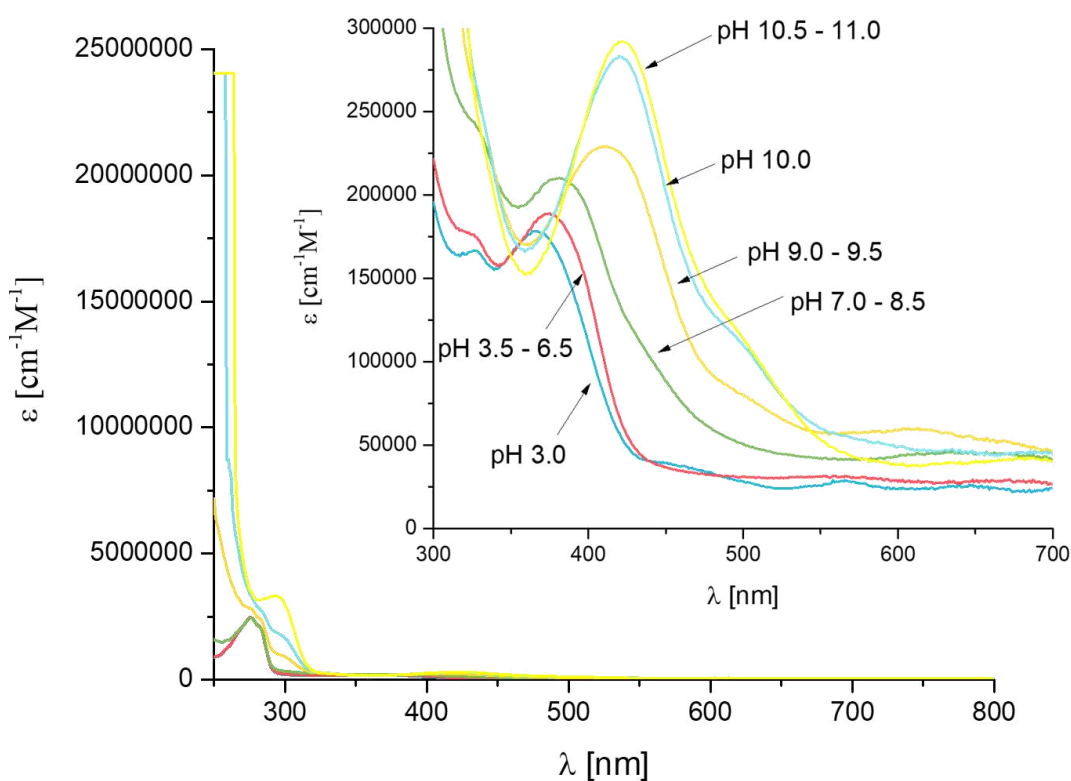


Figure 127 UV-Vis spectra of Ni(II) complex with the Ac-GGGHYHYIDGKAVFHAG-NH₂ fragment from AztC protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

3. Secondary structure analysis

The addition of Zn(II) and Ni(II) to the Ac-HEHEHEHEHEHEHEHEH-NH₂ (Figure 128), Ac-GGEHEHEHEHEHEHEHEHEHDGHHGAEEQAHHDDHSG-NH₂ (Figure 129) and Ac-GGGHYHYIDGKAVFHAG-NH₂ (Figure 130) do not significant influence on the secondary structure of ligands. In the whole pH range (3.5 - 11.5), the structure remains disordered (random coil).

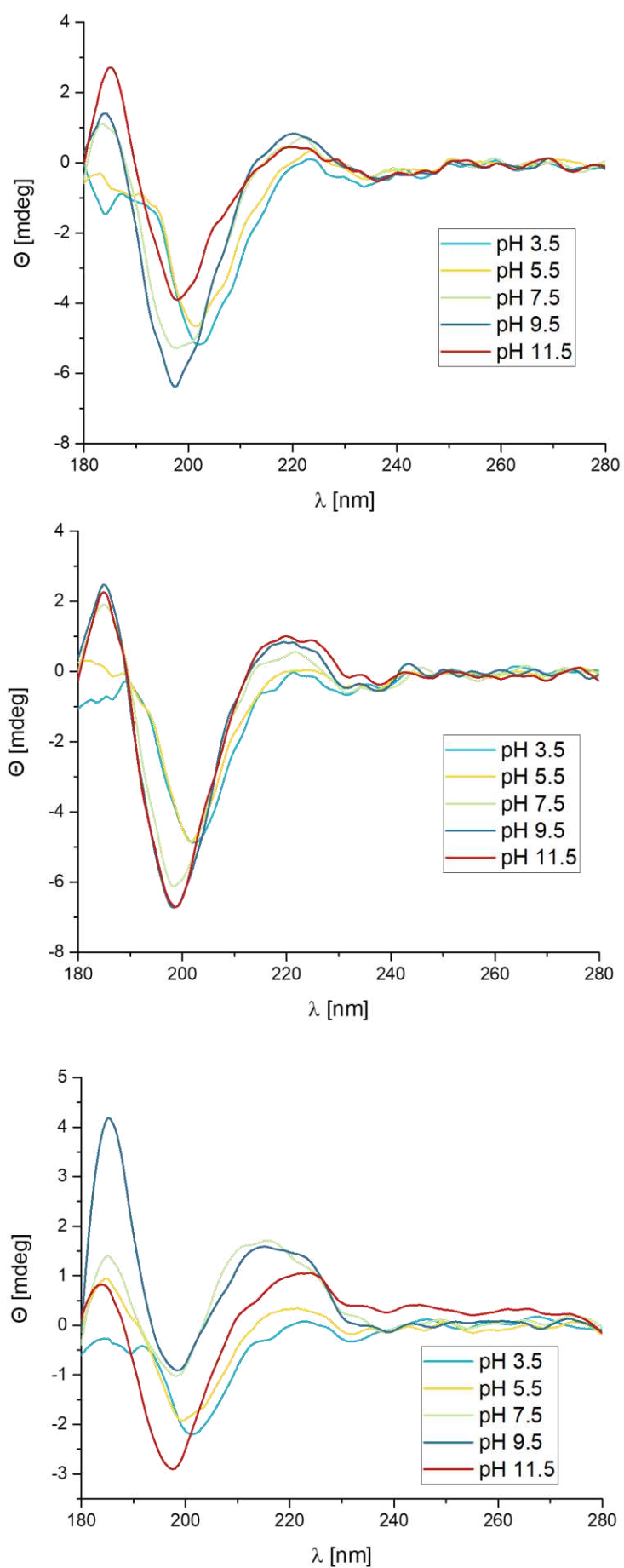


Figure 128 CD spectra of A) Ac-HEHEHEHEHEHEHEHEH-NH₂ and its complexes with b) Zn(II) and C) Ni(II); optical path 0.1 mm, Ni(II)/L molar ratio = 1 : 1

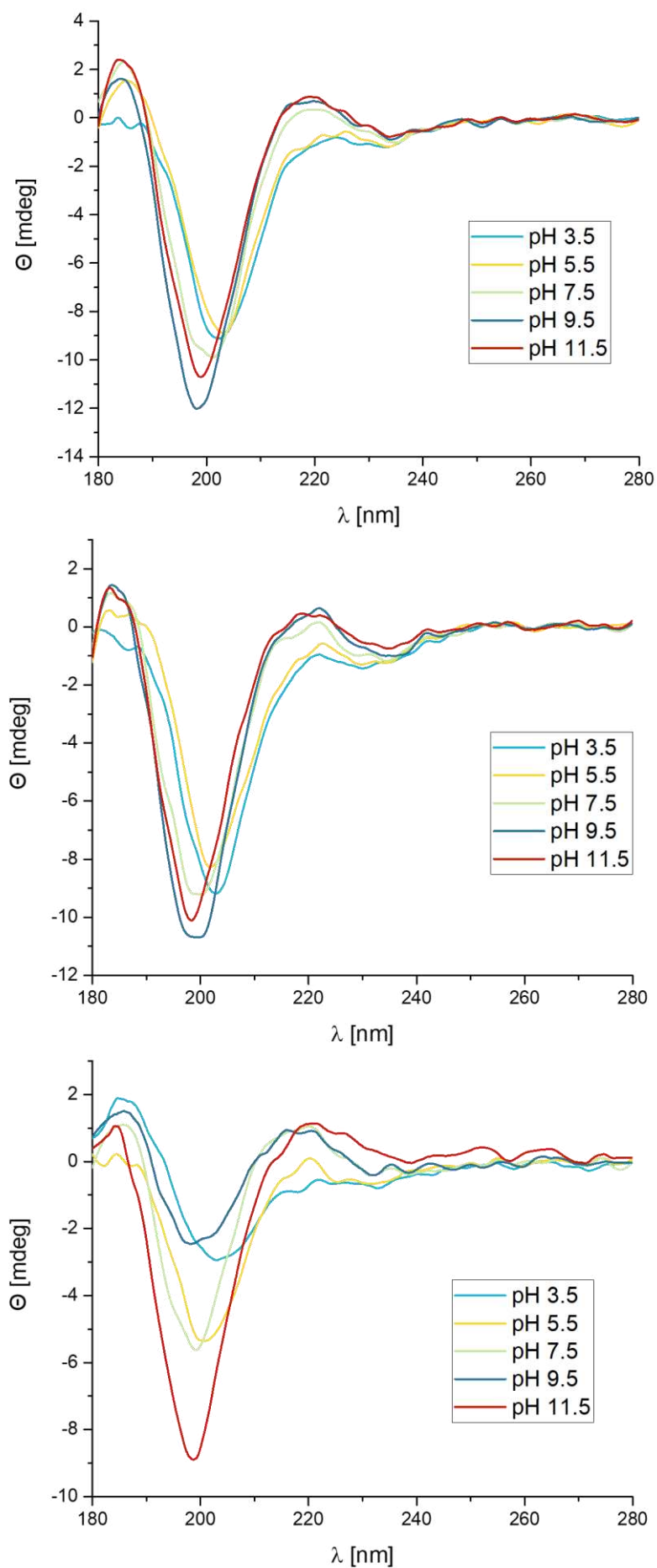


Figure 129 CD spectra of A) Ac-GGEHEHEHEHEHEHEHEHDGHHGAEQAHHDHDHSG-NH₂ and its complexes with b) Zn(II) and C) Ni(II); optical path 0.1 mm, Ni(II)/L molar ratio = 1 : 1

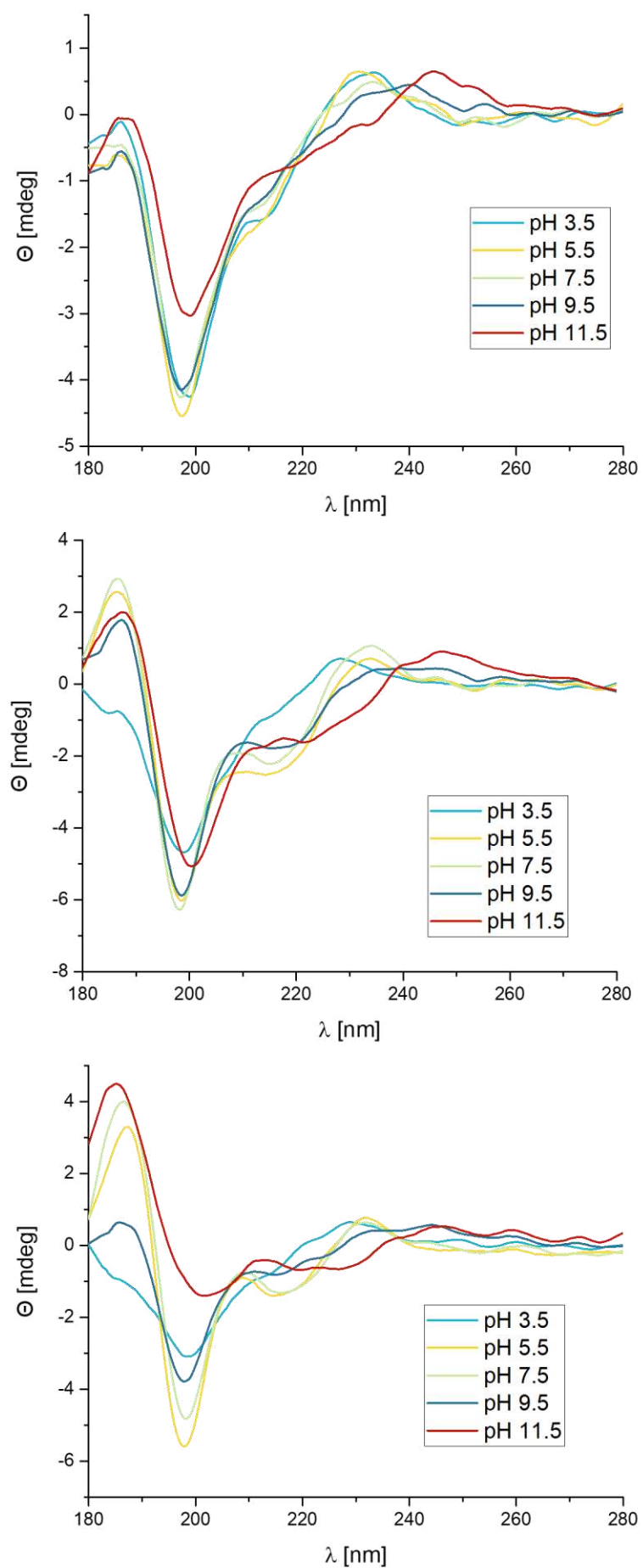


Figure 130 CD spectra of A) Ac-GGGHYHYIDGKAVFHAG-NH₂ and its complexes with b) Zn(II) and C) Ni(II); optical path 0.1 mm, Ni(II)/L molar ratio = 1 : 1

4. Comparison of the stability of Zn(II) and Ni(II) complexes

In order to compare the strength of Zn(II) binding to two bacterial Zn(II) transporters, ZnuA and AztC from *Paracoccus denitrificans*, we used the thermodynamic data for Zn(II) complexes to simulate a theoretical situation in which equimolar concentrations of Zn(II) and all the studied peptides were mixed. This allows a direct comparison of the binding ability at different pH values (Figure 131). At acidic pH, the strongest ligand is the longest Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ fragment, due to its large number of glutamic and aspartic acid residues acting as anchoring sites for Zn(II). Interestingly, this peptide, which has also the largest number of histidine residues in the sequence, is preferred over both its own fragments, the histidine-rich loop, Ac-HEHEHEHEHEHEHEHEH-NH₂, and the AztC fragment, Ac-GGGHYHYIDGKAVFHAG-NH₂, throughout the acidic pH range. At physiological pH (7.4), more than 40% of the metal is still coordinated to the longest ZnuA region, being bound to four imidazole nitrogens of histidine residues (Figure 131) belonging to different sets of imidazole rings, due to the polymorphic character of this binding sequence. It is worth noting that at this point, the stabilities of Zn(II) complexes with both multi-His ligands from ZnuA are comparable. Above pH 8.4, the Ac-GGGHYHYIDGKAVFHAG-NH₂ fragment becomes a slightly stronger Zn(II) chelator, with its {3N_{im}, OH⁻} binding mode (Figure 131). In this complex, the aromatic side chains of Tyr or Phe could also play an additional stabilizing role. The proximity of tyrosine and phenylalanine aromatic rings to the metal ion may result in the occurrence of a direct interaction between the d-electrons of the metal ion and the π -ring system of the aromatic side chains, which provides an additional driving force for the folding and stability of integral membrane proteins [613], [614].

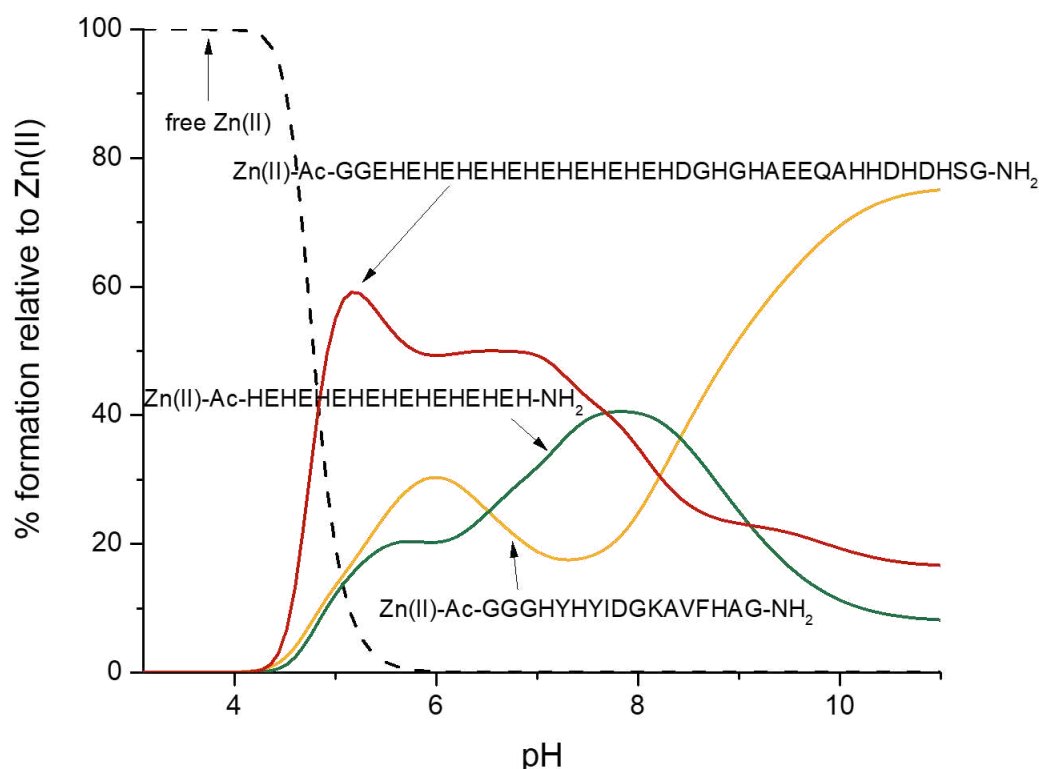


Figure 131 A competition plot between ZnuA fragments: Ac-HEHEHEHEHEHEHEHEH-NH₂, Ac-GGEHEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂, AztC fragment: Ac-GGGHYHYIDGKAVFHAG-NH₂ and Zn(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 9. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ac-HEHEHEHEHEHEHEHEH-NH₂] = [Ac-GGEHEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂] = [Ac-GGGHYHYIDGKAVFHAG-NH₂] = 0.001 M

Nickel's preferences towards the tested fragments are more pronounced than those of Zn(II). In the entire pH range, Ni(II) binds with the highest affinity to Ac-GGEHEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ (Figure 132). Above pH 7.2, more than 99% of Ni(II) is bound to this sequence with a {6N_{im}} binding mode (Figure 113) and, at higher pH values, with a {3N⁻, N_{im}} donor set. In the same pH range, Ni(II) is also bound to six histidine residues of Ac-HEHEHEHEHEHEHEHEH-NH₂, but the affinity of this region towards Ni(II) is much lower than that of the longer loop. The Ac-GGGHYHYIDGKAVFHAG-NH₂ ligand has the lowest affinity towards Ni(II) among the three tested fragments in the whole pH range. At alkaline pH, the sets of metal binding donors are the same for all studied peptides, but it is clear that the large number of histidine residues of the Ac-GGEHEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ region additionally stabilizes the formed complexes. It is also worth noting that the higher the number of imidazoles in the analyzed peptide, the higher the pH value at which amide nitrogens start to participate in the binding of Ni(II). In the case of Ac-GGGHYHYIDGKAVFHAG-NH₂, (the proposed coordination mode is depicted in Figure 125), amide coordination starts at around pH 9.0, while for Ac-GGEHEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂, amides are only bound around pH

above 10. From the chemical point of view, the obtained results confirm our assumption that a larger number of histidine residues in the sequence increases the efficiency of metal ion binding.

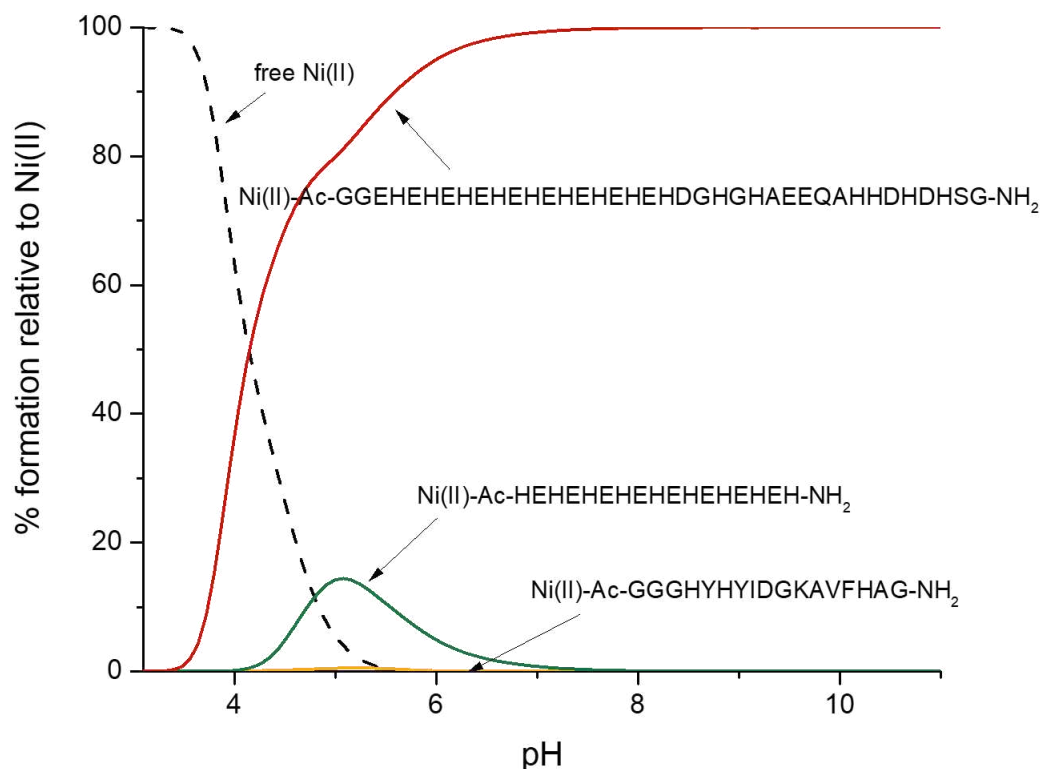


Figure 132 A competition plot between ZnuA fragments: Ac-HEHEHEHEHEHEHEHEH-NH₂, Ac-GGEHEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂, AztC fragment: Ac-GGGHYHYIDGKAVFHAG-NH₂, and Ni(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 9. Conditions: T=298 K, I=0.1 M NaClO₄, [Ni(II)] = [Ac-HEHEHEHEHEHEHEHEH-NH₂] = [Ac-GGEHEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂] = [Ac-GGGHYHYIDGKAVFHAG-NH₂] = 0.001 M

How stable are the described Zn(II) complexes compared to those of other metallophores? In comparison with the Zn(II)- binding Asp2 zincophore region from *Aspergillus fumigatus* (Ac-PNCHTHEGGQLHCT), ZnuA-(117–154) has a much higher affinity for Zn(II) in the acidic pH range 4.8– 6.8 (Figure 133). In a hypothetical situation in which equimolar amounts of Zn(II) and all peptides are present, at pH 5.2, more than 50% of Zn(II) would be bound to this sequence. At higher pH values this situation changes, and at physiological pH (7.4), more than 60% of Zn(II) would be bound to the C-terminal part of the Asp2 *Aspergillus fumigatus* zincophore, Ac- PNCHTHEGGQLHCT. The comparison of these stabilities shows that the {2N_{im}, 2S⁻} binding mode, typical of zinc fingers and present in Ac-PNCHTHEGGQLHCT, is more stable than not only the {3N_{im}, 1H₂O} mode detected in the Ac-GGGHYHYIDGKAVFHAG-NH₂ fragment of the AztC protein, but also the {4N_{im}} mode detected in the Ac-GGEHEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ and Ac-HEHEHEHEHEHEHEHEH-NH₂ fragments of the ZnuA protein.

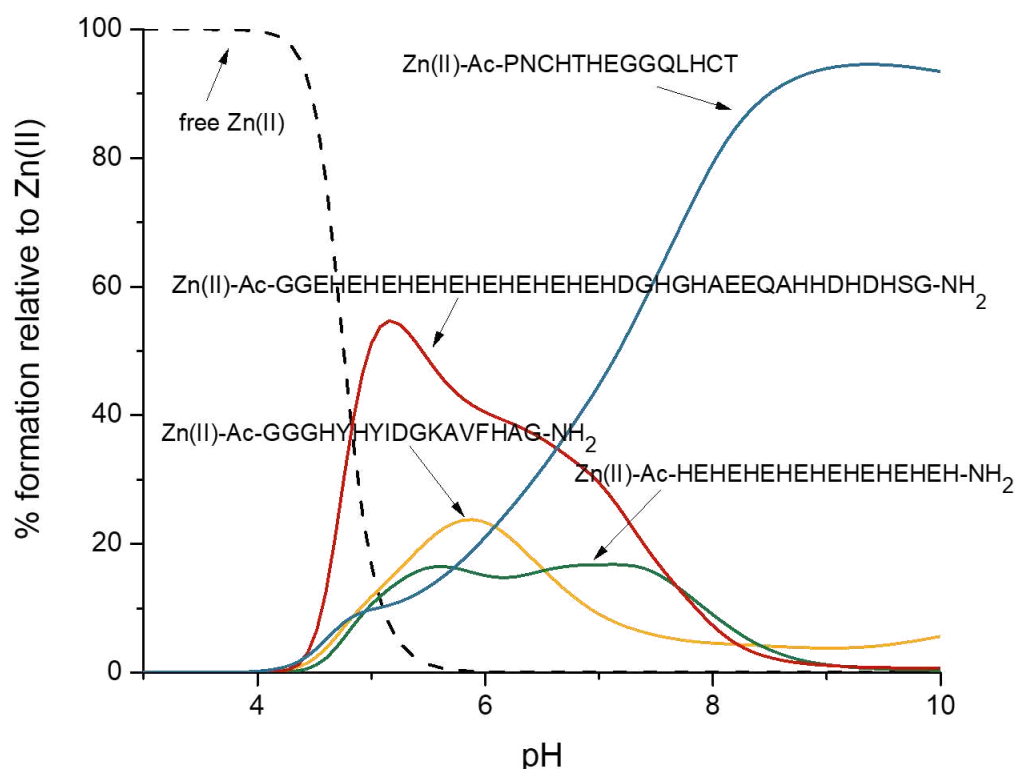


Figure 133 A competition plot between ZnuA fragments: Ac-HEHEHEHEHEHEHEHEH-NH₂, Ac-GGEHEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂, AztC fragment: Ac-GGGHYHYIDGKAVFHAG-NH₂, AspF2 fragment: Ac-PNCHTHEGGQLHCT and Zn(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 9 and [493]. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ac-HEHEHEHEHEHEHEHEH-NH₂] = [Ac-GGEHEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂] = [Ac-GGGHYHYIDGKAVFHAG-NH₂] = [Ac-PNCHTHEGGQLHCT] = 0.001 M

The sequences of His-rich antimicrobial peptides - clavanins, which could theoretically outcompete Zn(II) transporters from binding this metal nutrient in a process called 'nutritional immunity' (clavanins A, B, C, D and E; sequences are shown in Figure 134), have a much higher affinity for Zn(II) than the metallophores discussed in this work. In a hypothetical situation in which equimolar amounts of Zn(II) and all peptides are present, at pH 7.4, more than 40% of Zn(II) would be bound to the Ac-GGEHEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ fragment (Figure 134). Zn(II) coordination to clavanins would, in this case, be negligible [615].

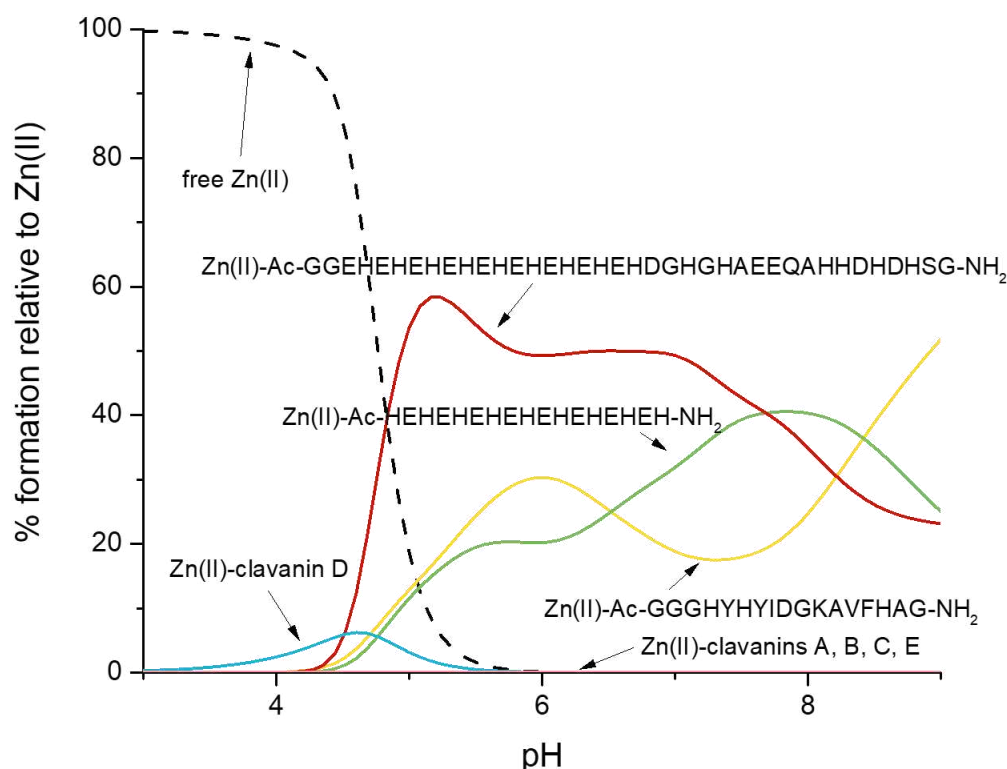


Figure 134 A competition plot between ZnuA fragments: Ac-HEHEHEHEHEHEHEH-NH₂, Ac-GGEHEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂, AztC fragment: Ac-GGGHYHYIDGKAVFHAG-NH₂, clavanin A: VFQFLGKIIHHVGNFVHGFSHF-COOH, clavanin B: VFQFLGRIIHHVGNFVHGFSHF-COOH, clavanin C: VFHLLGKIIHHVGNFVHGFSHF-COOH, clavanin D: AFKLLGRIIHHVGNFVHGFSHF-COOH, clavanin E: LFKLLGKIIHHVGNFVHGFSHF-COO and Zn(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 9 and [615]. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ac-HEHEHEHEHEHEHEH-NH₂] = [Ac-GGEHEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂] = [Ac-GGGHYHYIDGKAVFHAG-NH₂] = [VFQFLGKIIHHVGNFVHGFSHF-COOH] = [VFQFLGRIIHHVGNFVHGFSHF-COOH] = [VFHLLGKIIHHVGNFVHGFSHF-COOH] = [AFKLLGRIIHHVGNFVHGFSHF-COOH] = [LFKLLGKIIHHVGNFVHGFSHF-COOH]

5. Conclusions

The most probable Zn(II) binding sites of ZnuA and AztC Zn(II) transport proteins are most likely located in unstructured His-rich loops. The Ac-GGEHEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ region binds Zn(II) and Ni(II) with the highest affinity and the most probable Zn(II) coordination mode in this protein involves four histidine imidazoles. In the analyzed metal-peptide systems, which contain a large number of histidines, the presence of so-called polymorphic binding sites is observed. The Ac-GGEHEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ fragment is also an efficient Ni(II) binder, coordinating Ni(II) with the highest affinity in the whole studied pH range. At pH 7.4, this ZnuA region binds Ni(II) *via* a set of six histidine residues in a quite rare octahedral geometry. At higher pH values, the coordination mode changes, and Ni(ii) is bound by three amide nitrogens

and one histidine imidazole, forming a square-planar nickel complex. Both studied ZnuA fragments, Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEQAHHDHDSG-NH₂ and Ac-HEHEHEHEHEHEHEHEH-NH₂, strongly prefer to bind Ni(II) over Zn(II) (Figure 135A and Figure 135B). The numerous glutamic acid side chains of the latter fragment may play an additional stabilizing role in the case of Ni(II) complexes (the numerous carboxylates may fill the coordination zone of Ni(II)), making them more stable than Zn(II) ones.

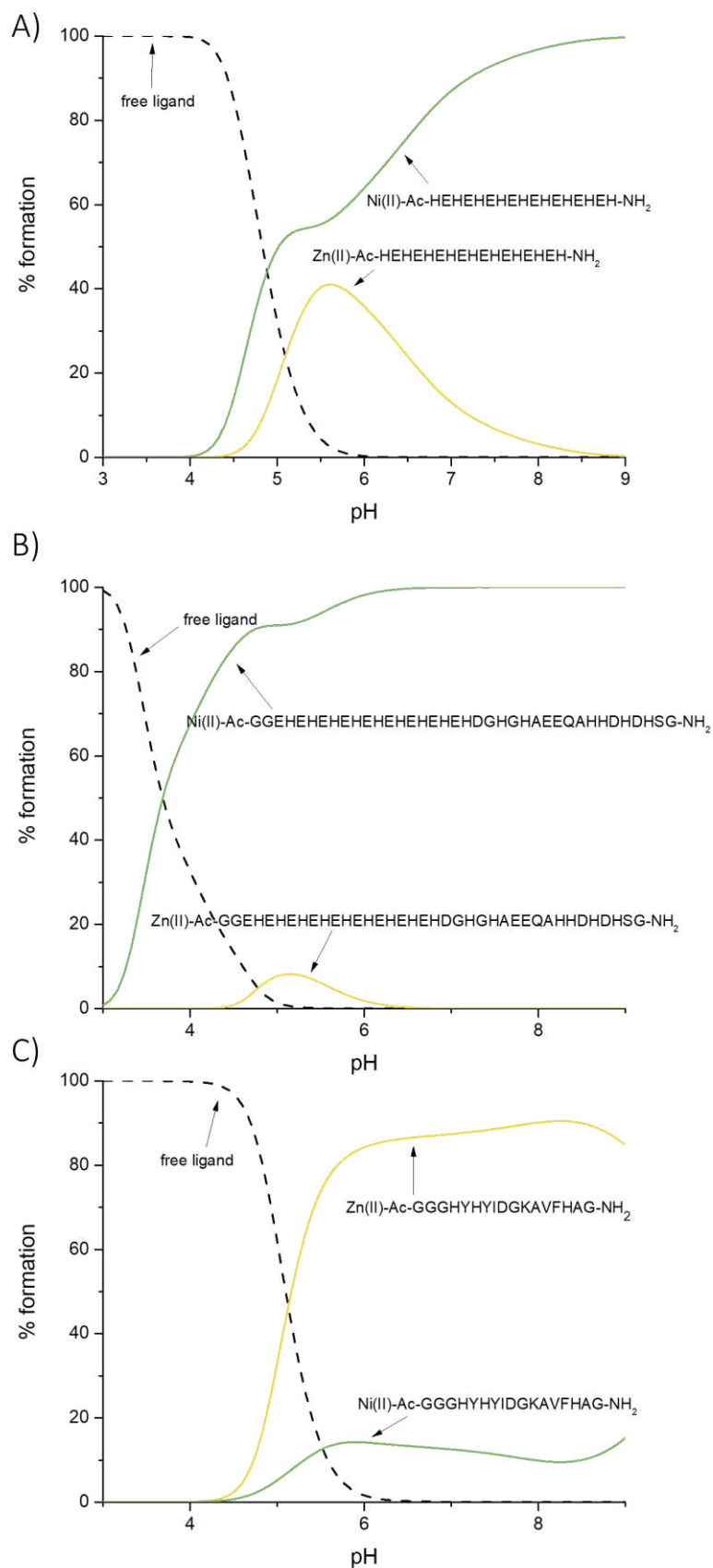


Figure 135 A competition plot between ZnuA fragments: A) Ac-HEHEHEHEHEHEHEHEH-NH₂, B) Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂, C) AztC fragment: Ac-GGGHYHYIDGKAVFHAG-NH₂

and Zn(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 9. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ac-HEHEHEHEHEHEHEH-NH₂] = [Ac-GGEHEHEHEHEHEHEHHDGHGHAEEQAHHDDHDSG-NH₂] = [Ac-GGGHYHYIDGKAVFHAG-NH₂] = 0.001 M

The AztC SBP fragment, on the other hand, prefers to bind Zn(II) over Ni(II) (Figure 135C). In a theoretical situation in which equimolar amounts of Zn(II) and Ni(II) would be available, at pH 7.4, more than 85% of the peptide would bind Zn(II). From the biological point of view, this suggests the Zn(II) specificity of AztC.

E. Thermodynamic and structural properties of Zn(II) and Ni(II) complexes with unstructured fragments of AdcA transporter

The main causative agent of dental caries, *Streptococcus mutans*, and the mechanism of its Zn(II) transport are presented in point B.3. of the Introduction (Zn(II) transport in *Streptococcus mutans*) and in the article [616]. To understand the transport of Zn(II) in *Streptococcus mutans*, I focus on Zn(II) and Ni(II) complexes of two AdcA regions (Ac-EGHGHKGGHHHA-NH₂ and Ac-HGIKSQKAEHFH-NH₂). The amino acid sequence of the AdcA transporter is presented in Figure 136. The probable Zn(II)-binding sites are highlighted in different colors. I explain their structural and thermodynamic properties, using a variety of complementary methods: mass spectrometry, potentiometry, UV-Vis spectroscopy and CD spectroscopy. Again, the AdcA regions are chosen on the basis of three criteria:

- (i) having probable Zn(II)-binding residues,
- (ii) being unstructured,
- (iii) being evolutionally conserved sites,

and therefore, they are appropriate for our peptide-based approach. The structure was predicted by Phyre2, a remote homology recognition technique, capable of generating reliable protein models [593]. I also check the thermodynamic parameters of Ni(II) binding sites, keeping in mind that Ni(II) is a biologically relevant metal ion, essential for pathogens, and could theoretically compete with Zn(II) for the binding sites in its transporter. It is important to keep in mind that in the full-length protein, the metal binding sites might also occur in locally structured regions.

10	20	30	40	50
MRKKPFIIVS	LLLVILAVVI	AFLAKDGEK	RSNGKLNVT	TFYPMYEFTK
60	70	80	90	100
NVVGDDQGVK	LLIKAGTEVH	DFEPSTKDVT	RIQEADTFVY	DSDSMETWVK
110	120	130	140	150
SVKKSVDVTQ	VPFVKATGNM	ILAPGVTEEE	GHGHKGHHHA	YDPHVWLSPK
160	170	180	190	200
RAIKLVENIR	DALSKKFPHR	AKIFKKNAAN	YIDKLQTLTK	EYAEGLANAK
210	220	230	240	250
QKSFVTQHAA	FGYLALDYGL	TQIPITGLTA	ESEPSAKRLA	ELSKYVKEYG
260	270	280	290	300
INYIYFEENA	SSAVSKTLAD	ETGVKTAVLS	PLESLTQKQM	DAGENYFSVM
310	320	330	340	350
RANLKALKKT	TDSAGKEIKP	EMDSKTVAN	GYFKDKSVKN	RKLSDWSGKW
360	370	380	390	400
QSIYPYLENG	TLDSVWDYKA	KSKKDMTAQE	YKEYYTKGYK	TDVEKITIDG
410	420	430	440	450
KKNTITFVQK	GKEHKYTYKY	VGKILTYKK	GNRGVRYLFE	TKDKGAGEFK
460	470	480	490	500
YVQFSDHGIK	SQKAEHFH	LFW	GSESQDKLL	EEMGNWPTY
510				
QEIIVAH				

Figure 136 Amino acid sequence of AdcA transporter; probable Zn(II) binding sites are highlighted in different colors

1. Methods of Zn(II) and Ni(II) coordination

a) Ac-EGHGHKGHHHA-NH₂

The Ac-EGHGHKGHHHA-NH₂ peptide behaves as an LH₇²⁺ acid with the protonating groups corresponding to the deprotonation of carboxylic side chain of glutamic acid (pK_a = 3.87), five histidine imidazole side chain groups (pK_a values of 5.48, 5.73, 6.47, 6.51 and 7.46, respectively) and to the deprotonation of lysine side chain group with pK_a value of 10.07 (Table 10).

Table 10 Potentiometric data for the proton, Zn(II) and Ni(II) complexes of the Ac-EGHGHKGHHHA-NH₂, Ac-HGIKSQKAEHFH-NH₂ in a water solution of 4 mM HClO₄ with I = 0.1 M NaClO₄, T = 298 K. Standard deviations are presented in parentheses

Species	Ac-EGHGHKGHHHA-NH ₂			Ac-HGIKSQKAEHFH-NH ₂		
	logβ	pK _a	residue	Logβ	pK _a	residue
H ₇ L	45.59 (3)	3.87	(E)			
H ₆ L	41.72 (3)	5.48	(H)	43.60 (2)	3.90	(E)
H ₅ L	36.25 (3)	5.73	(H)	39.71 (2)	5.84	(H)
H ₄ L	30.52 (3)	6.47	(H)	33.86 (1)	6.35	(H)
H ₃ L	24.05 (3)	6.51	(H)	27.51 (1)	7.08	(H)
H ₂ L	17.54 (3)	7.46	(H)	20.43 (1)	9.88	(K)
HL	10.07 (1)	10.07	(K)	10.56 (1)	10.56	(K)

Zn(II) complexes

ZnH ₃ L	29.00 (1)			30.88 (1)		
ZnH ₂ L	22.85 (1)	6.14	(H)	25.13 (1)	5.75	(H)
ZnHL	15.92 (2)	6.93	(H)	17.37 (2)	7.76	(H ₂ O)
ZnL	7.46 (2)	8.46	(H ₂ O)	9.24 (2)	8.13	(H ₂ O)
ZnH ₋₁ L	-2.11 (3)	9.58	(K)	-0.60 (3)	9.84	(K)
ZnH ₋₂ L				-11.19 (4)	10.51	(K)

Ni(II) complexes

NiH ₄ L				36.56 (8)		
NiH ₃ L	29.02 (1)			31.45 (1)	5.11	(H)
NiH ₂ L	22.87 (1)	6.15	(H)	25.48 (1)	5.98	(H)
NiHL	16.00 (1)	6.87	(H)			
NiL	7.61 (2)	8.39	(am)	8.61 (1)		
NiH ₋₁ L	-1.90 (2)	9.51	(am)	-0.78 (1)	9.38	(am)
NiH ₋₂ L				-11.04 (1)	10.27	(K)
NiH ₋₃ L	-23.65 (4)		(K)	-21.59 (1)	10.55	(K)

(1) Zn(II)-Ac-EGHGHKGHHHA-NH₂

The first Zn(II)-Ac-EGHGHKGHHHA-NH₂ complex form, ZnH₃L, has its maximum at pH 5.8 (Figure 137). It involves one glutamic acid residue and two histidine residues in the binding. The loss of one proton leads to the ZnH₂L⁻ form, which dominates in the solution around pH 6.5 and is most probably related to the deprotonation of non-coordinating histidine residue (due to no significant changes in the pK_a values – 6.51 and 6.14 for the free and complexed one, respectively) (Table 10). The next form, ZnHL²⁻, has a maximum at pH 7.7 and is most likely related to the deprotonation and coordination of the next imidazole nitrogen of histidine residue. Loss of the next proton leads to the formation of the ZnL³⁻ form, related to the proton loss of a water molecule bound to the Zn(II) atom. The last form, ZnH₋₁L⁴⁻, is connected with the deprotonation of non-coordinating lysine residue.

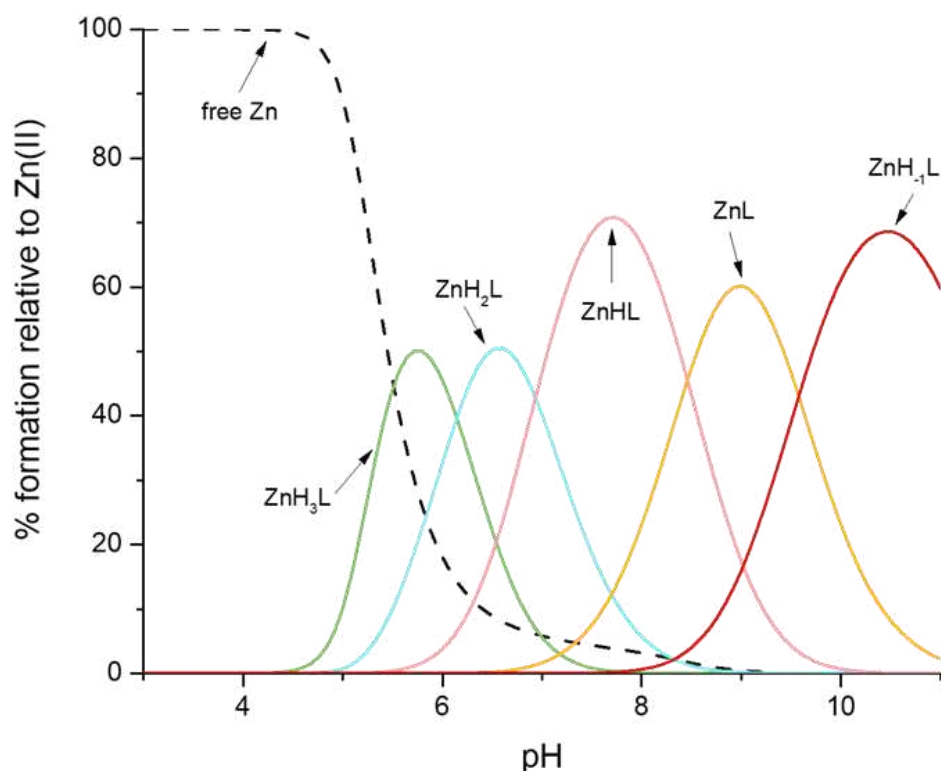


Figure 137 Species distribution diagram for the formation of Zn(II) complex with the Ac-EGHGHKGHHHA-NH₂ AdcA fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

(2) Ni(II)-Ac-EGHGHKGHHHA-NH₂

For the Ac-EGHGHKGHHHA-NH₂, we observe six complex forms with Ni(II) ions (Figure 138). The first one is NiH₃L with a maximum at pH 5.7. Most probably, it engages three histidine residues in binding. The increase of pH leads to the NiH₂L⁻ form, which corresponds to the deprotonation of subsequent histidine residue, which does not participate in binding (the low pK_a difference for this group between the free ligand (6.57) and the complexed one (6.15) confirms this theory). In this pH, the coordination mode does not change. The next form, NiHL²⁻, dominates in the solution above pH 6.9. The lowered pK_a value (7.46 and 6.87 for the free ligand and the complexed one, respectively) indicates the deprotonation and engagement of the last available histidine residue in Ni(II) binding. The following NiL³⁻ and NiH₋₁L⁴⁻ forms, with a maximum at around pH 8.9 and 10.2, respectively, are connected with the deprotonation and binding of two amide nitrogens. Therefore, the Ni(II) coordination sphere includes two histidine imidazole groups and two amide nitrogens. Around pH 8, a CD band (Figure 139), which appears near 415.9 nm (a positive Cotton effect) and 497.2 nm (a negative Cotton effect) can be assigned to the N⁻ → Ni(II) transition [587] and confirms this finding. Moreover, in the pH range from 8.0 to 11.0, the intensity of the bands increases. The involvement of two amide nitrogens in binding is also confirmed by UV-Vis spectroscopy (Figure 140), where from pH 9.0, a band with a maximum around 438.4 nm is observed. At very basic pH, the NiH₃L⁶⁻ complex is detected, and

it can be assigned to the deprotonation of lysine residue, which most probably does not participate in binding.

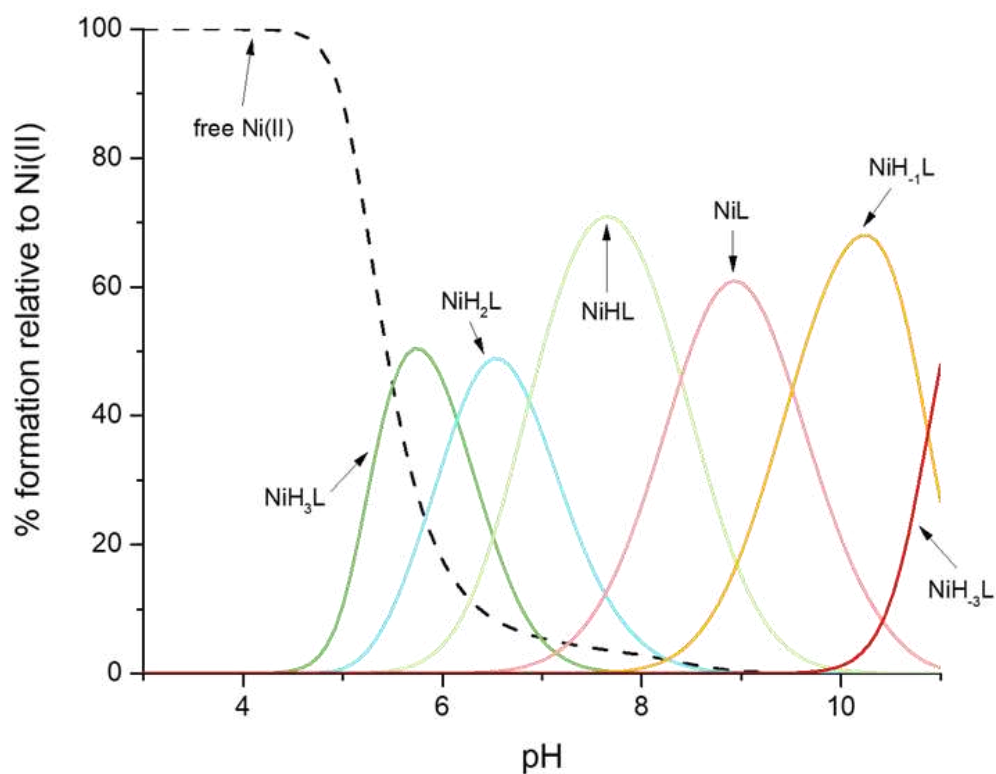


Figure 138 Species distribution diagram for the formation of Ni(II) complex with the Ac-EGHGHKGHHHA-NH₂ AdcA fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

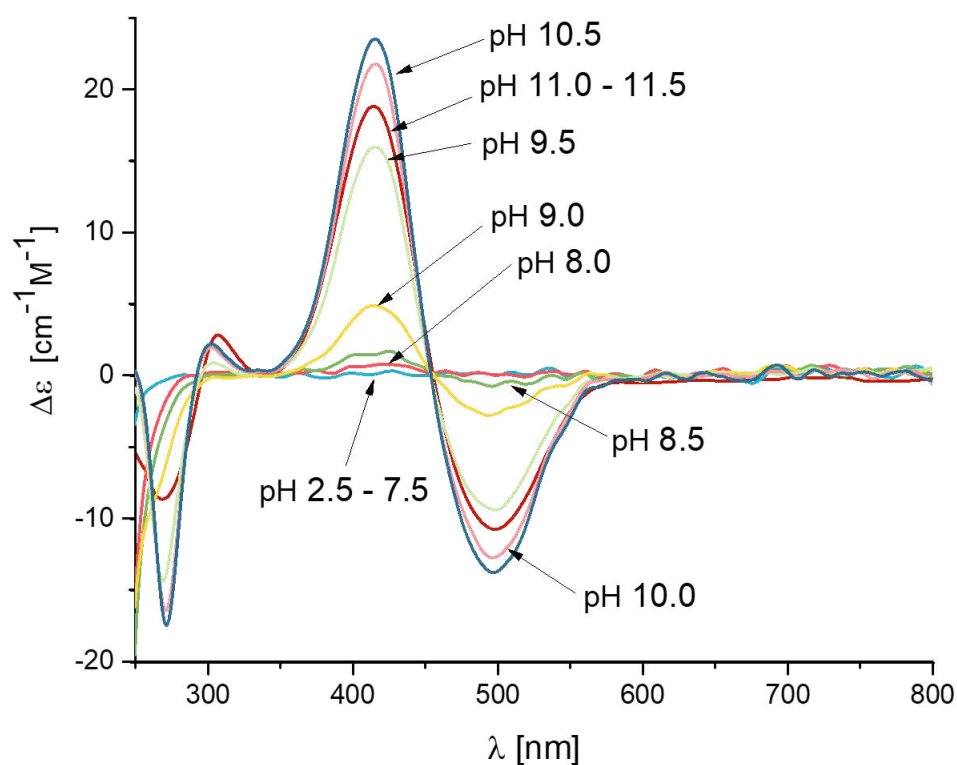


Figure 139 CD spectra of Ni(II) complex with the Ac-EGHGHKGHHHA-NH₂ fragment from AdcA protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

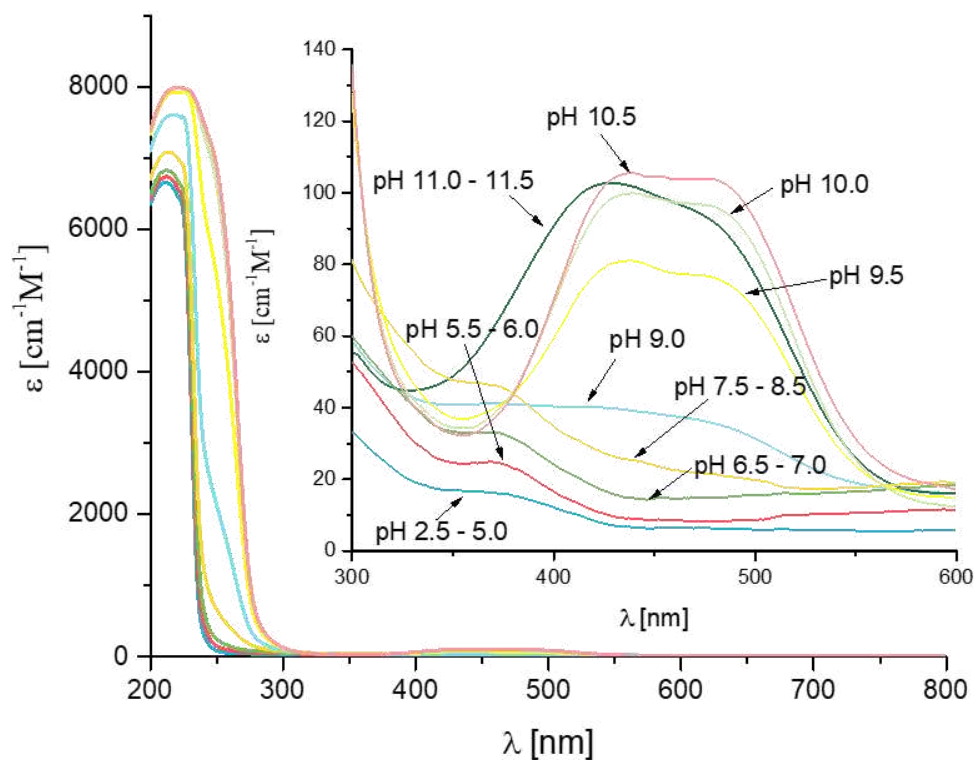


Figure 140 UV-Vis spectra of Ni(II) complex with the Ac-EGHGHKGHHHA-NH₂ fragment from AdcA protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

b) *Ac-HGIKSQKAEHFH-NH₂*

The *Ac-HGIKSQKAEHFH-NH₂* peptide has six protonation constants. The first ($pK_a = 3.90$) can be assigned to the deprotonation of carboxylic side chain of glutamic acid. The next three constants ($pK_a = 5.84$, 6.35 and 7.08) are related to the deprotonation of three histidine imidazole groups and the last two ($pK_a = 9.88$ and 10.56) correspond to the deprotonation of two lysine side chain groups (Table 10).

(1) *Zn(II)-Ac-HGIKSQKAEHFH-NH₂*

The first acidic *Zn(II)-Ac-HGIKSQKAEHFH-NH₂* complex is ZnH_3L^+ , with a maximum at pH 6.0 (Figure 141) and most probably involves two imidazole groups in coordination. The next form, ZnH_2L , with a maximum at pH 7.0 is related to the proton loss and coordination of the next histidine residue. A significant decrease of the pK_a values calculated for the complexes ($pK_a = 5.75$) and free imidazole ($pK_a = 7.08$) suggest that histidine residue is involved in *Zn(II)* binding (Table 10). The next two observed forms, $ZnHL^-$ and ZnL^{2-} , correspond to the deprotonation of two water molecules and the remaining two deprotonations are most likely related to the proton loss of two non-coordinating lysine residues.

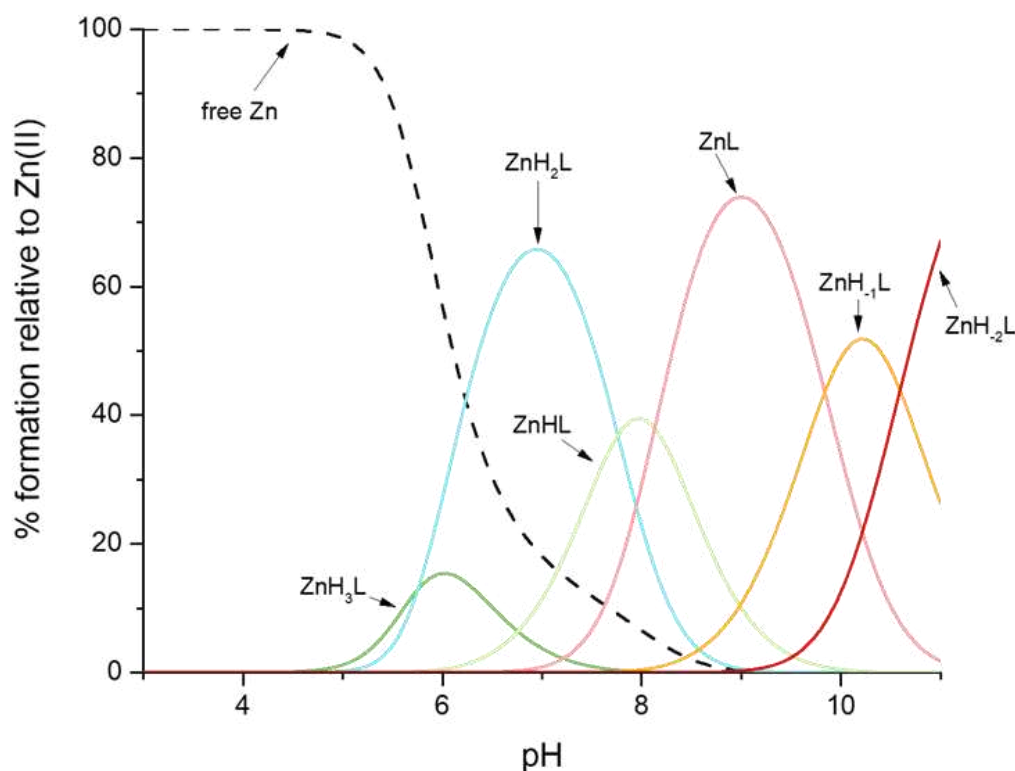


Figure 141 Species distribution diagram for the formation of *Zn(II)* complex with the *Ac-HGIKSQKAEHFH-NH₂* AdcA fragment, $T = 298\text{ K}$, $I = 0.1\text{ M NaClO}_4$, $[L] = 0.0005\text{ M}$, $Zn(II)/L$ molar ratio = $1 : 1$

In the case of Ac-HGIKSQKAEHFH-NH₂, Ni(II) coordination starts at pH 4.0 and the first observed form is NiH₄L²⁺ with a maximum at pH 5.5 (Figure 142). Most probably, it involves one histidine residue in binding. The next two forms, NiH₃L⁺ and NiH₂L, with maxima at pH 6.0 and 7.4, respectively, are both related to the proton loss and coordination of histidine residues. A significant decrease of the pK_a values calculated for the complexed and free imidazole (pK_a = 5.11 and 6.35; pK_a = 5.98 and 7.08, respectively) suggest that both residues are involved in Ni(II) binding. Above pH 8.5, the NiL²⁻ form dominates in solution. Around this pH, a CD band at 260.0 nm, that can be related to the N⁻ → Ni(II) charge transfer transitions [587] is observed (Figure 143). The increase of pH leads to the NiH₋₁L³⁻ form, in which most probably the Ni(II) coordination sphere includes two histidine imidazole groups and two amide nitrogens – the CD band with negative Cotton effect at 425.0 nm and positive Cotton effect at 525.0 nm and UV-Vis band at 420.0 nm confirm this finding and can be assigned to the square planar nickel complex (Figure 144). The last two forms, NiH₋₂L⁴⁻ and NiH₋₃L⁵⁻, correspond to the deprotonation of two lysine residues, which do not precipitate in coordination.

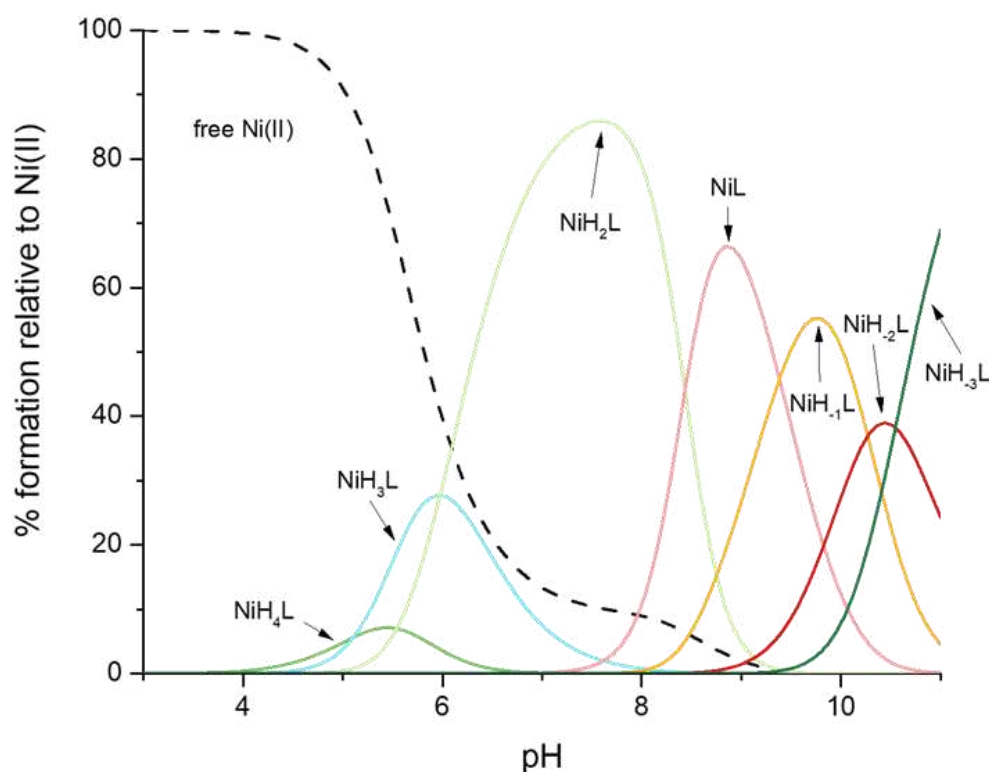


Figure 142 Species distribution diagram for the formation of Ni(II) complex with the Ac-HGIKSQKAEHFH-NH₂ AdcA fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

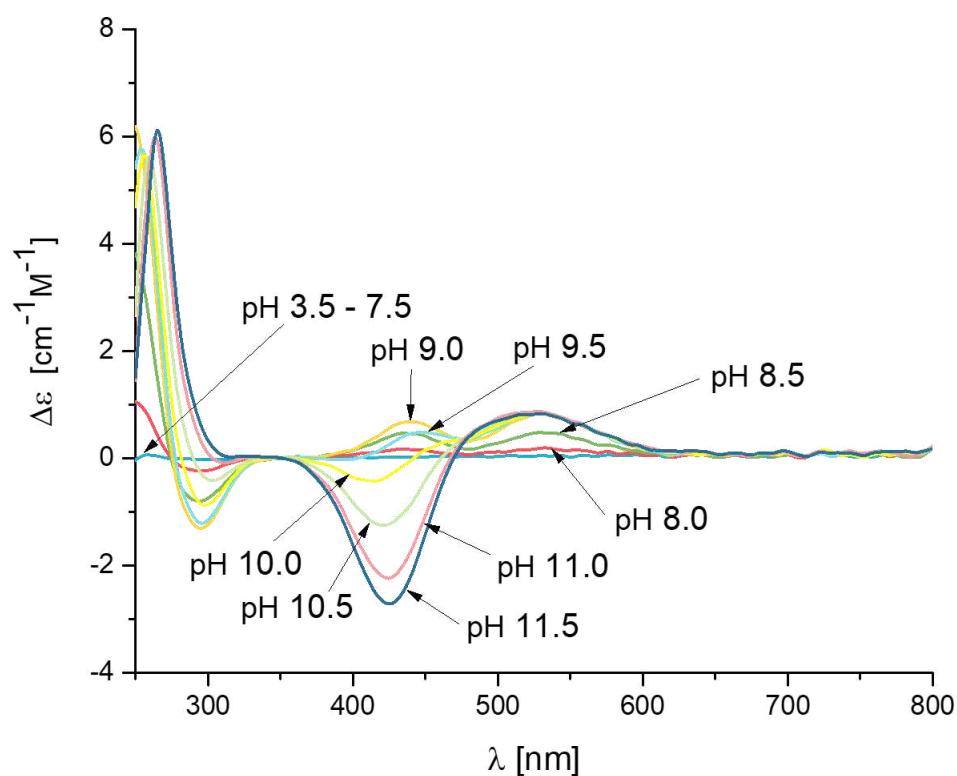


Figure 143 CD spectra of Ni(II) complex with the Ac-HGIKSQKAEHFH-NH₂ fragment from AdcA protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

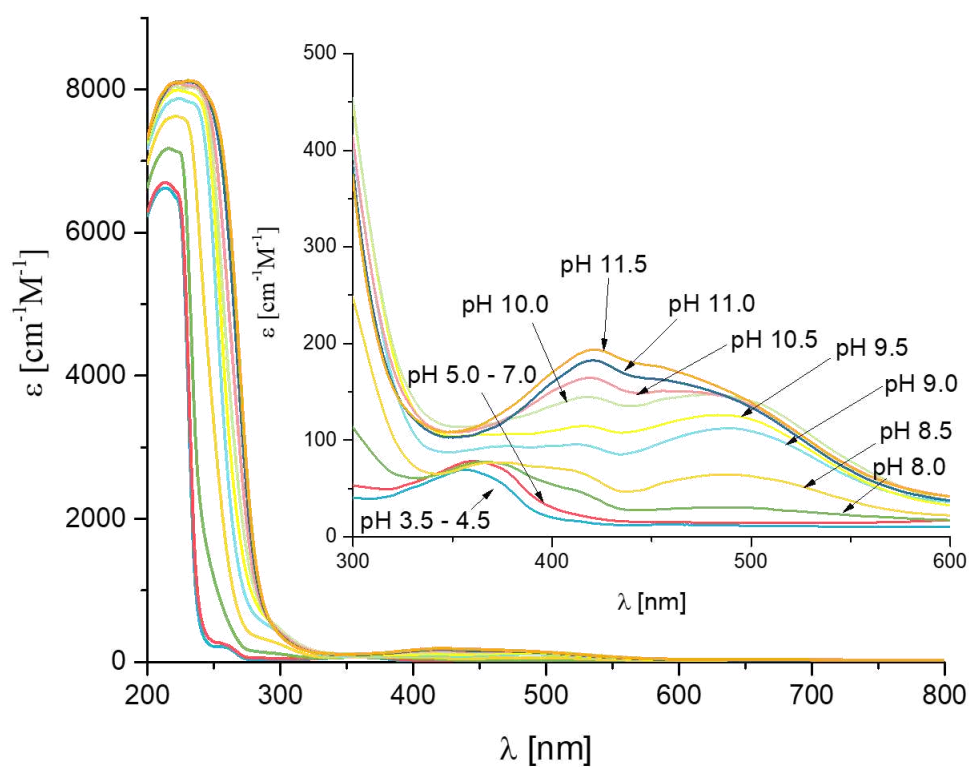


Figure 144 UV-Vis spectra of Ni(II) complex with the Ac-HGIKSQKAEHFH-NH₂ fragment from AdcA protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

2. Secondary structure analysis

The coordination of Zn(II) influence pronounced secondary structure changes of both Ac-EGHGHKGHHHA-NH₂ and Ac-HGIKSQKAEHFH-NH₂ AdcA regions (Figure 145 and Figure 146). At pH 7.4, both peptides are random coils, with a positive absorption band around 215 nm and a negative one below 200 nm. Metal coordination changes the CD spectra and therefore also the secondary structure of the Zn(II) complexes, the intensity of the spectral minima become smaller and the minima and maxima shifts to ca. 195 nm and 210 nm, respectively. Although these parameters are not typical for any particular structure (they are sometimes inadequately assigned to PPII like helixes in the literature), it is clear that metal coordination significantly induces structural changes.

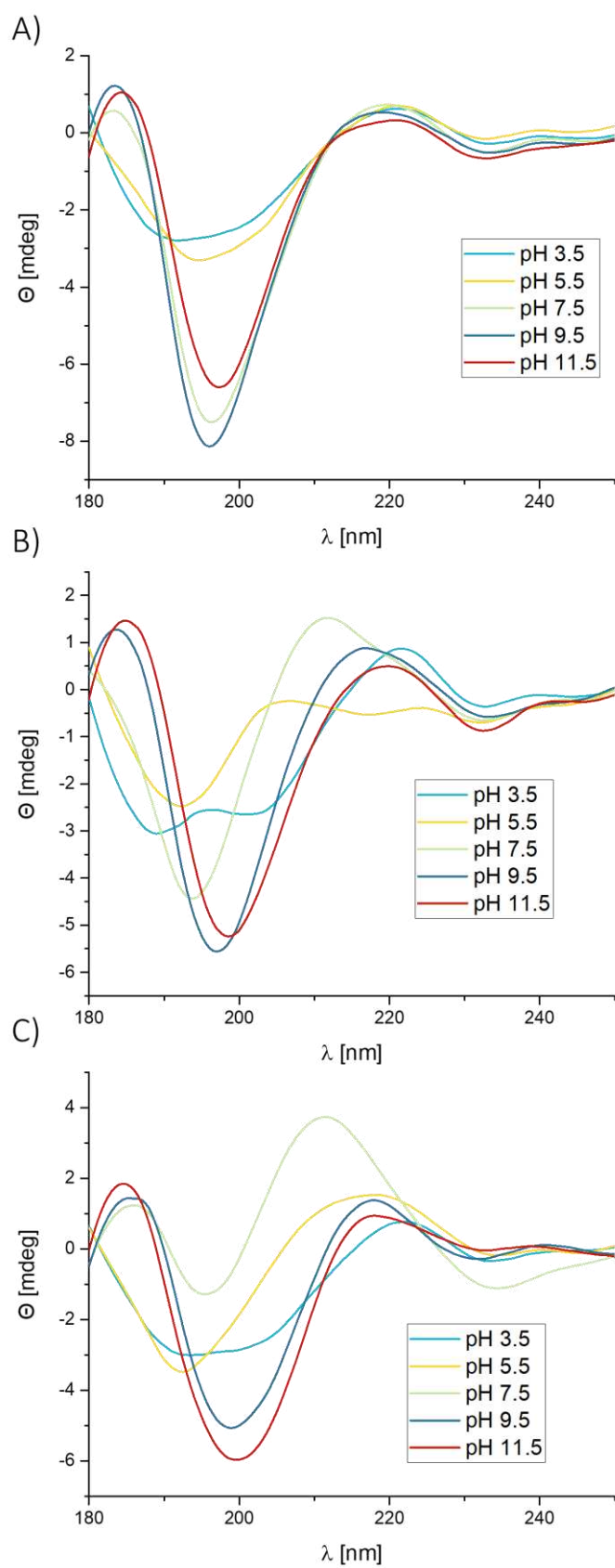


Figure 145 CD spectra of A) Ac-GIKSQQAEHFH-NH₂ and its complexes with b) Zn(II) and C) Ni(II); optical path 0.1 mm, Ni(II)/L molar ratio = 1 : 1

Ni(II) coordination is an interesting case – its binding appears to have almost no structural impact on the overall structure of Ac-HGIKSQKAEHFFH-NH₂, while in the case of Ac-EGHGHKGHHHA-NH₂, it induces a significant structural rearrangement, enhancing and shifting the CD maximum at 215 nm and making the spectral minimum at 195 nm much more shallow (Figure 145C and 146C). Those structural changes indicate that the secondary structure of peptide changes in the presence of metal ions. The secondary structure changes in terms of random coil. The addition of metal ions is important from the chemical point of view because it can change the mode of action, both due to the structural changes and the final charge.

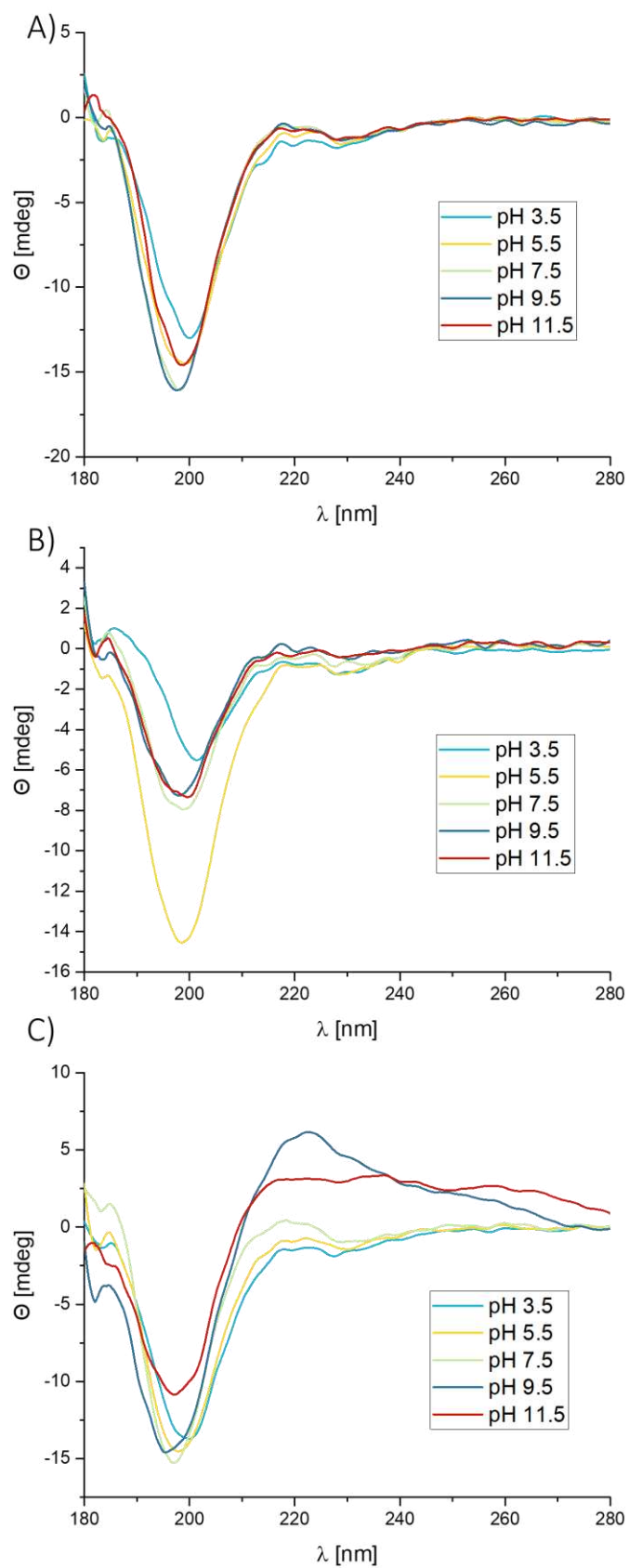


Figure 146 CD spectra of A) Ac-HGIKSQKAEHFH-NH₂ and its complexes with b) Zn(II) and C) Ni(II); optical path 0.1 mm, Ni(II)/L molar ratio = 1 : 1

3. Comparison of the stability of Zn(II) and Ni(II) complexes

Is it theoretically possible for other biologically relevant metal ions, Ni(II), necessary for proper growth and replication of microorganisms, to compete with Zn(II) for the binding sites in the AdcA protein?

In order to identify the AdcA fragment that binds Zn(II) and Ni(II) with the highest affinity, we used the thermodynamic data for the metal complexes to simulate a theoretical situation in which equimolar amounts of Zn(II) and both studied unstructured AdcA fragments were mixed. This allows a direct comparison of the calculated constants at different pH values (Figure 147) and shows that in almost the entire pH range, the Ac-EGHGHKGHHHA-NH₂ ligand is preferred over the Ac-HGIKSQKAEHFH-NH₂ one, which is not surprising, since the Ac-EGHGHKGHHHA-NH₂ region contains more histidine residues (five vs. three in the Ac-HGIKSQKAEHFH-NH₂ ligand). At pH 7.4, more than 70% of available Zn(II) would be bound to Ac-EGHGHKGHHHA-NH₂.

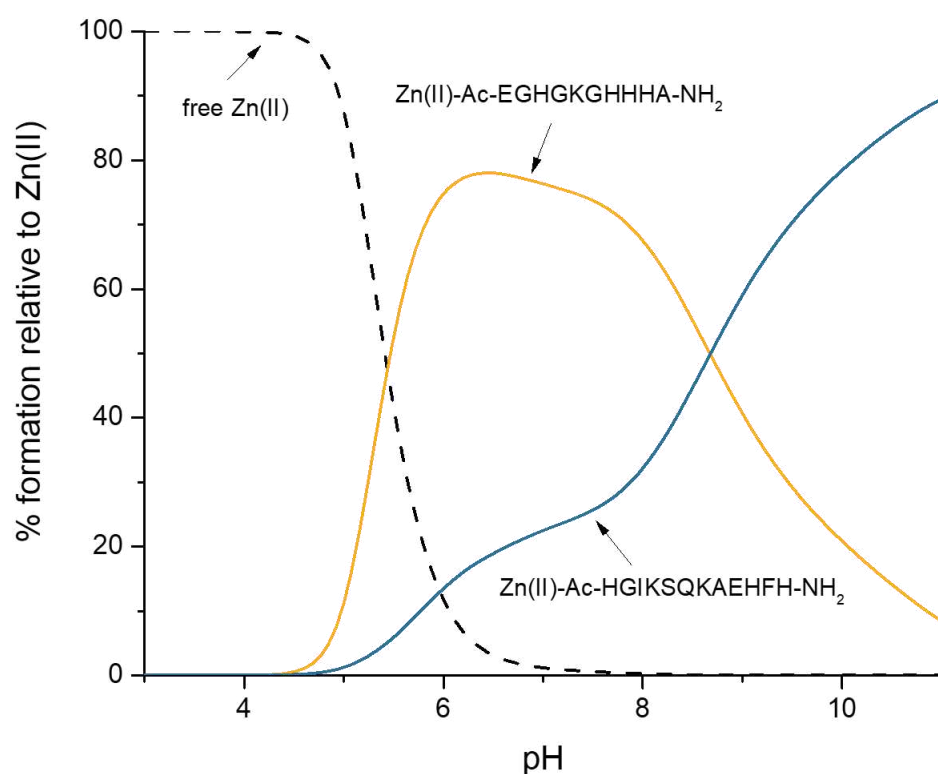


Figure 147 A competition plot between AdcA fragments: Ac-EGHGHKGHHHA-NH₂, Ac-HGIKSQKAEHFH-NH₂ and Zn(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 10. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ac-EGHGHKGHHHA-NH₂] = [Ac-HGIKSQKAEHFH-NH₂] = 0.001 M

Ni(II) also chooses Ac-EGHGHKGHHHA-NH₂ over Ac-HGIKSQKAEHFH-NH₂ almost in the entire pH range, in which His imidazoles are the metal binding sites; at pH 7.4, in our hypothetical situation, more than 85% of the metal would be bound to the His-rich Ac-EGHGHKGHHHA-NH₂. The situation changes dramatically in alkaline environment, when amide nitrogens are involved in coordination – the Ni(II)-Ac-HGIKSQKAEHFH-NH₂ complex becomes

more stable (Figure 148). To summarize, poly-His complexes are more tempting metal binding sites at lower pH values, while at higher pH, when amide nitrogens are involved in Ni(II) coordination, the affinity of poly-His sites turns out to be lower than that of the sequence with a lower number of His residues, which is more likely to involve an amide in binding.

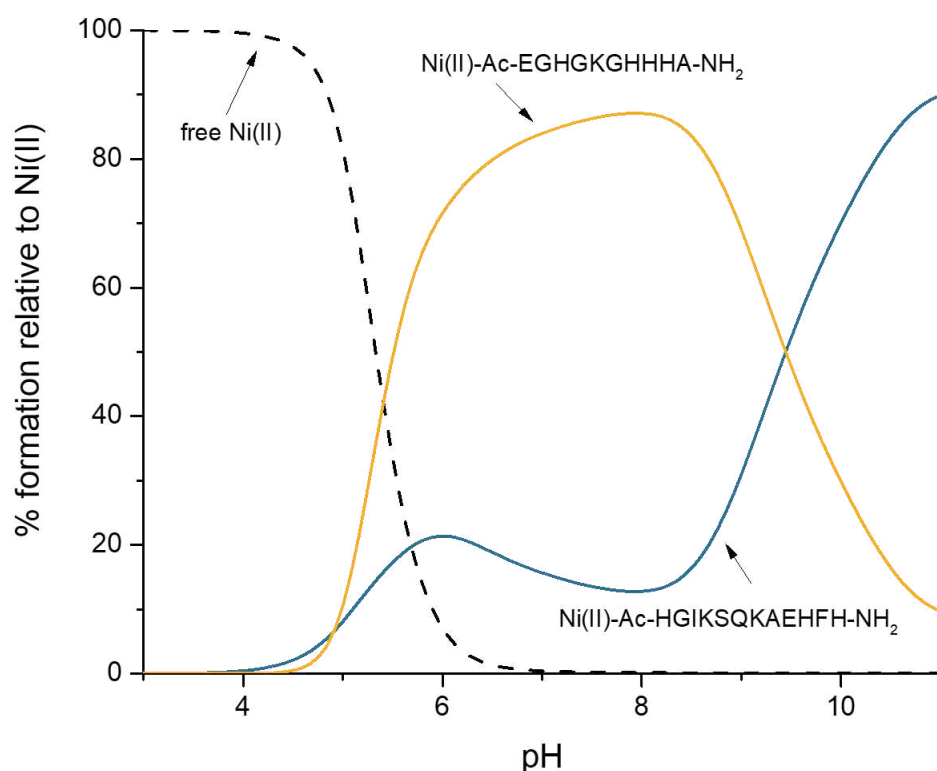


Figure 148 A competition plot between AdcA fragments: Ac-EGHGHKGHHHA-NH₂, Ac-HGKSQKAEHFH-NH₂ and Ni(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 10. Conditions: T=298 K, I=0.1 M NaClO₄, [Ni(II)] = [Ac-EGHGHKGHHHA-NH₂] = [Ac-HGKSQKAEHFH-NH₂] = 0.001 M

For the Ac-EGHGHKGHHHA-NH₂ ligand, Zn(II) and Ni(II) binding preferences are concerned, up to pH 7.4, no significant differences in the stability of both complexes are observed – the two metal bind with almost identical affinity, which is quite surprising. The situation changes in alkaline environment, when amide nitrogens are involved in the coordination of Ni(II), and the Ni(II)-Ac-EGHGHKGHHHA-NH₂ complex becomes significantly more stable than the Zn(II) one (Figure 149).

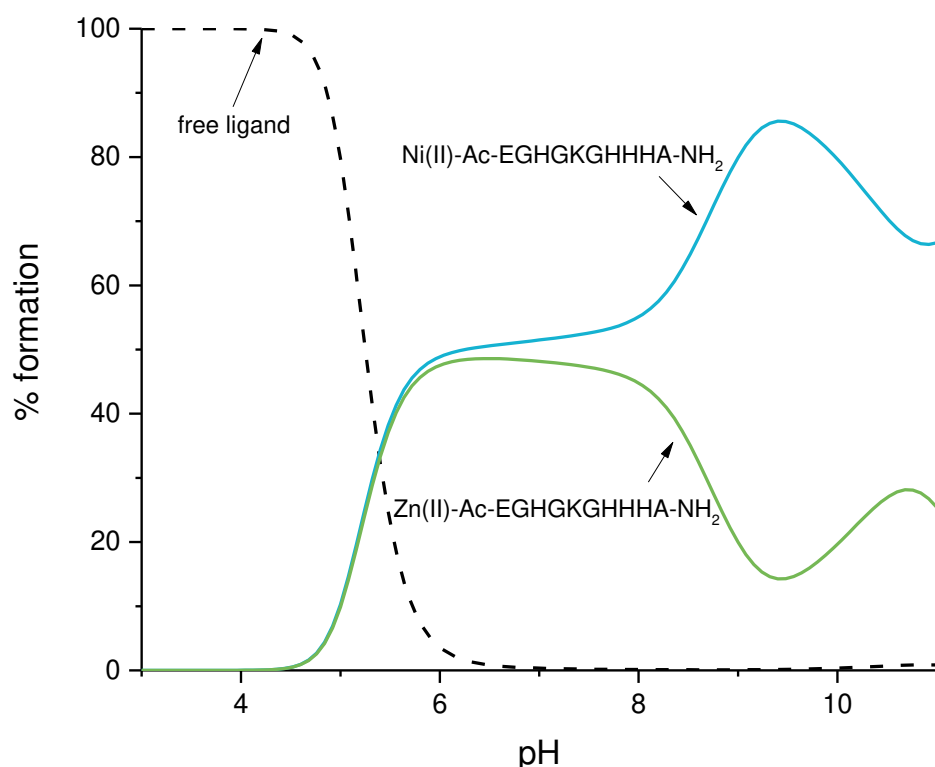


Figure 149 A competition plot between AdcA fragment: Ac-EGHGKGGHHA-NH₂, Zn(II) and Ni(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 10. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ni(II)] = Ac-EGHGKGGHHA-NH₂ = 0.001 M

A very analogous situation is observed for Ac-HGIKSQKAEHFH-NH₂: as for the Zn(II) and Ni(II) specificity of this region, it seems to have no strong metal preference up to pH 9. At basic pH, the coordination of amide nitrogens in Ni(II) complexes gives them greater stability than Zn(II) complexes (Figure 150), as in the case of the previous ligand (Figure 149).

In general, the much higher than expected affinity of Ni(II) towards regions that should be specific for Zn(II) was biologically difficult to explain. Analyzing this phenomenon, I went back to the literature in order to understand with which situation we are dealing with in a living organism – what are the physiological concentrations of Zn(II) and Ni(II) and does it matter how much of the appropriate metals is available?

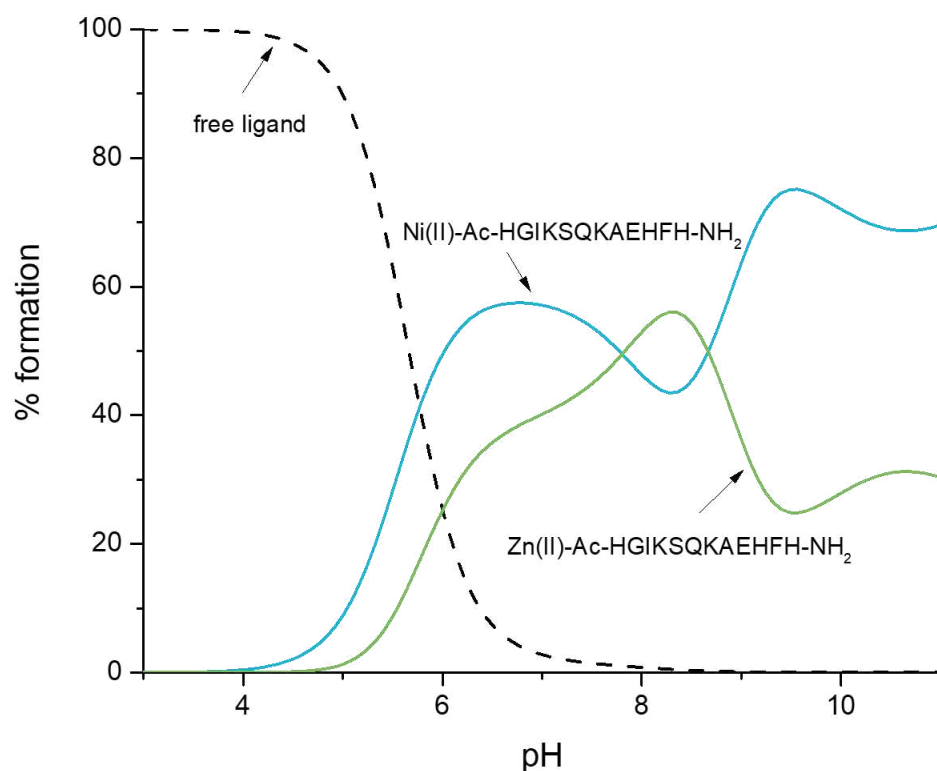


Figure 150 A competition plot between AdcA fragment: Ac-HGIKSQKAEHFH-NH₂, Zn(II) and Ni(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 10. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ni(II)] = [Ac-HGIKSQKAEHFH-NH₂] = 0.001 M

Metal ions like Zn(II) and Ni(II) are found naturally in the saliva, teeth and dental plaque and to compare the stability of Zn(II) and Ni(II) complexes to naturally found in the human body, we simulated a hypothetical situation in which the studied sequences: Ac-EGHGHKGHHHA-NH₂, Ac-HGIKSQKAEHFH-NH₂ and metals were mixed in appropriate concentrations in which the metal ions could be found in the oral cavity (Figure 151). Their concentrations are quite variable and depend on the diet, occupational exposure, salivary flow rate, exposure to toxic metals, diseases states or quantity of metal-binding proteins. However, the Zn(II) concentration in saliva is within the range of 0.01 – 0.25 µg/ml [472]. In dental plaque, Zn(II) concentration is higher and fluctuates from 6 to 20 µg/ml, Ni(II) concentration in saliva has also been tested and varies between numbers as low as 0.001 and 0.0019 µg/ml, but may even reach 0.19 µg/ml in the case of orthodontic treatment [617].

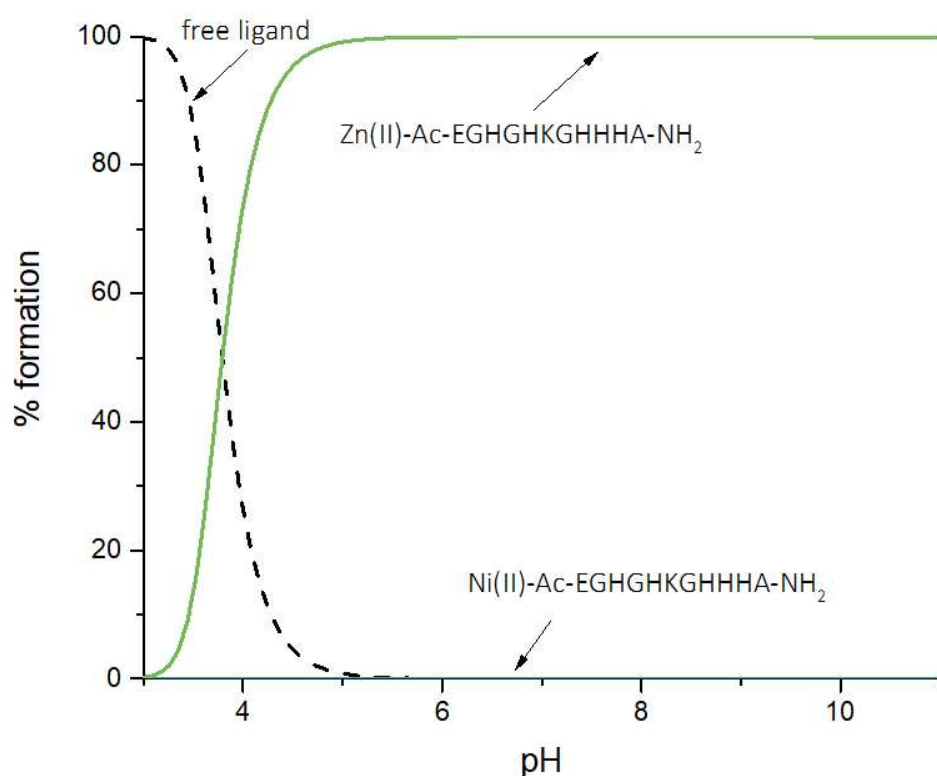


Figure 151 A competition plot between AdcA fragment: Ac-EGHGHKGGHHA-NH₂, Zn(II) and Ni(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 10; hypothetical metal concentrations are taken from the literature [472], [617] and equal: [Zn(II)] = 0.25 µg/ml, [Ni(II)] = 0.0019 µg/ml (in people without orthodontic treatment). Conditions: T=298 K, I=0.1 M NaClO₄

The results clearly indicate that in the physiological environment, both ligands (Ac-EGHGHKGGHHA-NH₂ (Figure 151) and Ac-HGKSQKAEHFH-NH₂ (Figure 152)) will be found bound to Zn(II) rather than Ni(II). The saliva and dental plaque Zn(II) concentrations are much higher than those of Ni(II), thus the trend is constant over the entire pH range. This guarantees an efficient Zn(II) transport in *S. mutans* using the AdcA protein, despite the affinities towards Ni(II) being quite high for both studied regions.

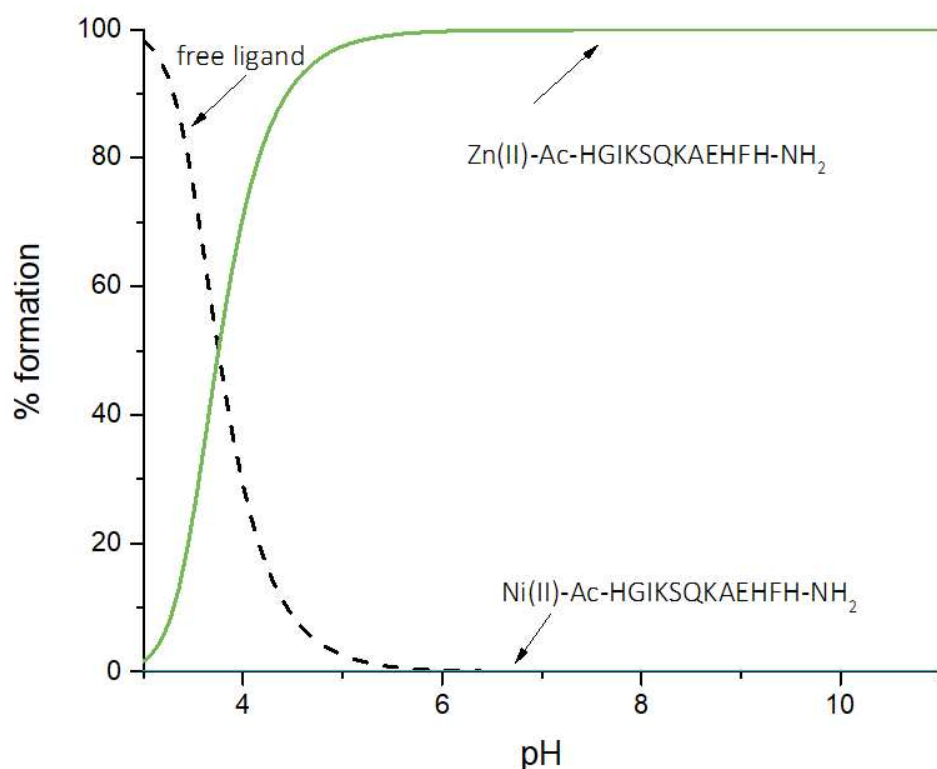


Figure 152 A competition plot between AdcA fragment: Ac-HGIKSQKAEHFH-NH₂, Zn(II) and Ni(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 10; hypothetical metal concentrations are taken from the literature [472], [617] and equal: [Zn(II)] = 0.25 µg/ml, [Ni(II)] = 0.0019 µg/ml (in people without orthodontic treatment). Conditions: T=298 K, I=0.1 M NaClO₄

4. Conclusions

I specified two probable binding sites in the AdcA lipoprotein from *Streptococcus mutans*. At physiological pH, the Ac-EGHGHKGHHHA-NH₂ region binds Zn(II) with a higher affinity than the Ac-HGIKSQKAEHFH-NH₂ one. The binding preferences of Ni(II), metal ions that could potentially compete with Zn(II) binding to AdcA, involve not only histidine imidazoles, but also an amide nitrogen, but the stability of Zn(II) and Ni(II) complexes is comparable. However, considering the high Zn(II) concentration in human saliva and dental plaque, the amount of Zn(II)-AdcA complexes present in the oral cavity would be much greater than those of Ni(II), that is why the ligand affinity towards Ni(II) most likely does not matter under the physiological conditions in the natural habitat of *Streptococcus mutans*.

These findings are not only an input to the basic chemistry of Zn(II), Cu(II) and Ni(II), and an enhancement of our understanding of metal transport in pathogens, but, quite importantly, they show how important it is to keep in mind the physiological concentration of the metal ions which could compete for the same biologically important ligands – in other words, “high affinity is not enough, high concentration is a lot better”.

VII. CHAPTER V: FINAL DISCUSSION

The most important finding of this doctoral thesis is understanding the bioinorganic chemistry of unstructured regions of bacterial and fungal zincophores and zinc transporters. The protein regions were selected based on three main criteria: (i) having probable Zn(II)-binding residues, (ii) being unstructured, and (iii) being evolutionally conserved sites. The work elucidated the bioinorganic chemistry and determined the relationship between the coordination mode, structure and thermodynamic stability of Zn(II) and Ni(II) complexes (since Ni(II) is a potential competitor for Zn(II) ions, having high affinity towards similar binding sites) with the following peptides:

(i) Ac-ARHAKAH-NH₂, Ac-MHRLYHVP-NH₂, Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂, and Ac-PNCHTHEGGQLHCT from AspF2 zincophore (*Aspergillus fumigatus*) [493],

(ii) Ac-TGCHSHGS-NH₂ and Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ from ZrFC transporter (*Aspergillus fumigatus*) [494],

(iii) Ac-HEHEHEHEHEHEHEHEH-NH₂ and Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHSG-NH₂ from ZnuA solute binding protein (*Paracoccus denitrificans*) [492],

(iv) Ac-GGGHYHYIDGKAVFHAG-NH₂ from AztC solute binding protein (*Paracoccus denitrificans*) [492],

(v) Ac-EGHGHKGHHHA-NH₂ and Ac-HGIKSQKAEHFH-NH₂ from AdcA lipoprotein (*Streptococcus mutans*) [616],

(vi) Ac-ACHA-NH₂ and Ac-AHCA-NH₂ (model sequences) [604].

Thanks to the use of a number of physicochemical methods (MS, potentiometry, UV-Vis spectroscopy, CD spectroscopy and NMR spectroscopy) and the possibility of conducting some work in cooperation with the University of Ferrara, the objectives of the work (outlined in detail in Chapter II) were fully achieved. Based on the results presented in this work, the following main conclusions can be drawn:

(i) The number of amino acids in the sequence and their position strongly influence the properties of the Zn(II) complexes with unstructured fragments of the AspF2 zincophore, ZrFC transporter, ZnuA and AztC solute binding proteins and AdcA lipoprotein. Common Zn(II) ligands found within proteins include the sulfur atom of cysteine residue, imidazole nitrogen of histidine residues, and the carboxyl group of aspartate or glutamate residues. All tested ligands possess different sets of donor atoms, differently arranged in the sequence. The ligands with the biggest affinity toward Zn(II) have ligands with Cys residues (Ac-PNCHTHEGGQLHCT from AspF2 zincophore and Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ ZrFC transporter). It is not a surprise, because Cys residues perform essential functions within proteins, including metal binding, like Zn(II). In particular, Cys residues can display high affinity toward Zn(II), and their complexes are critical mediators of protein structure, but also catalysis or regulation. The Ac-

GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ fragment from the ZnuA solute binding protein present in *Paracoccus denitrificans* also has a high affinity towards Zn(II). At pH 7.4, the binding mode includes four His imidazoles. It also is not a surprise, because ligands with the {4N_{im}} binding mode will have more temping Zn(II) binding sites than ligands with the {3N_{im}}, {2N_{im}}, or {1N_{im}} binding modes. Moreover, due to the presence of large number of histidine residues, on the basis of potentiometric titration, I indicated the polymorphic binding sites for that ligand, which can additionally stabilize the complexes.

The coordination properties of tested ligands toward Zn(II) and Ni(II) depend on the pH and amino acid sequence. At pH 7.4, discussed ligands bind Zn(II) and Ni(II) with the binding modes presented in the Table 11. The coordination geometries of Zn(II) and Ni(II) complexes with unstructured fragments of Aspf2, ZrfC, ZnuA, AztC and AdcA proteins are: tetrahedral for Zn(II), and square planar or octahedral for Ni(II).

Table 11 Summary of proposed Zn(II)- and Ni(II)-binding models for unstructured fragments of Aspf2 zincophore, ZrfC transporter, ZnuA solute binding protein, AztC solute binding protein, AdcA lipoprotein at physiological pH (pH = 7.4) with their geometrical shapes

Aspf2 zincophore [493]	
Ac-ARHAKAH-NH ₂	
Zn(II) binding mode: {2N _{im} }	Ni(II) binding mode: {2N _{im} }
Ac-MHRLYHVP-NH ₂	
Zn(II) binding mode: {2N _{im} }	Ni(II) binding mode: {2N _{im} }
Ac-MHRLYHVPVGGQWVDHFAD-NH ₂	
Zn(II) binding mode: {3N _{im} , COO ⁻ }, tetrahedral	Ni(II) binding mode: {3N _{im} }
Ac-PNCHTHEGGQLHCT	
Zn(II) binding mode: {2N _{im} , 2S ⁻ }, tetrahedral	Ni(II) binding mode: {2N _{im} , 2S ⁻ }, square planar
ZrfC transporter [494]	
Ac-TGCHSHGS-NH ₂	
Zn(II) binding mode: {2N _{im} , S ⁻ }	Ni(II) bindings mode: {2N _{im} , S ⁻ }
Ac-MNCHFHAGVEHCIGAGESEGSSQ-NH ₂	
Zn(II) binding mode: {2N _{im} , 2S ⁻ }, tetrahedral	Ni(II) binding mode: {3N _{im} , S ⁻ } / {N _{im} , 2S ⁻ , N ⁻ }, square planar
ZnuA solute binding protein [492]	
Ac-HEHEHEHEHEHEHEHEH-NH ₂	
Zn(II) binding mode: {4N _{im} }, tetrahedral	Ni(II) binding mode: {6N _{im} }, octahedral
Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH ₂	
Zn(II) binding mode: {4N _{im} }, tetrahedral	Ni(II) binding mode: {6N _{im} }, octahedral
AztC solute binding protein [492]	
Ac-GGGHYHYIDGKAVFHAG-NH ₂	

Zn(II) binding mode: {3 _{im} }	Ni(II) binding mode: {3N _{im} }
AdcA lipoprotein [616]	
Ac-EGHGHKGHHHA-NH ₂	
Zn(II) binding mode: {3N _{im} }	Ni(II) binding mode: {3N _{im} }
Ac-HGKSQKAEHFH-NH ₂	
Zn(II) binding mode: {3N _{im} }	Ni(II) binding mode: {3N _{im} }

(ii) I compared the stabilities of all formed complexes, pointing out the most probable metal binding sites, basing on the protonation constants of the ligands and the formation constants of their Zn(II) and Ni(II) complexes. Based on them, I prepared competition plots that present a hypothetical situation in which equimolar amounts of individual reactants would be mixed. They allowed me to compare the thermodynamic stability of the resulting complexes. The most probable Zn(II)-binding sites within the studied proteins are as follows:

- 1) Ac-MNCHFHAGVEHCIGAGESESGSSQ-NH₂ fragment of the ZrfC transporter,
- 2) C-terminal region of the Aspf2 zincophore, Ac-PNCHTHEGGQLHCT,
- 3) Ac-GGEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ fragment of the ZnuA solute binding protein,
- 4) and the Ac-EGHGHKGHHHA-NH₂ fragment of the AdcA lipoprotein.

These places also constitute the most probable Ni(II)-binding sites within the studied proteins:

- 1) Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ fragment of the ZnuA solute binding protein,
- 2) Ac-MNCHFHAGVEHCIGAGESESGSSQ-NH₂ fragment of the ZrfC transporter,
- 3) Ac-HEHEHEHEHEHEHEHEH-NH₂ fragment of the ZnuA solute binding protein,
- 4) the C-terminal region of the Aspf2 zincophore, Ac-PNCHTHEGGQLHCT,

However, after binding the metal ion in the primary binding site, the conformation of the native protein may slightly change, and the availability of the other binding sites may differ from the affinity obtained for model sequences.

To compare the thermodynamic stability of Aspf2, ZrfC, ZnuA, AztC and AdcA fragments, I prepared a competition plot showing the percentage of their Zn(II) complexes (Figure 153 and Figure 154) and Ni(II) complexes (Figure 155 and Figure 156) at pH 7.4. Keeping in mind the remaining two model sequences (Ac-AHCA-NH₂ and Ac-ACHA-NH₂), I prepared competition plots that present a hypothetical situation in which equimolar amounts of Zn(II), Ni(II) and all tested ligands were mixed (Figure 157 and Figure 158, respectively).

The work shows that the most effective way of coordinating Zn(II) ions at physiological pH is the coordination involving two histidines and two cysteines with {2N_{im}, 2S⁻} binding mode (motif quite common in chemistry from zinc fingers). More than 85% of Zn(II) bind Ac-MNCHFHAGVEHCIGAGESESGSSQ-NH₂ fragment of the ZrfC transporter (Figure 153). The C-terminal part of the Aspf2 zincophore, Ac-PNCHTHEGGQLHCT, has a much lower affinity for Zn(II),

but the highest of the other ligands (about 10%) and binds Zn(II) with the same set of donor atoms - {2N_{im}, 2S⁻} binding mode (Figure 153). These motifs are known for their structural functions in zinc fingers. The results clearly indicate, that Cys and His residues are the most effective Zn(II) binders.

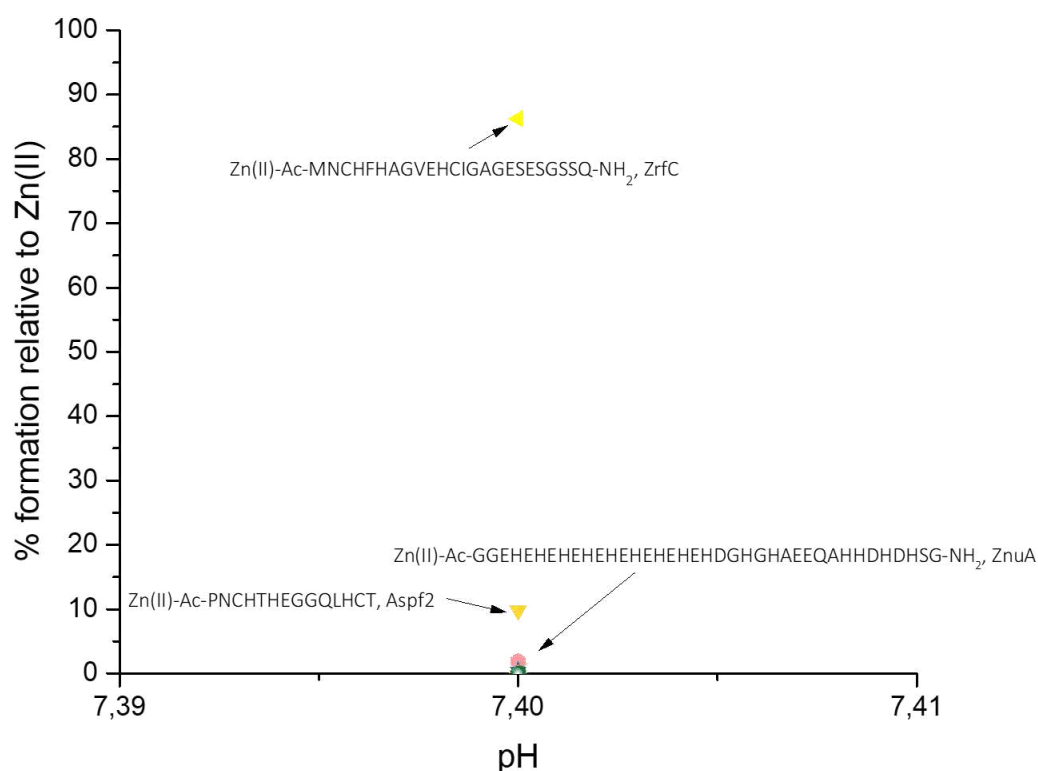


Figure 153 A competition plot between Zn(II) and AspF2, ZrfC, ZnuA, AztC and AdcA fragments, describes complex formation at pH 7.4 in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 6, Table 7, Table 9 and Table 10. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [AspF2, ZrfC, ZnuA, AztC and AdcA fragments] = 0.001 M

Subsequently, a large decrease in the stability of Zn(II) complexes can be observed for complexes in which different numbers of nitrogen atoms are involved in metal binding (Figure 154ABC). Complexes in which the donor atoms are four imidazole nitrogens, {4N_{im}}, are more stable, and their stability also depends on the number and nature of non-binding amino acid residues. The strongest ligand is the Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ fragment of the ZnuA solute binding protein, due to its large number of glutamic and aspartic acid residues acting as anchoring sites for Zn(II). Most probably due to the large amount of His residues, in the case of ZnuA fragments, in the coordination of Zn(II) participate different sets of imidazole rings (polymorphic binding sites). Thus, the Zn(II) affinity towards Ac-HEHEHEHEHEHEHEHEHEH-NH₂ is comparable (about 1.8% and 1.5%, respectively) (Figure 154A). Complexes in which the donor atoms are three imidazole nitrogens {3N_{im}} are less stable. Also complexes with {2N_{im}, S⁻} binding mode (Ac-TGCHSHGS-NH₂ fragment of the ZrfC transporter) has even less stability. However, ligands with Cys residues have greater affinity for Zn(II) than complexes with {3N_{im}} and {2N_{im}} binding modes. Therefore, Ac-EGHGHKGHHHA-NH₂ ({3N_{im}})

binding mode), Ac-HGIKSQKAEHFH-NH₂ ({3N_{im}} binding mode), Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂ ({3N_{im}} binding mode), Ac-ARHAKAH-NH₂ ({2N_{im}} binding mode), and Ac-MHRLYHVP-NH₂ ({2N_{im}} binding mode) and both Ac-AHCA-NH₂ and Ac-ACHA-NH₂ are far less stable.

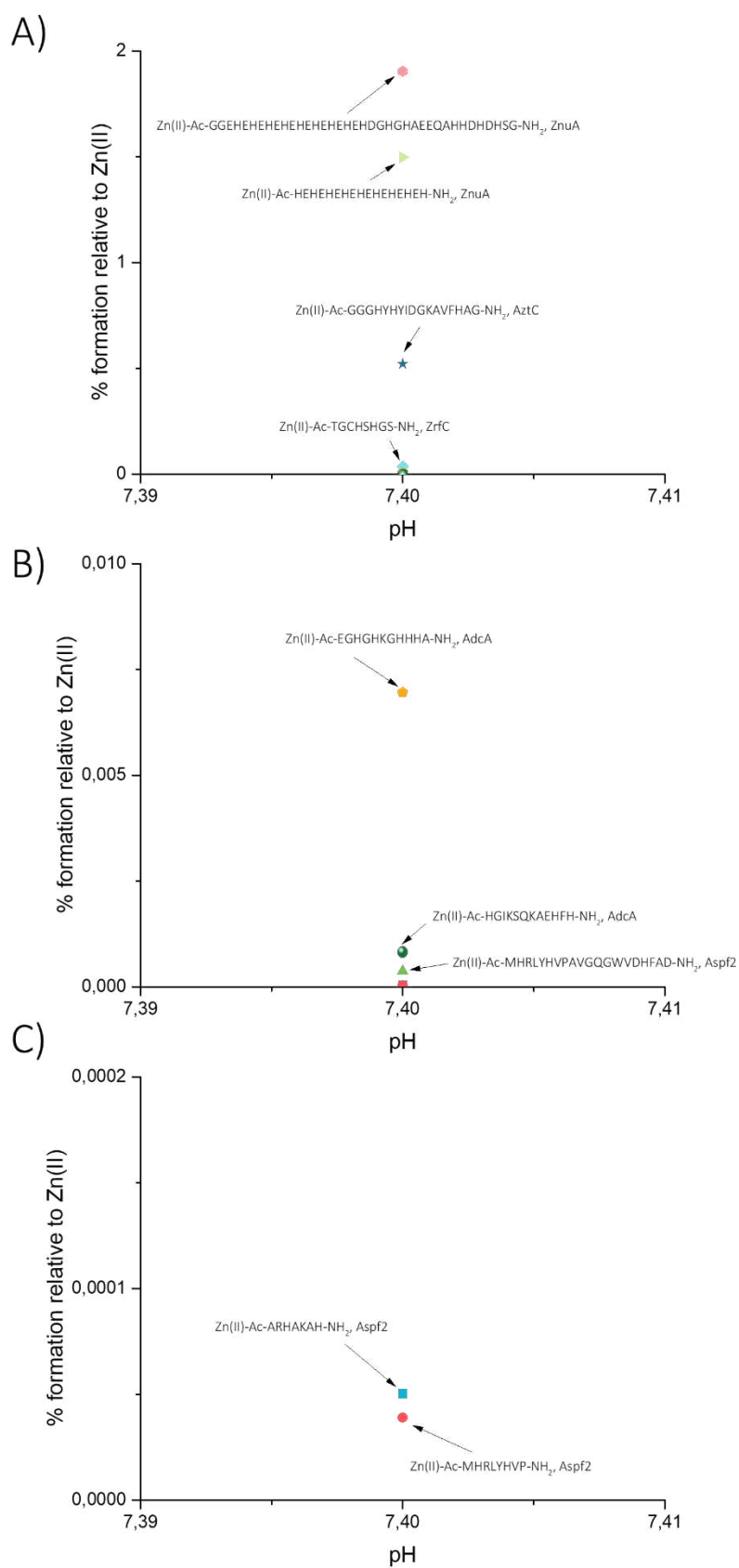


Figure 154 Enlargement of the competition plot between Zn(II) and AspF2, ZrFC, ZnuA, AztC and AdcA fragments, describes complex formation at pH 7.4 in a hypothetical situation in which equimolar amounts of all reagents are

mixed. Calculations are based on binding constants from Table 6, Table 7, Table 9 and Table 10. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Aspf2, ZrfC, ZnuAm AztC and AdcA fragments] = 0.001 M

The Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ fragment of the ZnuA protein has the highest affinity toward Ni(II) (the potential competitor of Zn(II) ions). Almost all available Ni(II) would bind to the indicated sequence with the more rare {6N_{im}} binding mode (Figure 155). At physiological pH, pH 7.4, Ni(II) is also bound to six histidine residues of the Ac-HEHEHEHEHEHEHEHEH-NH₂, but the affinity of this region towards Ni(II) is much lower than that of the longer loop- Figure 156A. Furthermore, even the fragment of the ZrfC transporter (Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂) binds Ni(II) with a higher affinity than the shorter fragment of the ZnuA loop, with {3N_{im}, S⁻} and {N_{im}, 2S⁻, N⁻} binding modes (Figure 156A). From the chemical point of view, it shows that nonbinding residues and the length of the peptide influence complex stability (in the case of fragments of the ZnuA solute binding protein), and Cys and amide donor atoms are more tempting binding sites for Ni(II) in the case of ZrfC and ZnuA fragments. Much less stable complexes are formed by Ac-PNCHTHEGGQLHCT from Aspf2 zincophore with the {2N_{im}, 2S⁻} binding mode, Ac-GGGHYHYIDGKAVFHAG-NH₂ from AztC solute binding protein, Ac-EGHGHKGHHHA-NH₂ and Ac-HGKSQKAEHFH-NH₂ from AdcA lipoprotein, Ac-MHRLYHVP-NH₂ and Ac-MHRLYHVPVGGQGWVDHFAD-NH₂ from Aspf2 zincophore, Ac-TGCHSHGS-NH₂ from ZrfC transporter, the Ac-ARHAKAH-NH₂ fragment of the Aspf2 zincophore and the two model sequences Ac-AHCA-NH₂ and Ac-ACHA-NH₂.

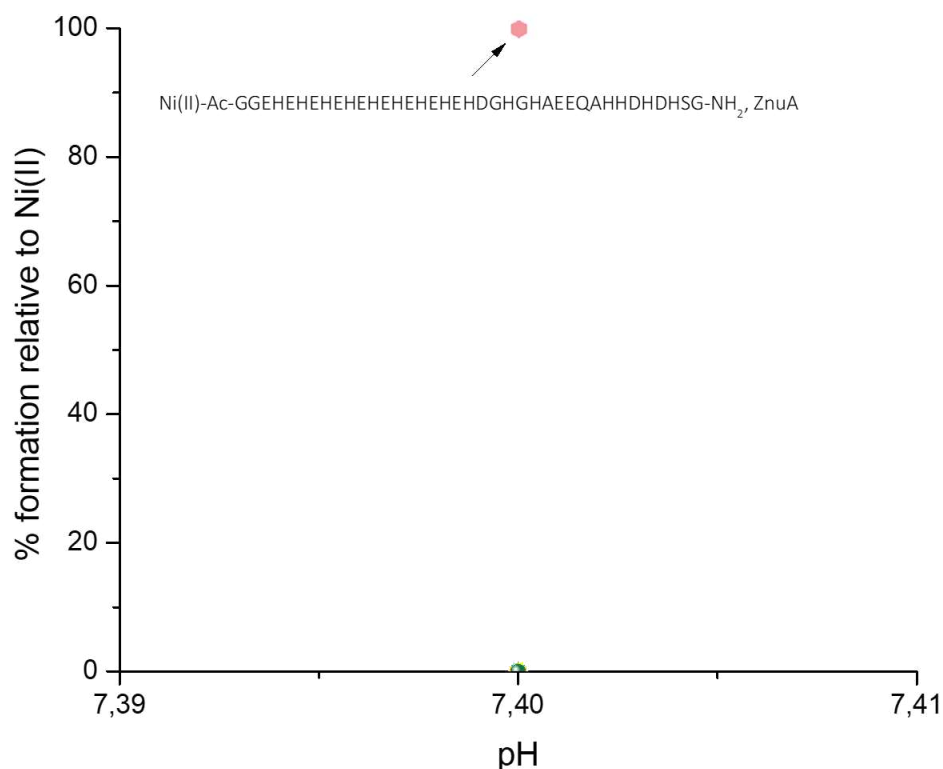


Figure 155 A competition plot between Ni(II) and Aspf2, ZrfC, ZnuA, AztC and AdcA fragments, describes complex formation at pH 7.4 in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations

are based on binding constants from Table 6, Table 7, Table 9 and Table 10. Conditions: $T=298\text{ K}$, $I=0.1\text{ M NaClO}_4$, $[\text{Ni(II)}] = [\text{Aspf2, ZrfC, ZnuA, AztC and AdcA fragments}] = 0.001\text{ M}$

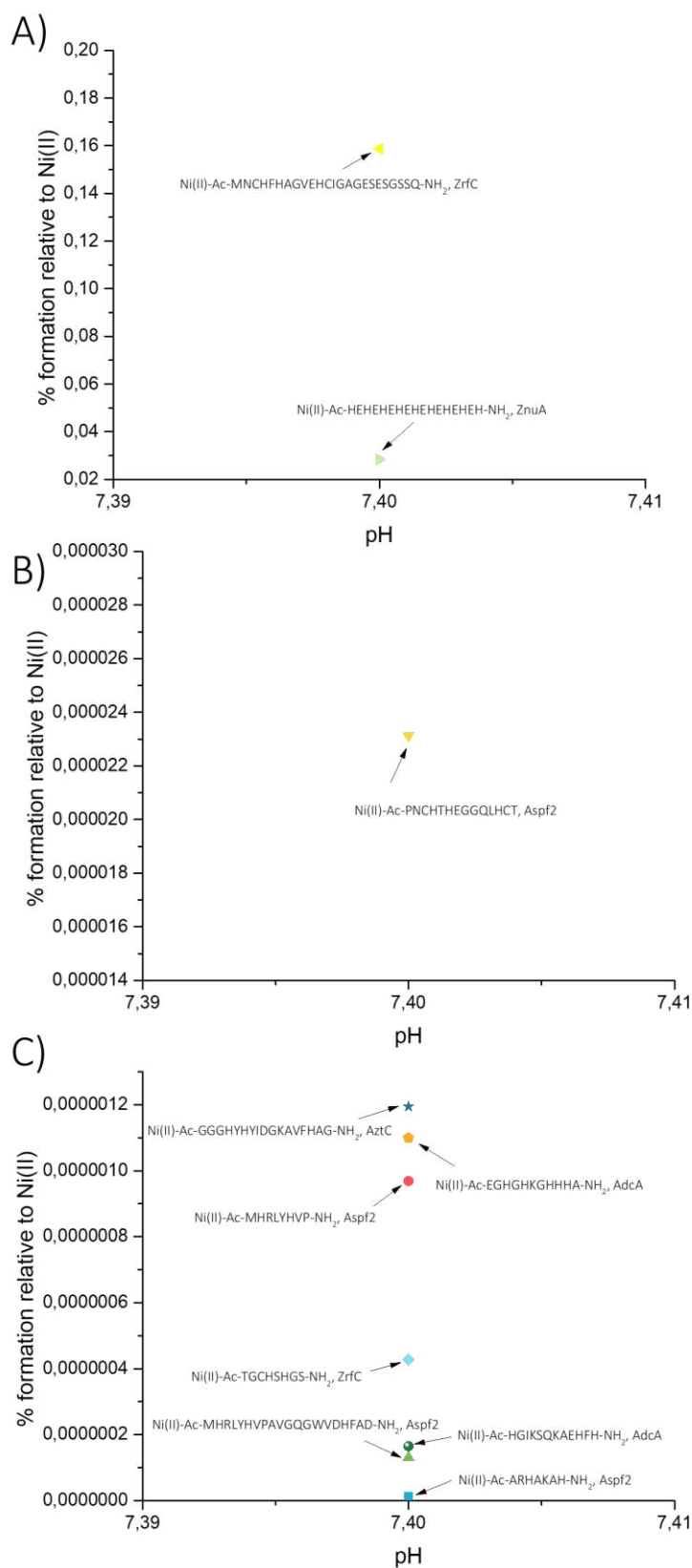


Figure 156 Enlargement of the competition plot between Ni(II) and Aspf2, ZrfC, ZnuA, AztC and AdcA fragments, describes complex formation at pH 7.4 in a hypothetical situation in which equimolar amounts of all reagents are

mixed. Calculations are based on binding constants from Table 6, Table 7, Table 9 and Table 10. Conditions: T=298 K, I=0.1 M NaClO₄, [Ni(II)] = [Aspf2, ZrfC, ZnuA, AztC and AdcA fragments] = 0.001 M

(iii) In my work, I focused on unstructured regions of proteins involved in Zn(II) transport (Aspf2, ZrfC, ZnuA, AztC and AdcA proteins), which are their most probable Zn(II)-binding sites. I also checked the thermodynamic parameters and precise Ni(II)-binding sites, keeping in mind that Ni(II) is a biologically relevant metal ion, essential for Ni(II)-dependent enzymes and could theoretically compete with Zn(II) for the binding sites in its transporting proteins. The potentiometric measurements allowed me to determine the stability constants and draw the pH-dependent competition plots, which shows the hypothetical situation in which all tested ligands are mixed in equimolar amounts. That research allowed me to determine that at pH 7.4, the majority of all ligands prefer to bind Zn(II) than Ni(II). Among them, I can highlight: Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂ and Ac-PNCHTHEGGQLHCT from Aspf2 zincophore, Ac-TGCHSHGS-NH₂ from ZrfC transporter and Ac-GGGHYHYIDGKAVFHAG-NH₂ from AztC solute binding protein. For Ac-EGHGHKGHHHA-NH₂ and Ac-HGKSQKAEHFH-NH₂, at physiological pH, the Zn(II) and Ni(II) binding preferences are very similar. Thus, the Zn(II) transport is specific for this metal at pH 7.4, but the situation drastically changes at basic pH, when the coordination of amide nitrogens in Ni(II) complexes gives them greater stability than Zn(II) complexes. The situation is different in the case of ligands: Ac-ARHAKAH-NH₂ and Ac-MHRLYHVP-NH₂ from Aspf2 zincophore, Ac-MNCHFHAAGVEHCIGAGESESGSSQ-NH₂ from ZrfC transporter, and Ac-HEHEHEHEHEHEHEHEHEH-NH₂ and Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ from ZnuA, where Ni(II) is a more tempting metal ion. However, in my work, I show how important are the physiological concentrations of Zn(II) and Ni(II). It turns out that “high affinity is not enough, and high concentration is a lot better” – since the physiological concentration of Zn(II) is much higher than that of Ni(II), under physiological conditions, the tested ligands would bind more Zn(II) than Ni(II).

VIII. CHAPTER VI: CONCLUSIONS AND PERSPECTIVES

In the scope of this work, I specified the binding sites of unstructured fragments of the Aspf2 zincophore from *Aspergillus fumigatus*, ZrfC transporter from *Aspergillus fumigatus*, ZnuA solute binding protein from *Paracoccus denitrificans*, AztC solute binding protein from *Paracoccus denitrificans*, AdcA from *Streptococcus mutans*, and two model sequences (Ac-ACHA-NH₂ and Ac-AHCA-NH₂) [493], [494], [492], [616], [604].

The most probable Zn(II) binding site on the Aspf2 zincophore from *Aspergillus fumigatus* is its C-terminal region (PNCHTHEGGQLHCT). At physiological, pH 7.4, that part of the zincophore binds Zn(II) via a {2N_{im}, 2S} coordination mode. At the same time, that part of the protein binds Ni(II) in the analogous binding mode. However, the affinity toward Ni(II) is smaller than that toward Zn(II), which provides specific selectivity of Zn(II) capture through the Aspf2 zincophore. To transport Zn(II), *Aspergillus fumigatus* uses the ZrfC transporter. The most probable binding

site for this protein is the MNCHFAGVEHCIGAGESESGSSQ fragment. At physiological pH, that fragment binds Zn(II) via the same set of donor atoms as Aspf2 zincophore - {2N_{im}, 2S⁻} binding mode. The Zn(II) binding site on ZrfC coordinates Zn(II) with higher affinity than the Zn(II) binding site on the Aspf2 zincophore. It allows the Zn(II) transfer from zincophore to the transporter. The binding preferences of ZrfC fragment are more complicated than those of C-terminal part of the Aspf2 zincophore. The MNCHFAGVEHCIGAGESESGSSQ from ZrfC transporter binds Ni(II) with comparable affinity to Zn(II).

To acquire Zn(II) from the host, *Paracoccus denitrificans* uses ZnuABC and AztABCD transporters. ZnuA and AztC are two solute binding proteins, responsible for Zn(II) acquisition in those systems. Their unstructured, His-rich loops bind both Zn(II) and Ni(II). The Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ fragment of the ZnuA protein has the highest affinity toward those metal ions, and I determine the so-called polymorphic binding sites for those complexes with a {4N_{im}} binding mode. That fragment is also an efficient Ni(II) binder (strongly prefer to bind Ni(II) over Zn(II)). At pH 7.4, this ZnuA region binds Ni(II) via a set of six histidine residues, {6N_{im}} binding mode and octahedral geometry. An analogous situation is observed for the Ac-HEHEHEHEHEHEHEHEH-NH₂ fragment. On the other hand, the AztC fragment (Ac-GGGHYHYIDGKAVFHAG-NH₂) prefers to bind Zn(II) over Ni(II).

Streptococcus mutans, like other bacteria, has developed a number of mechanisms that allow Zn(II) uptake. One of the strategies is based on the AdcA lipoprotein. I characterized two unstructured fragments of this protein; both bind Zn(II) and Ni(II) with comparable affinity. However, considering the high Zn(II) concentration in human saliva and dental plaque, the amount of Zn(II)-AdcA complexes found in physiological conditions would be much greater than that of Ni(II).

To better understand the bioinorganic chemistry of Zn(II) transporters, I also checked the coordination properties of two model sequences: Ac-ACHA-NH₂ and Ac-AHCA-NH₂. The results clearly indicate that the position of His and Cys residues affects the stability of Ni(II) complexes, but not that of Zn(II) ones. Ni(II) will more likely bind to Ac-AHCA-NH₂. Moreover, both ligands prefer to bind Zn(II) over Ni(II).

In response to the phenomenon of 'nutritional resistance', fungi and bacteria have developed a number of mechanisms that allow them to uptake essential metal ions, such as Zn(II). One of the strategies to minimize the deficiency of Zn(II) are the zincophore based transport and ABC transporters. In this work, I specified four probable binding sites in the Aspf2 zincophore, two probable binding sites in the ZrfC transporter, two probable binding sites in the ZnuA solute binding protein, one probable binding site in the AztC solute binding protein, two probable bindings sites in the AdcA lipoprotein and two model sequences. A precise understanding of Zn(II) transport mechanisms in *Aspergillus fumigatus*, *Paracoccus denitrificans* and *Streptococcus mutans* could be an important milestone towards the understanding of metal transport systems present in other pathogens and the rational design of new, highly specific therapeutics against antibiotic-resistant bacterial and fungi species. Therefore, these findings are not only an input to the basic chemistry of Zn(II) and Ni(II), and an enhancement of our understanding of metal transport in pathogens, but quite importantly, they constitute a starting point for the design of zincophore mimetics. The results presented in Chapter IV and Chapter

VII clearly indicate that two His imidazoles and two Cys thiolates constitute the best binding sites for Zn(II). The $\{2N_{im}, 2S^-\}$ binding mode is also typical for zinc fingers, which perform structural functions in many proteins. This knowledge can be used to design molecules that bind Zn(II) with high affinity and search for probable binding sites in other proteins involved in its transport. Moreover, the results presented in the Chapter IV (B4) clearly indicates that non-binding residues and the placement of binding motifs influence the metal binding affinity (Ac-MNCHFHAGVEHCIGAGESEGSSQ-NH₂ from ZrFC transporter binds Zn(II) with a higher affinity than Ac-PNCHTHEGGQLHCT from AspF2 zincophore, although the binding mode of Zn(II) at pH 7.4 is the same in both cases). This knowledge constitutes the basis for the search for zincophore mimetics and allows research with the determined C-terminal fragment of the AspF2 zincophore (for *Aspergillus fumigatus*). The comparison of the Zn(II) binding affinity and determination of the most probable binding sites in the ZnuA, AztC and AdcA proteins allow to design zincophore mimetics for *Paracoccus denitrificans* and *Streptococcus mutans*.

I do believe that these finding will be a stepping stone towards determining general trends in the design of this type of cheating molecules.

The knowledge collected in this work should be used when designing new, highly specific drugs. The phenomenon of still increasing antibiotic resistance (resistance of microorganisms to antimicrobial drugs that were originally effective for treatment) prompts the scientific world to look for new drug classes. The STAMP technology, specifically targeted antimicrobial peptides, is based on two regions: targeting and killing domains, which are connected with a short flexible linker [618]. The schematic model of compounds based on STAMP technology is presented in Figure 159.

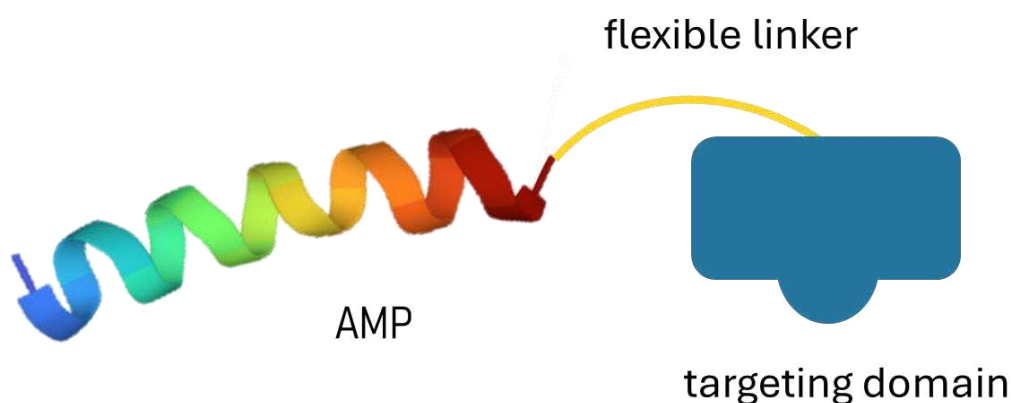


Figure 159 Schematic model of drugs based on STAMP technology. STAMPs are composed of two domains: AMP domain and a targeting domain.

Both regions are independent, and the targeting region is responsible for to enhance of the antimicrobial peptide affinity, thus the accumulation of the killing substance in the neighborhood of pathogens. Thus, specifically targeted antimicrobial peptides are designed to target and kill pathogens with high selectivity, and one of their biggest advantages is the minimization of side effects for the normal microflora [619]. A schematic of the selective activity against pathogens is presented in the Figure 160. The conventional STAMPs usually contain one targeting and one killing domain. However, there are also STAMPs with multiple-targeting regions that target two different bacteria [620].

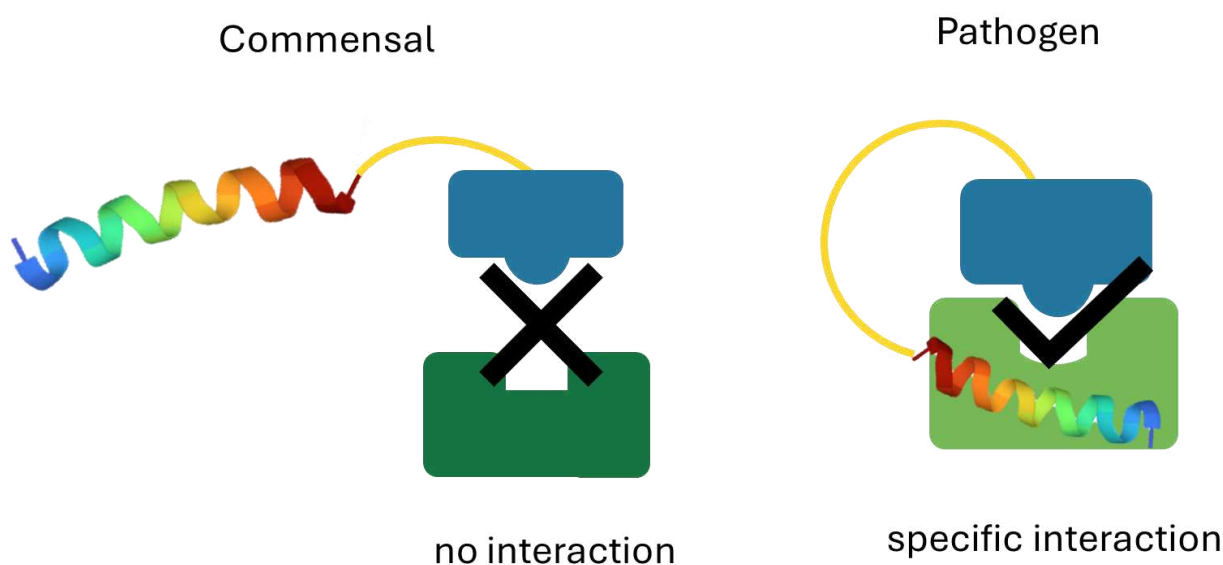


Figure 160 The STAMP-based drugs A) do not interact with commensal cells, but B) accumulate around the targeted pathogenic cells and kill them.

One of the promising compounds for the antimicrobial domain are antimicrobial peptides (AMPs). AMPs are known as small host defense peptides that form part of the innate immune response shared by a variety of living species like for example plants or vertebrates [621]. They have a broad spectrum of inhibitory effects against bacteria, fungi, parasites and viruses [622], which include membrane-targeting mechanisms (Wormhole, Barrel-Stove, Carpet-like) and extramembrane targeting mechanisms (inhibition of protein biosynthesis, nucleic acids, inhibition of protease activity, or inhibition of cell division) [621]. The activity of AMP depends largely on the presence of transition metal ions, such as Zn(II) or Cu(II) [623]. They take part in many biological processes, which makes them necessary for the survival of all living organisms. Some antimicrobial peptides form stable complexes with metal ions, thus blocking their access to pathogens [624]. Moreover, the binding of a metal ion may result in a change in the physicochemical properties of the peptide, such as structure or charge, and result in enhanced antimicrobial activity [625]. Moreover, apart from a few cases, microorganisms have not developed resistance mechanisms against them [626], even though they have been a key element of host defense mechanisms for thousands of years. All tested the unstructured zincophore fragments and proteins involved in Zn(II) transport can serve as promising targeting domains. Attaching them via an appropriate linker, to a chosen AMP or other antimicrobial drug could be a fascinating field of future discoveries.

IX. LITERATURE

- [1] N. Lane, The unseen world: reflections on Leeuwenhoek (1677) 'Concerning little animals', *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 2015, 370, 20140344, DOI:10.1098/rstb.2014.0344.
- [2] S. N. Michaleas, K. Laios, A. Charalabopoulos, G. Samonis and M. Karamanou, Joseph Lister (1827-1912): A Pioneer of Antiseptic Surgery, *Cureus* 2022, 14, e32777, DOI:10.7759/cureus.32777.
- [3] C. Walsh and T. Wencewicz, Prospects for new antibiotics: a molecule-centered perspective, *The Journal of Antibiotics* 2014, 67, 7–22, DOI:10.1038/ja.2013.49.
- [4] E. P. Abraham, E. Chain, C. M. Fletcher, H. W. Florey, A. D. Gardner, N. G. Heatley and M. A. Jennings, Further observations on penicillin. 1941, *European journal of clinical pharmacology* 1992, 42, 3–9.
- [5] R. Gaynes, The Discovery of Penicillin—New Insights After More Than 75 Years of Clinical Use, *Emerging Infectious Diseases* 2017, 23, 849–853, DOI:10.3201/eid2305.161556.
- [6] G. G. Rao, Risk Factors for the Spread of Antibiotic-Resistant Bacteria, *Drugs* 1998, 55, 323–330, DOI:10.2165/00003495-199855030-00001.
- [7] N. Kresge, R. D. Simoni and R. L. Hil, Selman Waksman: the Father of Antibiotics, *Journal of Biological Chemistry* 2004, 279, e7, DOI:10.1016/S0021-9258(20)67861-9.
- [8] S. Jacquelyn and E. A. Grice, *The Human Microbiome, Genomic and Precision Medicine (Third Edition)* 2017, Chapter 4.
- [9] R. Urban-Chmiel, A. Marek, D. Stępień-Pyśniak, K. Wieczorek, M. Dec, A. Nowaczek and J. Osek, Antibiotic Resistance in Bacteria—A Review, *Antibiotics* 2022, 11, 1079, DOI: <https://doi.org/10.3390/antibiotics11081079>.
- [10] M. Huemer, S. Mairpady Shambat, S. D. Brugger and A. S. Zinkernagel, Antibiotic resistance and persistence—Implications for human health and treatment perspectives, *EMBO reports* 2020, 21, e51034, DOI:10.15252/embr.202051034.
- [11] J. Davies, Where have All the Antibiotics Gone?, *The Canadian journal of infectious diseases & medical microbiology* 2006, 17, 287–290, DOI:10.1155/2006/707296.
- [12] J. F. Prescott, The resistance tsunami, antimicrobial stewardship, and the golden age of microbiology, *Veterinary microbiology* 2014, 171, 273–278, DOI:10.1016/j.vetmic.2014.02.035.
- [13] S. Painuli, P. Semwal, R. Sharma and S. Akash, Superbugs or multidrug resistant microbes: A new threat to the society. *Health science reports* 2023, 6, e1480, DOI:10.1002/hsr2.1480.
- [14] L. Serwecińska, Antimicrobials and Antibiotic-Resistant Bacteria: A Risk to the Environment and to Public Health, *Water* 2020, 12, 3313, DOI:10.3390/w12123313.
- [15] S. Di Lodovico, T. Fasciana, M. Di Giulio, L. Cellini, A. Giammanco, G. M. Rossolini and A. Antonelli, Spread of Multidrug-Resistant Microorganisms, *Antibiotics* 2022, 11, 832, DOI:10.3390/antibiotics11070832.

- [16] J. Davies and D. Davies, Origins and evolution of antibiotic resistance. *Microbiology and molecular biology reviews* : MMBR 2010, 74, 417–433, DOI:10.1128/MMBR.00016-10.
- [17] D. I. Andersson, Persistence of antibiotic resistant bacteria, *Current opinion in microbiology* 2003, 6, 452–456, DOI:10.1016/j.mib.2003.09.001.
- [18] S. R. Hennigar and J. P. McClung, Nutritional Immunity: Starving Pathogens of Trace Minerals, *American journal of lifestyle medicine* 2016, 10, 170–173, DOI:10.1177/1559827616629117.
- [19] T. E. Kehl-Fie and E. P. Skaar, Nutritional immunity beyond iron: a role for manganese and zinc, *Current opinion in chemical biology* 2010, 14, 218–224, DOI:10.1016/j.cbpa.2009.11.008.
- [20] P. K. Walencik, J. Wątyły and M. Rowińska-Żyrek, Fungal Zinc Homeostasis - A Tug of War Between the Pathogen and Host, *Current medicinal chemistry* 2016, 23, 3717–3729, DOI:10.2174/0929867323666160817163834.
- [21] D. Wilson and G. S. Deepe Jr., The intersection of host and fungus through the zinc lens, *Current Opinion in Microbiology* 2019, 52, 35-40, DOI:10.1016/j.mib.2019.04.008.
- [22] M. Rohm, M. J. Grimm, A. C. D'Auria, N. G. Almyroudīs, B. H. Segal and C. F. Urban, NADPH Oxidase Promotes Neutrophil Extracellular Trap Formation in Pulmonary Aspergillosis, *Infection and Immunity* 2014, 82, 1766-1777, DOI:.
- [23] L. S. Cunden, A. Gaillard and E. M. Nolan, Calcium ions tune the zinc-sequestering properties and antimicrobial activity of human S100A12, *Chemical Science* 2016, 7:1338-1348, DOI:10.1039/C5SC03655K.
- [24] J. Wehkamp, B. O. Schroeder, J. M. Schroeder and E. Morita, Disulphide-reduced psoriasin is a human apoptosis-inducing broad-spectrum fungicide, *Proceedings of the National Academy of Sciences of the United States of America* 2015, 112, 13039–13044, DOI:10.1073/pnas.1511197112.
- [25] A. Brauner, C. Alvendal, M. Chromek, K. H. Stopsack, S. Ehrström, J. M. Schröder and N. Bohm-Stark, Psoriasin, a novel anti-Candida albicans adhesin, *Journal of molecular medicine* 2018, 96, 537–545, DOI:10.1007/s00109-018-1637-6.
- [26] N. F. Krebs, Overview of zinc absorption and excretion in the human gastrointestinal tract, *The Journal of nutrition* 2000, 130, 1374S–75, DOI:10.1093/jn/130.5.1374S.
- [27] W. Maret, Molecular aspects of human cellular zinc homeostasis: redox control of zinc potentials and zinc signals, *Biometals* 2009, 22, 149–157, DOI:10.1007/s10534-008-9186-z.
- [28] H. Haase, An element of life: competition for zinc in host-pathogen interaction, *Immunity* 2013, 39, 623–624, DOI:10.1016/j.immuni.2013.09.009.
- [29] A. H. Shankar and A. S. Prasad, Zinc and immune function: the biological basis of altered resistance to infection. *The American journal of clinical nutrition* 1998, 68, 447S–463S, DOI:10.1093/ajcn/68.2.447S.
- [30] T. Fukada, S. Yamasaki, K. Nishida, M. Murakami and T. Hirano, Zinc homeostasis and signaling in health and diseases: Zinc signaling, *Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry* 2011, 16, 1123–1134, DOI:10.1007/s00775-011-0797-4.
- [31] C. Andreini and I. Bertini, A bioinformatics view of zinc enzymes, *Journal of inorganic biochemistry* 2012, 111, 150–156, DOI:10.1016/j.jinorgbio.2011.11.020.
- [32] C. Andreini, Bertini and G. Cavallaro, Minimal functional sites allow a classification of zinc sites in proteins, *PloS One* 2011, 6, e26325, DOI:10.1371/journal.pone.0026325.

- [33] C. Andreini, I. Bertini and A. Rosato, Metalloproteomes: a bioinformatic approach, *Accounts of chemical research* 2009, 42, 1471–1479, DOI:10.1021/ar900015x.
- [34] F. W. Beck, A. S. Prasad, J. Kaplan, J. T. Fitzgerald and G. J. Brewer, Changes in cytokine production and T cell subpopulations in experimentally induced zinc-deficient humans, *The American journal of physiology* 1997, 272, E1002–E1007, DOI:10.1152/ajpendo.1997.272.6.E1002.
- [35] H. Haase and L. Rink, Functional significance of zinc-related signaling pathways in immune cells, *Annual review of nutrition* 2009, 29, 133–152, DOI:10.1146/annurev-nutr-080508-141119.
- [36] J. Wątył, S. Potocki and M. Rowińska-Żyrek, Zinc Homeostasis at the Bacteria/Host Interface—From Coordination Chemistry to Nutritional Immunity, *Chemistry* 2016, 22, 15992–16010, DOI:10.1002/chem.201602376.
- [37] W. Maret, Zinc coordination environments in proteins as redox sensors and signal transducers, *Antioxidants & redox signaling* 2006, 8, 1419–1441, DOI:10.1089/ars.2006.8.1419.
- [38] A. S. Prasad, Discovery of human zinc deficiency: 50 years later, *Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements* 2012, 26, 66–69, DOI:10.1016/j.jtemb.2012.04.004.
- [39] D. J. Eide, Zinc transporters and the cellular trafficking of zinc, *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 2006, 1763, 711–722, DOI:10.1016/j.bbamcr.2006.03.005.
- [40] J. H. Laity and G. K. Andrews, Understanding the mechanisms of zinc-sensing by metal-response element binding transcription factor-1 (MTF-1), *Archives of Biochemistry and Biophysics* 2007, 463, 201–210, DOI:10.1016/j.abb.2007.03.019.
- [41] Y. Li and W. Maret, Human metallothionein metallomics, *Journal of Analytical Atomic Spectrometry* 2008, 8, 1055–1062, DOI:10.1039/B802220H.
- [42] G. K. Andrews, H. Wang, S. K. Dey and R. D. Palmiter, Mouse zinc transporter 1 gene provides an essential function during early embryonic development, *Genesis* 2004, 40, 74–81, DOI:10.1002/gene.20067.
- [43] S. Antala and R. E. Dempsey, The human ZIP4 transporter has two distinct binding affinities and mediates transport of multiple transition metals, *Biochemistry* 2012, 51, 963–973, DOI:10.1021/bi201553p.
- [44] C. Andreini, L. Banci, I. Bertini and A. Rosato, Zinc through the Three Domains of Life, *Journal of Proteome Research* 2006, 5, 3173–3178, DOI:10.1021/pr0603699.
- [45] C. Andreini, L. Banci, I. Bertini and A. Rosato, Counting the zinc-proteins encoded in the human genome, *Journal of proteome research* 2006, 5, 196–201, DOI:10.1021/pr050361j.
- [46] D. Kandari, H. Joshi and R. Bhatnagar, Zur: Zinc-Sensing Transcriptional Regulator in a Diverse Set of Bacterial Species, *Pathogens* 2021, 10, 344, DOI:10.3390/pathogens10030344.
- [47] Z. R. Lonergan and E. P. Skaar, Nutrient Zinc at the Host-Pathogen Interface, *Trends in biochemical sciences* 2019, 44, 1041–1056, DOI:10.1016/j.tibs.2019.06.010.
- [48] T. Kambe, T. Tsuji, A. Hashimoto and N. Itsumura, The Physiological, Biochemical, and Molecular Roles of Zinc Transporters in Zinc Homeostasis and Metabolism, *Physiological reviews* 2015, 95, 749–784, DOI:10.1152/physrev.00035.2014.
- [49] J. C. King, D. M. Shames and L. R. Woodhouse, Zinc Homeostasis in Humans, *The Journal of Nutrition* 2000, 130, 1360S–1366S, DOI:10.1093/jn/130.5.1360S.

- [50] T. Hara, T. A. Takeda, T. Takagishi, K. Fukue, T. Kambe and T. Fukada, Physiological roles of zinc transporters: molecular and genetic importance in zinc homeostasis, *The journal of physiological sciences : JPS*, 67, 283–301, DOI:10.1007/s12576-017-0521-4.
- [51] M. J. Jackson , *Physiology of Zinc: General Aspects*, *Zinc in Human Biology* 1989, 1-14, DOI:10.1007/978-1-4471-3879-2_1.
- [52] H. Haase and L. Rink, Zinc signals and immune function, *BioFactors* 2014, 40, 27–40, DOI:10.1002/biof.1114.
- [53] R. E. Thiers and B. L. Vallee, Distribution of metals in subcellular fractions of rat liver, *The Journal of biological chemistry* 1957, 226, 911–920.
- [54] T. Chikowore, P. T. Pisa, T. van Zyl, E. J. Feskens, E. Wentzel-Viljoen and K. R. Conradie, Nutrient Patterns Associated with Fasting Glucose and Glycated Haemoglobin Levels in a Black South African Population, *Nutrients* 2017, 9, 9, DOI:10.3390/nu9010009.
- [55] B. J. Scott and A. R. Bradwell, Identification of the serum binding proteins for iron, zinc, cadmium, nickel, and calcium, *Clinical chemistry* 1983, 29, 629–633.
- [56] C. E. Outten and T. V. O'Halloran, Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis, *Science* 2001, 292, 2488–2492, DOI:10.1126/science.1060331.
- [57] Y. Qin, P. J. Dittmer, J. G. Park, K. B. Jansen and A. E. Palmer, Measuring steady-state and dynamic endoplasmic reticulum and Golgi Zn²⁺ with genetically encoded sensors, *Proceedings of the National Academy of Sciences of the United States of America* 2011, 108, 7351–7356, DOI:10.1073/pnas.1015686108.
- [58] S. L. Sensi, L. M. Canzoniero, S. P. Yu, H. S. Ying, J. Y. Koh, G. A. Kerchner and D. W. Choi, Measurement of intracellular free zinc in living cortical neurons: routes of entry, *The Journal of neuroscience : the official journal of the Society for Neuroscience* 1997, 17, 9554–9564, DOI:10.1523/JNEUROSCI.17-24-09554.1997.
- [59] J. L. Vinkenborg, T. J. Nicolson, E. A. Bellomo, M. S. Koay, G. A. Rutter and M. Merckx, Genetically encoded FRET sensors to monitor intracellular Zn²⁺ homeostasis, *Nature methods* 2009, 6, 737–740, DOI:10.1038/nmeth.1368.
- [60] H. A. Katimba, R. Wang, C. Cheng, Y. Zhang, W. Lu and Y. Ma, Zinc Absorption & Homeostasis in the Human Body: A General Overview, *Food Reviews International* 2024, 40, 715–739, DOI:10.1080/87559129.2023.2195188.
- [61] A. B. Abdel-Mageed and F. W. Oehme, A review of the biochemical roles, toxicity and interactions of zinc, copper and iron: II. Copper, *Veterinary and human toxicology* 1990, 32, 230–234.
- [62] D. Son, S. Gu, J. W. Choi, D. J. Suh, J. Jae, J. Choi and J. M. Ha, Production of phenolic hydrocarbons from organosolv lignin and lignocellulose feedstocks of hardwood, softwood, grass and agricultural waste, *Journal of Industrial and Engineering Chemistry* 2019, 69, 304-314, DOI:10.1016/j.jiec.2018.09.009.
- [63] L. Sian, N. F. Krebs, J. E. Westcott, L. Fengliang, L. Tong, L. V. Miller, B. Sonko and M. Hambidge, Zinc homeostasis during lactation in a population with a low zinc intake, *The American journal of clinical nutrition* 2002, 75, 99–103, DOI:10.1093/ajcn/75.1.99.
- [64] A. E. Baum, M. Hamshere, E. Green, S. Cichon, M. Rietschel, M. M. Noethen, N. Craddock and F. J. McMahon, Meta-analysis of two genome-wide association studies of bipolar disorder reveals important points of agreement, *Molecular psychiatry* 2008, 13, 466–467, DOI:10.1038/mp.2008.16.
- [65] E. Bafaro, Y. Liu, Y. Xu and R. E. Dempski, The emerging role of zinc transporters in cellular homeostasis and cancer, *Signal Transduction and Targeted Therapy* 2017, 2, 17029, DOI:10.1038/sigtrans.2017.29.

- [66] L. A. Lichten and R. J. Cousins, Mammalian zinc transporters: nutritional and physiologic regulation, *Annual review of nutrition* 2009, 29, 153–176, DOI:10.1146/annurev-nutr-033009-083312.
- [67] S. Potocki, M. Rowińska-Żyrek, D. Witkowska, M. Pyrkosz, A. Szebesczyk, K. Krzywoszyńska and H. Kozłowski, Metal transport and homeostasis within the human body: toxicity associated with transport abnormalities, *Current medicinal chemistry* 2012, 19, 2738–2759, DOI:10.2174/092986712800609698.
- [68] S. Potocki, M. Rowińska-Żyrek, D. Valensin, K. Krzywoszyńska, D. Witkowska, M. Łuczowski and H. Kozłowski, Metal Binding Ability of Cysteine-Rich Peptide Domain of ZIP13 Zn²⁺ Ions Transporter, *Inorganic Chemistry* 2011, 50, 6135–6145, DOI:10.1021/ic200270p | *Inorg. Chem.* 2011, 50, 6135–6145.
- [69] H. Kozłowski, S. Potocki, M. Remelli, M. Rowińska-Żyrek and D. Valensin, Specific metal ion binding sites in unstructured regions of proteins, *Coordination Chemistry Reviews* 2013, 257, 2625–2638, DOI:10.1016/j.ccr.2013.01.024.
- [70] S. Potocki, D. Valensin and H. Kozłowski, The specificity of interaction of Zn²⁺, Ni²⁺ and Cu²⁺ ions with the histidine-rich domain of the TJNT1 ZIP family transporter, *Dalton Transactions* 2014, 43, 10215–10223, DOI:10.1039/C4DT00903G.
- [71] S. Küry, B. Dréno, S. Béziau, S. Giraudet, M. Kharfi, R. Kamoun and J. P. Moisan, Identification of SLC39A4, a gene involved in acrodermatitis enteropathica, *Nature genetics* 2002, 3, 239–240, DOI:10.1038/ng913.
- [72] K. Wang, B. Zhou, Y. M. Kuo, J. Zemansky and J. Gitschier, A novel member of a zinc transporter family is defective in acrodermatitis enteropathica, *American journal of human genetics* 2002, 71, 66–73, DOI:10.1086/341125.
- [73] R. J. McMahon and R. J. Cousins, Regulation of the zinc transporter ZnT-1 by dietary zinc, *Proceedings of the National Academy of Sciences of the United States of America* 1998, 95, 4841–4846, DOI:10.1073/pnas.95.9.4841.
- [74] Y. Zhang, Y. P. Zhang, K. Sun, Z. Y. Meng and L. G. Chen, The SLC transporter in nutrient and metabolic sensing, regulation, and drug development, *Journal of Molecular Cell Biology* 2019, 11, 1–13, DOI:.
- [75] W. Chowanadisai, D. M. Graham, C. L. Keen, R. B. Rucker and M. A. Messerli, Neurulation and neurite extension require the zinc transporter ZIP12 (slc39a12), *Proceedings of the National Academy of Sciences of the United States of America* 2013, 110, 9903–9908, DOI:10.1073/pnas.1222142110.
- [76] J. Dufner-Beattie, F. Wang, Y. M. Kuo, J. Gitschier, D. Eide and G. K. Andrews, The acrodermatitis enteropathica gene ZIP4 encodes a tissue-specific, zinc-regulated zinc transporter in mice, *The Journal of biological chemistry* 2003, 278, 33474–33481, DOI:10.1074/jbc.M305000200.
- [77] J. Dufner-Beattie, S. J. Langmade, F. Wang, D. Eide and G. K. Andrews, Structure, function, and regulation of a subfamily of mouse zinc transporter genes, *The Journal of biological chemistry* 2003, 278, 50142–50150, DOI:10.1074/jbc.M304163200.
- [78] L. A. Gaither and D. J. Eide, The human ZIP1 transporter mediates zinc uptake in human K562 erythroleukemia cells, *The Journal of biological chemistry* 2001, 276, 22258–22264, DOI:10.1074/jbc.M101772200.
- [79] Y. Higashi, S. Segawa, T. Matsuo, S. Nakamura, Y. Kikkawa, K. Nishida and K. Nagasawa, Microglial zinc uptake via zinc transporters induces ATP release and the activation of microglia, *Glia* 2011, 59, 1933–1945, DOI:10.1002/glia.21235.
- [80] L. A. Gaither and D. J. Eide, Functional expression of the human hZIP2 zinc transporter, *The Journal of biological chemistry* 2000, 275, 5560–5564, DOI:10.1074/jbc.275.8.5560.

- [81] Y. Inoue, S. Hasegawa, S. Ban, T. Yamada, Y. Date, H. Mizutani, S. Nakata, M. Tanaka and N. Hirashima, ZIP2 protein, a zinc transporter, is associated with keratinocyte differentiation, *The Journal of biological chemistry* 2014, 289, 21451–21462, DOI:10.1074/jbc.M114.560821.
- [82] S. L. Kelleher and B. Lönnerdal, Zn transporter levels and localization change throughout lactation in rat mammary gland and are regulated by Zn in mammary cells, *The Journal of nutrition* 2003, 133, 3378–3385, doi:10.1093/jn/133.11.3378.
- [83] S. L. Kelleher and B. Lönnerdal, Zip3 plays a major role in zinc uptake into mammary epithelial cells and is regulated by prolactin. *American journal of physiology, Cell physiology* 2005, 288, C1042–C1047, DOI:10.1152/ajpcell.00471.2004.
- [84] S. L. Kelleher, V. Lopez, B. Lönnerdal, J. Dufner-Beattie and G. K. Andrews, Zip3 (Slc39a3) functions in zinc reuptake from the alveolar lumen in lactating mammary gland, *American journal of physiology. Regulatory, integrative and comparative physiology* 2009, 297, R194–R201. DOI:10.1152/ajpregu.00162.2009.
- [85] R. Giacconi, M. Malavolta, L. Costarelli, F. Busco, R. Galeazzi, G. Bernardini, N. Gasparini and E. Mocchegiani, Comparison of intracellular zinc signals in nonadherent lymphocytes from young-adult and elderly donors: role of zinc transporters (Zip family) and proinflammatory cytokines, *The Journal of nutritional biochemistry* 2012, 23, 1256–1263,, DOI:10.1016/j.jnutbio.2011.07.005.
- [86] M. M. Desouki, J. Geradts, B. Milon, R. B. Franklin and L. C. Costello, hZip2 and hZip3 zinc transporters are down regulated in human prostate adenocarcinomatous glands, *Molecular cancer* 2007, 6, 37, DOI:10.1186/1476-4598-6-37.
- [87] J. Geiser, K. J. Venken, R. C. De Lisle and G. K. Andrews, A mouse model of acrodermatitis enteropathica: loss of intestine zinc transporter ZIP4 (Slc39a4) disrupts the stem cell niche and intestine integrity, *PLoS genetics* 2012, 8, e1002766, DOI:10.1371/journal.pgen.1002766.
- [88] S. Y. Assaf and S. H. Chung, Release of endogenous Zn²⁺ from brain tissue during activity, *Nature* 1984, 308, 734–736, DOI:10.1038/308734a0.
- [89] R. J. Cousins, Gastrointestinal factors influencing zinc absorption and homeostasis. *International journal for vitamin and nutrition research* 2010, 80, 243–248, DOI:10.1024/0300-9831/a000030.
- [90] M. van Boekel, V. Fogliano, N. Pellegrini, C. Stanton, G. Scholz, S. Lalljie, V. Somoza, D. Knorr, P. R. Jasti and G. Eisenbrand, A review on the beneficial aspects of food processing, *Molecular nutrition & food research* 2010, 54, 1215–1247, DOI:10.1002/mnfr.200900608.
- [91] J. Klepacka, A. Najda and K. Klimek, Effect of Buckwheat Groats Processing on the Content and Bioaccessibility of Selected Minerals, *Foods* 2020, 9, 832, DOI:10.3390/foods9060832.
- [92] S. Kasana, J. Din and W. Maret, Genetic causes and gene–nutrient interactions in mammalian zinc deficiencies: acrodermatitis enteropathica and transient neonatal zinc deficiency as examples, *Journal of trace elements in medicine and biology: organ of the Society for Minerals and Trace, Elements* 2015, 29, 47–62, DOI:10.1016/j.jtemb.2014.10.003.
- [93] J. Dufner-Beattie, Y. M. Kuo, J. Gitschier and G. K. Andrews, The adaptive response to dietary zinc in mice involves the differential cellular localization and zinc regulation of the zinc transporters ZIP4 and ZIP5, *The Journal of biological chemistry* 2004, 279, 49082–49090, DOI:10.1074/jbc.M409962200.
- [94] J. P. Liuzzi, J. A. Bobo, L. A. Lichten, D. A. Samuelson and R. J. Cousins, Responsive transporter genes within the murine intestinal-pancreatic axis form a basis of zinc homeostasis, *Proceedings of the National Academy of Sciences of the United States of America* 2004, 101, 14355–14360, DOI:10.1073/pnas.0406216101.

- [95] C. Huang, X. Cui, X. Sun, J. Yang and M. Li, Zinc transporters are differentially expressed in human non-small cell lung cancer, *Oncotarget* 2016, 7, 66935–66943, DOI:10.18632/oncotarget.11884.
- [96] E. M. Bafaro, S. Antala, T. V. Nguyen, S. P. Dzul, B. Doyon, T. L. Stemmler and R. E. Dempski, The large intracellular loop of hZIP4 is an intrinsically disordered zinc binding domain, *Metallomics: integrated biometal science* 2015, 7, 1319–1330, DOI:10.1039/c5mt00066a.
- [97] B. P. Weaver, Y. Zhang, S. Hiscox, G. L. Guo, U. Apte, K. M. Taylor, C. T. Sheline, L. Wang and G. K. Andrews, Zip4 (Slc39a4) expression is activated in hepatocellular carcinomas and functions to repress apoptosis, enhance cell cycle and increase migration, *PloS one* 2010, 5, e13158, DOI:10.1371/journal.pone.0013158.
- [98] M. Maares and H. Haase, A Guide to Human Zinc Absorption: General Overview and Recent Advances of In Vitro Intestinal Models, *Nutrients* 2020, 12, 762, DOI:10.3390/nu12030762.
- [99] H. Guo, X. Jin, T. Zhu, T. Wang, P. Tong, L. Tian, Y. Peng, L. Sun, A. Wan, J. Chen, Y. Liu, Y. Li, Q. Tian, L. Xia, L. Zhang, Y. Pan, L. Lu, Q. Liu, Shen L., Y. Li and K. Xia, SLC39A5 mutations interfering with the BMP/TGF- β pathway in non-syndromic high myopia, *Journal of medical genetics* 2014, 5, 518–525, DOI:10.1136/jmedgenet-2014-102351.
- [100] B. Y. Kong, F. E. Duncan, E. L. Que, A. M. Kim, T. V. O'Halloran and T. K. Woodruff, Maternally-derived zinc transporters ZIP6 and ZIP10 drive the mammalian oocyte-to-egg transition, *Molecular human reproduction* 2014, 20, 1077–1089, DOI:10.1093/molehr/gau066.
- [101] K. M. Taylor, H. E. Morgan, A. Johnson and R. I. Nicholson, Structure-function analysis of a novel member of the LIV-1 subfamily of zinc transporters, ZIP14, *FEBS letters* 2005, 579, 427–432, DOI:10.1016/j.febslet.2004.12.006.
- [102] C. Hogstrand, P. Kille, M. L. Ackland, S. Hiscox and K. M. Taylor, A mechanism for epithelial-mesenchymal transition and anoikis resistance in breast cancer triggered by zinc channel ZIP6 and STAT3 (signal transducer and activator of transcription 3), *The Biochemical journal* 2013, 455, 229–237, DOI:10.1042/BJ20130483.
- [103] Z. Liu, H. Li, M. Soleimani, K. Girijashanker, J. M. Reed, L. He, T. P. Dalton and D. W. Nebert, Cd²⁺ versus Zn²⁺ uptake by the ZIP8 HCO₃⁻-dependent symporter: Kinetics, electrogenicity and trafficking, *Biochemical and Biophysical Research Communications* 2008, 365, 814–820, DOI:10.1016/j.bbrc.2007.11.067.
- [104] J. Unno, K. Satoh, M. Hirota, A. Kanno, S. Hamada, H. Ito, A. Masamune, N. Tsukamoto, F. Motoi, S. Egawa, M. Unno, A. Horii and T. Shimosegawa, LIV-1 enhances the aggressive phenotype through the induction of epithelial to mesenchymal transition in human pancreatic carcinoma cells, *International Journal of Oncology* 2009, 35, 813–821, DOI:10.3892/ijo_00000394.
- [105] D. L. Manning, R. J. Daly, P. G. Lord, K. F. Kelly and C. D. Green, Effects of oestrogen on the expression of a 4.4 kb mRNA in the ZR-75-1 human breast cancer cell line, *Molecular and cellular endocrinology* 1988, 59, 205–212, DOI:10.1016/0303-7207(88)90105-0.
- [106] S. Tozlu, I. Girault, S. Vacher, J. Vendrell, C. Andrieu, F. Spyrtos, P. Cohen, R. Lidereau and I. Bieche, Identification of novel genes that co-cluster with estrogen receptor alpha in breast tumor biopsy specimens, using a large-scale real-time reverse transcription-PCR approach, *Endocrine-related cancer* 2006, 13, 1109–1120, DOI:10.1677/erc.1.01120.
- [107] R. A. McClelland, D. L. Manning, J. M. Gee, P. Willsher, J. F. Robertson, I. O. Ellis, R. W. Blamey and R. I. Nicholson, Oestrogen-regulated genes in breast cancer: association of pLIV1 with response to endocrine therapy, *British journal of cancer* 1998, 77, 1653–1656, DOI:10.1038/bjc.1998.271.
- [108] H. Kitamura, H. Morikawa, H. Kamon, M. Iguchi, S. Hojyo, T. Fukada, S. Yamashita, T. Kaisho, S. Akira, M. Murakami and T. Hirano, Toll-like receptor-mediated regulation of zinc homeostasis influences dendritic cell function, *Nature immunology* 2006, 7, 971–977, DOI:10.1038/ni1373.

- [109] M. Yu, W. W. Lee, D. Tomar, S. Pryshchep, M. Czesnikiewicz-Guzik, D. L. Lamar, G. Li, K. Singh, L. Tian, C. M. Weyand and J. J. Goronzy, Regulation of T cell receptor signaling by activation-induced zinc influx, *The Journal of experimental medicine* 2011, 208, 775–785, DOI:10.1084/jem.20100031.
- [110] L. Huang, C. P. Kirschke, Y. Zhang and Y. Y. Yu, The ZIP7 gene (Slc39a7) encodes a zinc transporter involved in zinc homeostasis of the Golgi apparatus, *The Journal of biological chemistry* 2005, 280, 15456–15463, DOI:10.1074/jbc.M412188200.
- [111] K. M. Taylor, H. E. Morgan, A. Johnson and R. I. Nicholson, Structure-function analysis of HKE4, a member of the new LIV-1 subfamily of zinc transporters, *The Biochemical journal* 2004, 377, 131–139, DOI:10.1042/BJ20031183.
- [112] K. M. Taylor, S. Hiscox, R. I. Nicholson, C. Hogstrand and P. Kille, Protein kinase CK2 triggers cytosolic zinc signaling pathways by phosphorylation of zinc channel ZIP7, *Science signaling* 2012, 5, ra11, DOI:10.1126/scisignal.2002585.
- [113] A. Grubman, G. E. Lidgerwood, C. Duncan, L. Bica, J. L. Tan, S. J. Parker, A. Caragounis, J. Meyerowitz, I. Volitakis, D. Moujalled, J. R. Liddell, J. L. Hickey, M. Horne, S. Longmuir, J. Koistinaho, P. S. Donnelly, P. J. Crouch, I. Tammen, A. R. White and K. M. Kanninen, Deregulation of subcellular biometal homeostasis through loss of the metal transporter, Zip7, in a childhood neurodegenerative disorder, *Acta neuropathologica communications* 2014, 2, 25, DOI:10.1186/2051-5960-2-25.
- [114] M. J. Liu, S. Bao, M. Gálvez-Peralta, C. J. Pyle, A. C. Rudawsky, R. E. Pavlovicz, D. W. Killilea, C. Li, D. W. Nebert, M. D. Wewers and D. L. Knoell, ZIP8 regulates host defense through zinc-mediated inhibition of NF- κ B, *Cell reports* 2013, 3, 386–400, DOI:10.1016/j.celrep.2013.01.009.
- [115] L. He, K. Girijashanker, T. P. Dalton, J. Reed, H. Li, M. Soleimani and D. W. Nebert, ZIP8, member of the solute-carrier-39 (SLC39) metal-transporter family: characterization of transporter properties, *Molecular pharmacology* 2006, 70, 171–180, DOI: 10.1124/mol.106.024521.
- [116] T. B. Aydemir, J. P. Liuzzi, S. McClellan and R. J. Cousins, Zinc transporter ZIP8 (SLC39A8) and zinc influence IFN-gamma expression in activated human T cells, *Journal of leukocyte biology* 2009, 86, 337–348, DOI:10.1189/jlb.1208759.
- [117] N. A. Begum, M. Kobayashi, Y. Moriwaki, M. Matsumoto, K. Toyoshima and T. Seya, Mycobacterium bovis BCG cell wall and lipopolysaccharide induce a novel gene, BIGM103, encoding a 7-TM protein: identification of a new protein family having Zn-transporter and Zn-metalloprotease signatures, *Genomics* 2002, 80, 630–645,, DOI:10.1006/geno.2002.7000.
- [118] J. Ovesen, B. Møller-Madsen, P. T. Nielsen, P. H. Christensen, O. Simonsen, H. C. Hoeck, M. B. Laursen and J. S. Thomsen, Differences in zinc status between patients with osteoarthritis and osteoporosis, *Journal of trace elements in medicine and biology: organ of the Society for Minerals and Trace Elements* 2009, 23(1), 1–8, DOI:10.1016/j.jtemb.2008.12.001.
- [119] J. H. Kim, J. Jeon, M. Shin, Y. Won, M. Lee, J. S. Kwak, G. Lee, J. Rhee, J. H. Ryu, C. H. Chun and J. S. Chun, egulation of the catabolic cascade in osteoarthritis by the zinc-ZIP8-MTF1 axis, *Cell* 2014, 156, 730–743, DOI:10.1016/j.cell.2014.01.007.
- [120] H. Fujishiro, Y. Yano, Y. Takada, M. Tanihara and S. Himeno, Roles of ZIP8, ZIP14, and DMT1 in transport of cadmium and manganese in mouse kidney proximal tubule cells, *Metallomics: integrated biometal science* 2012, 4, 700–708, DOI:10.1039/c2mt20024d.
- [121] K. Girijashanker, L. He, M. Soleimani, J. M. Reed, H. Li, Z. Liu, B. Wang, T. P. Dalton and D. W. Nebert, Slc39a14 gene encodes ZIP14, a metal/bicarbonate symporter: similarities to the ZIP8 transporter, *Molecular pharmacology* 2008, 73, 1413–1423, DOI:10.1124/mol.107.043588.

- [122] L. A. Gaither and D. J. Eide, Eukaryotic zinc transporters and their regulation, *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* 2001, 14, 251–270, DOI:10.1023/a:1012988914300.
- [123] T. Kambe, T. Suzuki, M. Nagao and Y. Yamaguchi-Iwai, Sequence similarity and functional relationship among eukaryotic ZIP and CDF transporters, *Genomics, proteomics & bioinformatics* 2006, 4, 1–9, DOI:10.1016/S1672-0229(06)60010-7.
- [124] W. Matsuura, T. Yamazaki, Y. Yamaguchi-Iwai, S. Masuda, M. Nagao, G. K. Andrews and T. Kambe, SLC39A9 (ZIP9) regulates zinc homeostasis in the secretory pathway: characterization of the ZIP subfamily I protein in vertebrate cells, *Bioscience, biotechnology, and biochemistry* 2009, 73, 1142–1148, DOI:10.1271/bbb.80910.
- [125] P. Thomas, Y. Pang, J. Dong and A. H. Berg, Identification and characterization of membrane androgen receptors in the ZIP9 zinc transporter subfamily:II. Role of human ZIP9 in testosterone-induced prostate and breast cancer cell apoptosis, *Endocrinology* 2014, 155, 4250–4265, DOI:10.1210/en.2014-120.
- [126] M. Taniguchi, A. Fukunaka, M. Hagihara, K. Watanabe, S. Kamino, T. Kambe, S. Enomoto and M. Hiromura, Essential role of the zinc transporter ZIP9/SLC39A9 in regulating the activations of Akt and Erk in B-cell receptor signaling pathway in DT40 cells, *PloS one* 2013, 8, e58022, DOI:10.1371/journal.pone.0058022.
- [127] A. H. Berg, C. D. Rice, M. S. Rahman, J. Dong and P. Thomas, Identification and characterization of membrane androgen receptors in the ZIP9 zinc transporter subfamily: I. Discovery in female atlantic croaker and evidence ZIP9 mediates testosterone-induced apoptosis of ovarian follicle cells, *Endocrinology* 2014,, 155, 4237–4249, DOI:10.1210/en.2014-1198.
- [128] L. A. Lichten, M. S. Ryu, L. Guo, J. Embury and R. J. Cousins, MTF-1-mediated repression of the zinc transporter Zip10 is alleviated by zinc restriction, *PloS one* 2011, 6, e21526, DOI:10.1371/journal.pone.0021526.
- [129] S. Hojyo, T. Miyai, H. Fujishiro, M. Kawamura, T. Yasuda, A. Hijikata, B. H. Bin, T. Irié, J. Tanaka, T. Atsumi, M. Murakami, M. Nakayama, O. Ohara, S. Himeno, H. Yoshida, H. Koseki, T. Ikawa, K. Mishima and T. Fukada, Zinc transporter SLC39A10/ZIP10 controls humoral immunity by modulating B-cell receptor signal strength, *Proceedings of the National Academy of Sciences of the United States of America* 2014, 111, 11786–11791, DOI:doi.org/10.1073/pnas.1323557111.
- [130] T. Miyai, S. Hojyo, T. Ikawa, M. Kawamura, T. Irié, H. Ogura, A. Hijikata, B. H. Bin, T. Yasuda, H. Kitamura, M. Nakayama, O. Ohara, H. Yoshida, H. Koseki, K. Mishima and T. Fukada, Zinc transporter SLC39A10/ZIP10 facilitates antiapoptotic signaling during early B-cell development. *Proceedings of the National Academy of Sciences of the United States of America* 2014, 111, 11780–11785, DOI:10.1073/pnas.1323549111.
- [131] N. Kagara, N. Tanaka, S. Noguchi and T. Hirano, Zinc and its transporter ZIP10 are involved in invasive behavior of breast cancer cells, *Cancer science* 2007, 98, 692–697, DOI:10.1111/j.1349-7006.2007.00446.x.
- [132] M. S. Ryu, L. A. Lichten, J. P. Liuzzi and R. J. Cousins, Zinc transporters ZnT1 (Slc30a1), Zip8 (Slc39a8), and Zip10 (Slc39a10) in mouse red blood cells are differentially regulated during erythroid development and by dietary zinc deficiency, *The Journal of nutrition* 200, 138, 2076–2083, DOI:10.3945/jn.108.0935.
- [133] S. L. Kelleher, V. Velasquez, T. P. Croxford, N. H. McCormick, V. Lopez and J. MacDavid, Mapping the zinc-transporting system in mammary cells: molecular analysis reveals a phenotype-dependent zinc-transporting network during lactation, *Journal of cellular physiology* 2012, 227, 1761–1770, DOI:10.1002/jcp.22900.
- [134] A. B. Martin, T. B. Aydemir, G. J. Guthrie, D. A. Samuelson, S. M. Chang and R. J. Cousins, Gastric and colonic zinc transporter ZIP11 (Slc39a11) in mice responds to dietary zinc and exhibits nuclear localization, *The Journal of nutrition* 2013, 143, 1882–1888, DOI:10.3945/jn.113.184457.

- [135] F. Chimienti, S. Devergnas, F. Pattou, F. Schuit, R. Garcia-Cuenca, B. Vandewalle, J. Kerr-Conte, L. Van Lommel, D. Grunwald, A. Favier and M. Seve, In vivo expression and functional characterization of the zinc transporter ZnT8 in glucose-induced insulin secretion, *Journal of cell science* 2006, 119, 4199–4206, DOI:10.1242/jcs.03164.
- [136] B. H. Bin, T. Fukada, T. Hosaka, S. Yamasaki, W. Ohashi, S. Hojyo, T. Miyai, K. Nishida, . S. Yokoyama and T. Hirano, Biochemical characterization of human ZIP13 protein: a homo-dimerized zinc transporter involved in the spondylocheiro dysplastic Ehlers-Danlos syndrome, *The Journal of biological chemistry* 2011, 286, 40255–40265, DOI:10.1074/jbc.M111.256784.
- [137] J. Jeong, J. M. Walker, F. Wang, J. G. Park, A. E. Palmer, C. Giunta, M. Rohrbach, B. Steinmann and D. J. Eide, Promotion of vesicular zinc efflux by ZIP13 and its implications for spondylocheiro dysplastic Ehlers-Danlos syndrome, *Proceedings of the National Academy of Sciences of the United States of America* 2012, 109, E3530–E3538, DOI:10.1073/pnas.1211.
- [138] C. Giunta, N. H. Elçioğlu, B. Albrecht, G. Eich, C. Chambaz, A. R. Janecke, H. Yeowell, M. Weis, D. R. Eyre, M. Kraenzlin and B. Steinmann, Spondylocheiro dysplastic form of the Ehlers-Danlos syndrome--an autosomal-recessive entity caused by mutations in the zinc transporter gene SLC39A13, *American journal of human genetics* 2008, 82, 1290–1305, DOI:10.1016/j.ajhg.2008.05.001.
- [139] J. P. Liuzzi, L. A. Lichten, S. Rivera, R. K. Blanchard, T. B. Aydemir, M. D. Knutson, T. Ganz and R. J. Cousins, Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response, *Proceedings of the National Academy of Sciences of the United States of America* 2005, 102, 6843–6848, DOI:10.1073/pnas.0502257102.
- [140] K. Homma, T. Fujisawa, N. Tsuburaya, N. Yamaguchi, H. Kadowaki, K. Takeda, H. Nishitoh, A. Matsuzawa, I. Naguro and H. Ichijo, SOD1 as a molecular switch for initiating the homeostatic ER stress response under zinc deficiency, *Molecular cell* 2013, 52, 75–86, DOI:10.1016/j.molcel.2013.08.038.
- [141] S. Hojyo, T. Fukada, S. Shimoda, W. Ohashi, B. H. Bin, H. Koseki and T. Hirano, The zinc transporter SLC39A14/ZIP14 controls G-protein coupled receptor-mediated signaling required for systemic growth, *PLoS one* 2011, 6, e18059, DOI:10.1371/journal.pone.0018059.
- [142] J. J. Pinilla-Tenas, B. K. Sparkman, A. Shawki, A. C. Illing, C. J. Mitchell, N. Zhao, J. P. Liuzzi, R. J. Cousins, M. D. Knutson and B. Mackenzie, Zip14 is a complex broad-scope metal-ion transporter whose functional properties support roles in the cellular uptake of zinc and nontransferrin-bound iron. *American journal of physiology, Cell physiology* 2011, 301, C862–C871,, DOI:10.1152/ajpcell.00479.2010.
- [143] E. Ohana, E. Hoch, C. Keasar, T. Kambe, O. Yifrach, M. Hershfinkel and I. Sekler, Identification of the Zn²⁺ binding site and mode of operation of a mammalian Zn²⁺ transporter, *The Journal of biological chemistry* 2009, 284, 17677–17686, DOI:10.1074/jbc.M109.007203.
- [144] E. Shusterman, O. Beharier, L. Shiri, R. Zarivach, Y. Etzion, C. R. Campbell, I. H. Lee, K. Okabayashi, A. Dinudom, D. I. Cook, A. Katz and A. Moran, ZnT-1 extrudes zinc from mammalian cells functioning as a Zn(2+)/H(+) exchanger, *Metallomics: integrated biometal science* 2014, 6, 1656–1663, DOI:10.1039/c4mt00108g.
- [145] R. D. Palmiter and S. D. Findley, Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc, *The EMBO journal* 1995, 14, 639–649, DOI:10.1002/j.1460-2075.1995.tb07042.x.
- [146] X. Wang and B. Zhou, Dietary zinc absorption: A play of Zips and ZnTs in the gut, *IUBMB life* 2010, 62, 176–182, DOI:10.1002/iub.291.
- [147] M. Tsuda, K. Imaizumi, T. Katayama, K. Kitagawa, A. Wanaka, M. Tohyama and T. Takagi, Expression of zinc transporter gene, ZnT-1, is induced after transient forebrain ischemia in the gerbil, *The Journal of neuroscience : the official journal of the Society for Neuroscience* 1997, 17, 6678–6684, DOI:10.1523/JNEUROSCI.17-17-06678.1.

- [148] N. Itsumura, Y. Inamo, F. Okazaki, F. Teranishi, H. Narita, T. Kambe and H. Kodama, Compound heterozygous mutations in SLC30A2/ZnT2 results in low milk zinc concentrations: a novel mechanism for zinc deficiency in a breast-fed infant, *PLoS one* 2013, 8, e64045, DOI:10.1371/journal.pone.0064045.
- [149] J. M. Falcón-Pérez and E. C. Dell'Angelica, Zinc transporter 2 (SLC30A2) can suppress the vesicular zinc defect of adaptor protein 3-depleted fibroblasts by promoting zinc accumulation in lysosomes, *Experimental cell research* 2007, 313, 1473–1483, DOI:10.1016/j.yexcr.2007.02.006.
- [150] T. B. Cole, H. J. Wenzel, K. E. Kafer, P. A. Schwartzkroin and R. D. Palmiter, Elimination of zinc from synaptic vesicles in the intact mouse brain by disruption of the ZnT3 gene, *Proceedings of the National Academy of Sciences of the United States of America* 1999, 96, 1716–1721, DOI:10.1073/pnas.96.4.1716.
- [151] L. Huang, C. P. Kirschke and J. Gitschier, Functional characterization of a novel mammalian zinc transporter, ZnT6, *The Journal of biological chemistry* 2002, 277, 26389–26395, DOI:10.1074/jbc.M200462200.
- [152] I. Kukic, J. K. Lee, J. Coblenz, S. L. Kelleher and K. Kiselyov, Zinc-dependent lysosomal enlargement in TRPML1-deficient cells involves MTF-1 transcription factor and ZnT4 (Slc30a4) transporter, *The Biochemical journal* 2013, 451, 155–163, DOI:10.1042/BJ20121506.
- [153] J. P. Liuzzi, J. A. Bobo, L. Cui, R. J. McMahon and R. J. Cousins, Zinc transporters 1, 2 and 4 are differentially expressed and localized in rats during pregnancy and lactation, *The Journal of nutrition* 2003, 133, 342–351, DOI:10.1093/jn/133.2.342.
- [154] T. Suzuki, K. Ishihara, H. Migaki, W. Matsuura, A. Kohda, K. Okumura, M. Nagao, Y. Yamaguchi-Iwai and T. Kambe, Zinc transporters, ZnT5 and ZnT7, are required for the activation of alkaline phosphatases, zinc-requiring enzymes that are glycosylphosphatidylinositol-anchored to the cytoplasmic membrane, *The Journal of biological chemistry* 2005, 280, 637–643, DOI:10.1074/jbc.M411247200.
- [155] C. D. Ellis and C. W. Macdiarmid, Heteromeric protein complexes mediate zinc transport into the secretory pathway of eukaryotic cells, *The Journal of biological chemistry* 2005, 280, 28811–28818, DOI:10.1074/jbc.M505500200.
- [156] A. Fukunaka, T. Suzuki, Y. Kurokawa, T. Yamazaki, N. Fujiwara, K. Ishihara, H. Migaki, K. Okumura, S. Masuda, Y. Yamaguchi-Iwai, M. Nagao and T. Kambe, Demonstration and characterization of the heterodimerization of ZnT5 and ZnT6 in the early secretory pathway, *The Journal of biological chemistry* 2009, 284, 30798–30806, DOI:10.1074/jbc.M109.026435.
- [157] I. Lasry, Y. Golan, B. Berman, N. Amram, F. Glaser and Y. G. Assaraf, In situ dimerization of multiple wild type and mutant zinc transporters in live cells using bimolecular fluorescence complementation, *The Journal of biological chemistry* 2014, 289, 7275–7292, DOI:10.1074/jbc.M113.533786.
- [158] C. D. Ellis, F. Wang, C. W. MacDiarmid, S. Clark, T. Lyons and D. J. Eide, Zinc and the Msc2 zinc transporter protein are required for endoplasmic reticulum function, *The Journal of cell biology* 2004, 166, 325–335, DOI:10.1083/jcb.200401157.
- [159] K. Ishihara, T. Yamazaki, Y. Ishida, T. Suzuki, K. Oda, M. Nagao, Y. Yamaguchi-Iwai and T. Kambe, Zinc transport complexes contribute to the homeostatic maintenance of secretory pathway function in vertebrate cells, *The Journal of biological chemistry* 2006, 281, 17743–17750, DOI:10.1074/jbc.M602470200.
- [160] K. Nishida, A. Hasegawa, S. Nakae, K. Oboki, H. Saito, S. Yamasaki and T. Hirano, Zinc transporter Znt5/Slc30a5 is required for the mast cell-mediated delayed-type allergic reaction but not the immediate-type reaction, *The Journal of experimental medicine* 2009, 206, 1351–1364, DOI:10.1084/jem.20082533.
- [161] T. Kambe, Molecular architecture and function of ZnT transporters, *Current topics in membranes* 2012, 69, 199–220, DOI:10.1016/B978-0-12-394390-3.00008-2.

- [162] C. P. Kirschke and L. Huang, ZnT7, a novel mammalian zinc transporter, accumulates zinc in the Golgi apparatus, *The Journal of biological chemistry* 2003, 278, 4096–4102, DOI:10.1074/jbc.M207644200.
- [163] A. Fukunaka, Y. Kurokawa, F. Teranishi, I. Sekler, K. Oda, M. L. Ackland, V. Faundez, M. Hiromura, S. Masuda, M. Nagao, S. Enomoto and T. Kambe, Tissue nonspecific alkaline phosphatase is activated via a two-step mechanism by zinc transport complexes in the early secretory pathway, *The Journal of biological chemistry* 2011, 286, 16363–16373, DOI:10.1074/jbc.M111.227173.
- [164] T. Kambe, Methods to evaluate Zinc transport into and out of the secretory and endosomal-lysosomal compartments in DT40 cells, *Methods in enzymology* 2014, 534, 77–92, DOI:10.1016/B978-0-12-397926-1.00005-6.
- [165] L. Huang, C. P. Kirschke, Y. A. Lay, L. B. Levy, D. E. Lamirande and P. H. Zhang, Znt7-null mice are more susceptible to diet-induced glucose intolerance and insulin resistance, *The Journal of biological chemistry* 2012, 287, 33883–33896, DOI:10.1074/jbc.M111.309666.
- [166] F. Chimienti, S. Devergnas, A. Favier and M. Seve, Identification and cloning of a beta-cell-specific zinc transporter, ZnT-8, localized into insulin secretory granules, *Diabetes* 2004, 53, 2330–2337, DOI:10.2337/diabetes.53.9.2330.
- [167] B. Y. Besecker, M. C. Exline, J. Hollyfield, G. Phillips, R. A. Disilvestro, M. D. Wewers and D. L. Knoell, A comparison of zinc metabolism, inflammation, and disease severity in critically ill infected and noninfected adults early after intensive care unit admission, *The American journal of clinical nutrition* 2011, 93, 1356–1364, DOI:10.3945/ajcn.110.
- [168] N. Patrushev, B. Seidel-Rogol and G. Salazar, Angiotensin II requires zinc and downregulation of the zinc transporters ZnT3 and ZnT10 to induce senescence of vascular smooth muscle cells, *PloS one* 2012, 7, e33211, DOI:10.1371/journal.pone.0033211.
- [169] H. Fujishiro, M. Yoshida, Y. Nakano and S. Himeno, Interleukin-6 enhances manganese accumulation in SH-SY5Y cells: implications of the up-regulation of ZIP14 and the down-regulation of ZnT10, *Metallomics: integrated biometal science* 2014, 6, 944–949, DOI:10.1039/c3mt00362k.
- [170] L. J. Coneyworth, K. A. Jackson, J. Tyson, H. J. Bosomworth, E. van der Hagen, G. M. Hann, O. A. Ogo, D. C. Swann, J. C. Mathers, R. A. Valentine and D. Ford, Identification of the human zinc transcriptional regulatory element (ZTRE): a palindromic protein-binding DNA sequence responsible for zinc-induced transcriptional repression, *The Journal of biological chemistry* 2012, 287, 36567–36581,, DOI:10.1074/jbc.M112.397000.
- [171] D. L. Sim and V. T. Chow, The novel human HUEL (C4orf1) gene maps to chromosome 4p12-p13 and encodes a nuclear protein containing the nuclear receptor interaction motif, *Genomics* 1999, 59, 224–233, DOI:10.1006/geno.1999.5856.
- [172] V. Cherezov, N. Höfer, D. M. E. Szebenyi, O. Kolaj, J. G. Wall, R. Gillilan, V. Srinivasan, C. P. Jaroniec and M. Caffrey, Insights into the Mode of Action of a Putative Zinc Transporter CzcB in *Thermus thermophilus*, *Structure* 2008, 16, 1378-1388, DOI:10.1016/j.str.2008.05.014.
- [173] T. Hirano, M. Murakami, T. Fukada, K. Nishida, S. Yamasaki and T. Suzuki, Roles of zinc and zinc signaling in immunity: zinc as an intracellular signaling molecule, *Advances in immunology* 2008, 97, 149–176, DOI:10.1016/S0065-2776(08)00003-5.
- [174] M. Murakami and T. Hirano, Intracellular zinc homeostasis and zinc signaling, *Cancer science* 2008, 99, 1515–1522, DOI:10.1111/j.1349-7006.2008.00854.x.
- [175] S. L. Stafford, N. J. Bokil, M. E. Achard, R. Kapetanovic, M. A. Schembri, A. G. McEwan and M. J. Sweet, Metal ions in macrophage antimicrobial pathways: emerging roles for zinc and copper, *Bioscience reports* 2013, 33, e00049, DOI:10.1042/BSR20130014.

- [176] K. Subramanian Vignesh, J. A. Landero Figueroa, A. Porollo, J. A. Caruso and G. S. J. Deepe, Granulocyte macrophage-colony stimulating factor induced Zn sequestration enhances macrophage superoxide and limits intracellular pathogen survival, *Immunity* 2013, 39, 697–710, DOI:10.1016/j.immuni.2013.09.006.
- [177] G. K. Andrews, Regulation and function of Zip4, the acrodermatitis enteropathica gene, *Biochemical Society transactions* 2008, 36, 1242–1246, DOI:10.1042/BST0361242.
- [178] W. Maret, Zinc biochemistry: from a single zinc enzyme to a key element of life, *Advances in nutrition* 2013, 4, 82–91, DOI:10.3945/an.112.003038.
- [179] W. Chowanadisai, B. Lönnnerdal and S. L. Kelleher, Identification of a mutation in SLC30A2 (ZnT-2) in women with low milk zinc concentration that results in transient neonatal zinc deficiency, *The Journal of biological chemistry* 2006, 281, 39699–39707 DOI:10.1074/jbc.M605821200.
- [180] K. Tuschl, P. T. Clayton, S. M. J. Gospe, S. Gulab, S. Ibrahim, P. Singhi, R. Aulakh, R. T. Ribeiro, O. G. Barsottini, M. S. Zaki, M. L. Del Rosario, S. Dyack, V. Price, A. Rideout, K. Gordon, R. A. Wevers, W. K. Chong and P. B. Mills, Syndrome of Hepatic Cirrhosis, Dystonia, Polycythemia, and Hypermanganesemia Caused by Mutations in SLC30A10, a Manganese Transporter in Man, *American journal of human genetics* 2016, 99, 521, DOI:10.1016/j.ajhg.2016.07.015.
- [181] I. T. Paulsen and M. H. J. Saier, A novel family of ubiquitous heavy metal ion transport proteins, *The Journal of membrane biology* 1997, 156, 99–103, DOI:10.1007/s002329900192.
- [182] T. Kimura and T. Kambe, The Functions of Metallothionein and ZIP and ZnT Transporters: An Overview and Perspective, *International journal of molecular sciences* 2016, 17, 336, DOI:10.3390/ijms17030336.
- [183] H. Tapiero and K. D. Tew, Trace elements in human physiology and pathology: zinc and metallothioneins, *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* 2003, 57, 399–411, DOI:10.1016/s0753-3322(03)00081-7.
- [184] A. K. Baltaci, K. Yuce and R. Mogulkoc, Zinc Metabolism and Metallothioneins, *Biological trace element research* 2018, 183, 22–31, DOI:10.1007/s12011-017-1119-7.
- [185] J. Lu, T. Jin, G. Nordberg and M. Nordberg, Metallothionein gene expression in peripheral lymphocytes from cadmium-exposed workers, *Cell stress & chaperones* 2001, 6, 97–104, DOI:10.1379/1466-1268(2001)006<0097:mgeipl>2.0.co;2.
- [186] W. Maret, Thiol reactivity as a central aspect of metallothionein's mechanism of action, *Metallothioneins in Biochemistry and Pathology* 2008, 27-45, DOI:10.1142/9789812778949_0002.
- [187] M. Rowińska-Żyrek, S. Potocki, D. Witkowska, D. Valensin and H. Kozłowski, The zinc-binding fragment of HypA from *Helicobacter pylori*: a tempting site also for nickel ions, *Dalton Transactions* 2013, 42, 6012-6020, DOI:10.1039/C2DT32195E.
- [188] H. J. Blewett and C. G. Taylor, Dietary Zinc Deficiency in Rodents: Effects on T-Cell Development, Maturation and Phenotypes, *Nutrients* 2012, 4, 449-466, DOI:10.3390/nu4060449.
- [189] X. Zhang, H. Tamaru, S. I. Khan, J. R. Horton, L. J. Keefe, E. U. Selker and X. Cheng, Structure of the *Neurospora* SET Domain Protein DIM-5, a Histone H3 Lysine Methyltransferase, *Cell* 2002, 111, 117-127, DOI:10.1016/S0092-8674(02)00999-6.
- [190] U. Wimmer, Y. Wang, O. Georgiev and W. Schaffner, Two major branches of anti-cadmium defense in the mouse: MTF-1/metallothioneins and glutathione, *Nucleic Acids Research* 2005, 33, 5715–5727, DOI:10.1093/nar/gki881.
- [191] J. Gumulec, M. Raudenska, V. Adam, R. Kizek and M. Masarik, Metallothionein – Immunohistochemical Cancer Biomarker: A Meta-Analysis, *PLoS ONE* 2014, 9, e85346, DOI:10.1371/journal.pone.0085346.

- [192] D. L. Pountney, T. C. Dickson, J. H. C. Power, J. C. Vickers, A. J. West and W. P. Gai, Association of Metallothionein-III with Oligodendroglial Cytoplasmic Inclusions in Multiple System Atrophy, *Neurotoxicity Research* 2011, 19, 115–122, DOI:10.1007/s12640-009-9146-6.
- [193] W. Ohashi, T. Hara, T. Takagishi, K. Hase and T. Fukada, Maintenance of Intestinal Epithelial Homeostasis by Zinc Transporter, *Digestive diseases and sciences* 2019, 64, 2404–2415, DOI:10.1007/s10620-019-05561-2.
- [194] K. T. Smith and R. J. Cousins, Quantitative aspects of zinc absorption by isolated, vascularly perfused rat intestine, *The Journal of nutrition* 1980, 110, 316–323 DOI:10.1093/jn/110.2.316.
- [195] B. Hinskens, J. C. Philcox, P. Coyle and A. M. Rofo, Increased zinc absorption but not secretion in the small intestine of metallothionein-null mice, *Biological trace element research* 2000, 78, 231–240, DOI:10.1385/BTER:78:1-3:231.
- [196] S. R. Davis, R. J. McMahon and R. J. Cousins, Metallothionein knockout and transgenic mice exhibit altered intestinal processing of zinc with uniform zinc-dependent zinc transporter-1 expression, *The Journal of nutrition* 1998, 128, 825–831 DOI:10.1093/jn/128.5.825.
- [197] L. Rink and H. Haase, Zinc homeostasis and immunity, *Trends in immunology* 2007, 28, 1–4, DOI:10.1016/j.it.2006.11.005.
- [198] K. A. Jackson, R. A. Valentine, L. J. Coneyworth, J. C. Mathers and D. Ford, Mechanisms of mammalian zinc-regulated gene expression, *Biochemical Society transactions* 2008, 36, 1262–1266, DOI:10.1042/BST0361262.
- [199] T. B. Aydemir, S. M. Chang, G. J. Guthrie, A. B. Maki, M. S. Ryu, A. Karabiyik and R. J. Cousins, Zinc transporter ZIP14 functions in hepatic zinc, iron and glucose homeostasis during the innate immune response (endotoxemia), *PloS one* 2012, 7, e48679, DOI:10.1371/journal.pone.0048679.
- [200] K. H. Ibs and L. Rink, Zinc-altered immune function, *The Journal of nutrition* 2003, 133, 1452S–6S, DOI:10.1093/jn/133.5.1452S.
- [201] H. Hasegawa, K. Suzuki, Y. Suzuki, S. Nakaji and K. Sugawara, Effects of zinc on the reactive oxygen species generating capacity of human neutrophils and on the serum opsonic activity in vitro, *Luminescence : the journal of biological and chemical luminescence* 2000, 15, 321–327, DOI:10.1002/1522-7243(20000.
- [202] M. Stefanidou, C. Maravelias, A. Dona and C. Spiliopoulou, Zinc: a multipurpose trace element, *Archives of toxicology* 2006, 80, 1–9, DOI:10.1007/s00204-005-0009-5.
- [203] S. C. Liang, X. Y. Tan, D. P. Luxenberg, R. Karim, K. Dunussi-Joannopoulos, M. Collins and L. A. Fouser, Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides, *The Journal of experimental medicine* 2003, 203, 2271–2279, DOI:10.1084/jem.20061308.
- [204] M. Wolf and B. Moser, Antimicrobial activities of chemokines: not just a side-effect?, *Frontiers in immunology* 2012, 3, 213, DOI:10.3389/fimmu.2012.00213.
- [205] B. D. Corbin, E. H. Seeley, A. Raab, J. Feldmann, M. R. Miller, V. J. Torres, K. L. Anderson, B. M. Dattilo, P. M. Dunman, R. Gerads, R. M. Caprioli, W. Nacken, W. J. Chazin, E. P. Skaar, Metal chelation and inhibition of bacterial growth in tissue abscesses, *Science* 2008, 319, 962–965, DOI:10.1126/science.1152449.
- [206] R. Donato, B. R. Cannon, G. Sorci, F. Riuzzi, K. Hsu, D. J. Weber, C. L. Geczy, Functions of S100 proteins, *Current molecular medicine* 2013, 13, 24–57.
- [207] J. Amich, R. Vicentefranqueira, E. Mellado, A. Ruiz-Carmuega, F. Leal and J. A. Calera, The ZrfC alkaline zinc transporter is required for *Aspergillus fumigatus* virulence and its growth in the presence of the Zn/Mn-chelating protein calprotectin., *Cellular Microbiology*, 2014, 16, 548–564, DOI:10.1111/cmi.12238.

- [208] L. W. Soares, A. M. Bailão, C. M. de Almeida Soares and M. G. S. Bailão, Zinc at the Host–Fungus Interface: How to Uptake the Metal?, *Journal of Fungi*, 2020, 6, 305, DOI:10.3390/jof6040305.
- [209] T. Lech and J. T. Sadlik, Zinc in postmortem body tissues and fluids, *Biological Trace Element Research* 2011, 142, 11–17, DOI:10.1007/s12011-010-8747-5.
- [210] H. Zhao and D. Eide, The yeast ZRT1 gene encodes the zinc transporter protein of a high-affinity, *Proceedings of the National Academy of Sciences of the United States of America* 1996, 93, 2454–2458, DOI:10.1073/pnas.93.6.2454.
- [211] H. Zhao and D. Eide, The ZRT2 gene encodes the low affinity zinc transporter in *Saccharomyces cerevisiae*, *Journal of Biological Chemistry* 1996, 271, 23203, 23210, DOI:10.1074/jbc.271.38.23203.
- [212] G. D. Brown, D. W. Denning, N. A. Gow, S. M. Levitz, M. G. Netea and T. C. White, Hidden killers: human fungal infections, *Science translational medicine* 2012, 4, 165rv13, DOI:10.1126/scitranslmed.3004404.
- [213] J. Park, J. Park, S. Jang, S. Kim, S. Kong, J. Choi, K. Ahn, J. Kim, S. Lee, S. Kim, B. Park, K. Jung, S. Kim, S. Kang, Y. H. Lee, FTFD: an informatics pipeline supporting phylogenomic analysis of fungal transcription factors, *Bioinformatics* 2008, 24, 1024–1025, DOI:10.1093/bioinformatics/btn058.
- [214] K. Wang, O. Sitsel, G. Meloni, H. E. Autzen, M. Andersson, T. Klymchuk, A. M. Nielsen, D. C. Rees, P. Nissen, P. Gourdon, Structure and mechanism of Zn²⁺-transporting P-type ATPases, *Nature* 2014, 514, 518–522, DOI:10.1038/nature13618.
- [215] C. C. Staats, Kmetzsch, L., Schrank, A., Vainstein, M. H., Fungal zinc metabolism and its connections to virulence, *Frontiers in cellular and infection microbiology* 2013, 3, 65, DOI: 10.3389/fcimb.2013.00065.
- [216] W. H. Jung, The Zinc Transport Systems and Their Regulation in Pathogenic Fungi, *Mycobiology* 2015, 43(3), 179–183, DOI:10.5941/MYCO.2015.43.3.179.
- [217] S. Macpherson, M. Larochelle and B. Turcotte, A Fungal Family of Transcriptional Regulators: the Zinc Cluster Proteins, *Microbiology and Molecular Biology Reviews* 2006, 70, 583–604, DOI:10.1128/MMBR.00015-06.
- [218] A. J. Bird and S. Wilson, Zinc homeostasis in the secretory pathway in yeast, *Current opinion in chemical biology* 2020, 55, 145–150, DOI:10.1016/j.cbpa.2020.01.011.
- [219] L. Costello, Y. Liu, R. B. Franklin, M. C. Kennedy Zinc inhibition of mitochondrial aconitase and its importance in citrate metabolism of prostate epithelial cells, *The Journal of biological chemistry* 1997, 272, 28875–28881, DOI:10.1074/jbc.272.46.28875.
- [220] H. Botella, P. Peyron, F. Levillain, R. Poincloux, Y. Poquet, I. Brandli, C. Wang, L. Tailleux, S. Tilleul, G. M. Charrière, S. J. Waddell, M. Foti, G. Lugo-Villarino, Q. Gao, I. Maridonneau-Parini, P. D. Butcher, P. R. Castagnoli, B. Gicquel, C. de Chastellier, O. Neyrolles, Mycobacterial p(1)-type ATPases mediate resistance to zinc poisoning in human macrophages, *Cell host & microbe* 2011, 10, 248–259, DOI:10.1016/j.chom.2011.08.006.
- [221] I. Galván Márquez, M. Ghiyasvand, A. Massarsky, M. Babu, B. Samanfar, K. Omid, T. W. Moon, M. L. Smith, A. Golshani, Zinc oxide and silver nanoparticles toxicity in the baker's yeast, *Saccharomyces cerevisiae*, *PloS one* 2018, 13, e0193111, DOI:10.1371/journal.pone.0193111.
- [222] V. Sinisi, P. Pelagatti, M. Carcelli, A. Migliori, L. Mantovani, L. Righi, G. Leonardi, S. Pietarinen, C. Hubsch and D. A. Rogolino, A Green Approach to Copper-Containing Pesticides: Antimicrobial and Antifungal Activity of Brochantite Supported on Lignin for the Development of Biobased Plant Protection Products, *ACS Sustainable Chemistry & Engineering* 2019, 7, 3213–3221,, DOI:10.1021/acssuschemeng.8b05135.

- [223] L. He, Y. Liu, A. Mustapha, M. Lin, Antifungal activity of zinc oxide nanoparticles against *Botrytis cinerea* and *Penicillium expansum*, *Microbiological research* 2011, 166, 207–215, DOI:10.1016/j.micres.2010.03.003.
- [224] L. Lanfranco, R. Balsamo, E. Martino, S. Perotto, P. Bonfante, Zinc ions alter morphology and chitin deposition in an ericoid fungus, *European journal of histochemistry* 2002, 46, 341–350, DOI:10.4081/1746.
- [225] R. A. Colvin, W. R. Holmes, C. P. Fontaine, W. Maret, Cytosolic zinc buffering and muffling: their role in intracellular zinc homeostasis, *Metallomics : integrated biometal science* 2010, 2, 306–317, DOI:10.1039/b926662c.
- [226] P. Zhai, Y. Chai and L. Lu, Fungal Zinc Homeostasis and Its Potential as an Antifungal Target: A Focus on the Human Pathogen *Aspergillus fumigatus*, *Microorganisms*, 2022, 10, 2469, DOI: 10.3390/microorganisms10122469.
- [227] S. J. Luloff, Hahn, B. L., Sohnle, P. G., Fungal susceptibility to zinc deprivation, *The Journal of laboratory and clinical medicine* 2004, 144, 208–214, DOI:10.1016/j.lab.2004.07.007.
- [228] M. Garcia Silva-Bailão, K. Lobato Potenciano da Silva, L. Raniere Borges Dos Anjos, P. de Sousa Lima, M. de Melo Teixeira, S. Maria de Almeida Soares and A. Melo Bailão, Mechanisms of copper and zinc homeostasis in pathogenic black fungi, *Fungal Biology*, 2018, 122, 526–537, DOI:10.1016/j.funbio.2017.12.002.
- [229] I. Stríz and I. Trebichavský, Calprotectin - a pleiotropic molecule in acute and chronic inflammation, *Physiological research* 2004, 53, 245–253.
- [230] D. Wilson, F. Citiulo and B. Hube, Zinc Exploitation by Pathogenic Fungi, *PLOS Pathogens*, 2012, 8, e1003034, DOI:doi:10.1371/journal.ppat.1003034.
- [231] E. M. Zygiel and E. M. Nolan, Transition Metal Sequestration by the Host-Defense Protein Calprotectin, *Annual review of biochemistry* 2018, 87, 621–643, DOI:10.1146/annurev-biochem-062917-012312.
- [232] J. R. Robinson, O. S. Isikhuemhen and F. N. Anike, Fungal–Metal Interactions: A Review of Toxicity and Homeostasis, *Journal of Fungi* 2021, 7, 225, DOI:10.3390/jof7030225.
- [233] A. Kumánovics, K. E. Poruk, K. A. Osborn, D. M. Ward and J. Kaplan, YKE4 (YIL023C) encodes a bidirectional zinc transporter in the endoplasmic reticulum of *Saccharomyces cerevisiae*, *The Journal of biological chemistry* 2006, 281, 22566–22574, DOI:10.1074/jbc.M604730200.
- [234] J. Amich and J. A. Calera, Zinc acquisition: A key aspect in *Aspergillus fumigatus* virulence, *Mycopathologia*, 2014, 178, 379–385, DOI:10.1007/s11046-014-9764-2.
- [235] F. Citiulo, I. D. Jacobsen, P. Miramón, L. Schild, S. Brunke, P. Zipfel, M. Brock, B. Hube and D. Wilson, *Candida albicans* Scavenges Host Zinc via Pra1 during Endothelial Invasion, *PLoS Pathogens*, 2012, 8, e1002777, DOI:10.1371/journal.ppat.1002777.
- [236] D. J. Eide, The SLC39 family of metal ion transporters, *Pflügers Archiv : European journal of physiology* 2004, 447, 796–800, DOI:10.1007/s00424-003-1074-3.
- [237] S. Nishida, T. Mizuno and H. Obata, Involvement of histidine-rich domain of ZIP family transporter TjZNT1 in metal ion specificity, *Plant physiology and biochemistry : PPB* 2008, 46, 601–606, DOI:10.1016/j.plaphy.2008.02.011.
- [238] C. W. MacDiarmid, L. A. Gaither and D. Eide, Zinc transporters that regulate vacuolar zinc storage in *Saccharomyces cerevisiae*, *The EMBO journal* 2000, 19, 2845–2855, DOI:10.1093/emboj/19.12.2845.
- [239] Vacuolar zinc transporter Zrc1 is required for detoxification of excess intracellular zinc in the human fungal pathogen *Cryptococcus neoformans*, *Journal of microbiology* 2018, 56, 65–71, DOI:10.1007/s12275-018-7475-y.

- [240] A. Kamizono, M. Nishizawa, Y. Teranishi, K. Murata and A. Kimura, Identification of a gene conferring resistance to zinc and cadmium ions in the yeast *Saccharomyces cerevisiae*, *Molecular & general genetics* : MGG 1989, 219, 161–167, DOI:10.1007/BF00261172.
- [241] S. Miyabe, S. Izawa and Y. Inoue, The Zrc1 is involved in zinc transport system between vacuole and cytosol in *Saccharomyces cerevisiae*, *Biochemical and biophysical research communications* 2001, 282, 79–83, DOI:10.1006/bbrc.2001.4522.
- [242] D. S. Conklin, J. A. McMaster, M. R. Culbertson, and C. Kung, COT1, a gene involved in cobalt accumulation in *Saccharomyces cerevisiae*, *Molecular and cellular biology* 1992, 12 3678–3688, DOI:10.1128/mcb.12.9.3678-3688.1992.
- [243] J. S. de Curcio, M. G. Silva, M. G. Silva Bailão, S. N. Báó, L. Casaletti, A. M. Bailão and C. M. de Almeida Soares, Identification of membrane proteome of *Paracoccidioides lutzii* and its regulation by zinc, *Future science OA* 2017, 3, FSO232, DOI:10.4155/fsoa-2017-0044.
- [244] S. du Pré, T. Dogra and W. W. J. van de Sande, The putative role of zinc homeostasis in grain formation by *Madurella mycetomatis* during mycetoma infection, *Fungal Biology Reviews*, 2022, 39, 73–82, DOI:10.1016/j.fbr.2021.12.002.
- [245] L. M. Ramsay and G. M. Gadd, Mutants of *Saccharomyces cerevisiae* defective in vacuolar function confirm a role for the vacuole in toxic metal ion detoxification, *FEMS microbiology letters* 1997, 152, 293–298, DOI:10.1111/j.1574-6968.1997.tb10442.x.
- [246] G. DalCorso, F. Martini, E. Fasani, A. Manara, G. Visioli and A. Furini, Enhancement of Zn tolerance and accumulation in plants mediated by the expression of *Saccharomyces cerevisiae* vacuolar transporter ZRC1, *Planta* 2021, 253, 117, DOI:10.1007/s00425-021-03634-z.
- [247] O. Kolaj-Robin, D. Russell, K. A. Hayes, J. T. Pembroke and T. Soulimane, Cation Diffusion Facilitator family: Structure and function, *FEBS letters* 2015, 589, 1283–1295, DOI:10.1016/j.febslet.2015.04.007.
- [248] H. Zhao and D. J. Eide, Zap1p, a metalloregulatory protein involved in zinc-responsive transcriptional regulation in *Saccharomyces cerevisiae*, *Molecular and cellular biology* 1997, 17, 5044–5052, DOI:10.1128/MCB.17.9.5044.
- [249] C. Y. Wu, A. J. Bird, L. M. Chung, M. A. Newton, D. R. Winge and D. J. Eide, Differential control of Zap1-regulated genes in response to zinc deficiency in *Saccharomyces cerevisiae*, *BMC genomics* 2008, 9, 370 DOI:10.1186/1471-2164-9-370.
- [250] H. Zhao, E. Butler, J. Rodgers, T. Spizzo, S. Duesterhoeft and D. Eide, Regulation of zinc homeostasis in yeast by binding of the ZAP1 transcriptional activator to zinc-responsive promoter elements, *The Journal of biological chemistry* 1998, 273, 28713–28720, DOI:10.1074/jbc.273.44.28713.
- [251] R. S. Gitan, H. Luo, J. Rodgers, M. Broderius and D. Eide, Zinc-induced inactivation of the yeast ZRT1 zinc transporter occurs through endocytosis and vacuolar degradation, *The Journal of biological chemistry* 1998, 273, 28617–28624, DOI:10.1074/jbc.273.44.28617.
- [252] S. Wilson and A. J. Bird, Zinc sensing and regulation in yeast model systems, *Archives of biochemistry and biophysics* 2016, 611, 30–36, DOI:10.1016/j.abb.2016.02.031.
- [253] M. North, Steffen, J., Loguinov, A. V., Zimmerman, G. R., Vulpe, C. D., Eide, D. J., Genome-wide functional profiling identifies genes and processes important for zinc-limited growth of *Saccharomyces cerevisiae*, *PLoS genetics* 2012, 8, e1002699,, DOI:10.1371/journal.pgen.1002699.
- [254] R. Schneider, Fogaça, N.deS., Kmetzsch, L., Schrank, A., Vainstein, M. H., Staats, C. C., Zap1 regulates zinc homeostasis and modulates virulence in *Cryptococcus gattii*. *PloS one* 2012, 7, e43773, DOI:10.1371/journal.pone.0043773.

- [255] M. A. Moreno, O. Ibrahim-Granet, R. Vicente-franqueira, J. Amich, P. Ave, F. Leal, J. P. Latgé and J. A. Calera, The regulation of zinc homeostasis by the ZafA transcriptional activator is essential for *Aspergillus fumigatus* virulence, *Molecular Microbiology*, 2007, 64, 1182–1197, DOI:10.1111/j.1365-2958.2007.05726.x.
- [256] M. J. Kim, Kil, M., Jung, J. H., Kim, J., Roles of Zinc-responsive transcription factor Csr1 in filamentous growth of the pathogenic Yeast *Candida albicans*, *Journal of microbiology and biotechnology* 2008, 18, 242–247.
- [257] C. J. Nobile, Nett, J. E., Hernday, A. D., Homann, O. R., Deneault, J. S., Nantel, A., Andes, D. R., Johnson, A. D., Mitchell, A. P., Biofilm matrix regulation by *Candida albicans* Zap1. *PLoS biology* 2009, 7, e1000133, DOI:10.1371/journal.pbio.1000133.
- [258] S. J. Dainty, Kennedy, C. A., Watt, S., Bähler, J., Whitehall, S. K., Response of *Schizosaccharomyces pombe* to zinc deficiency, *Eukaryotic cell* 2008, 7, 454–464, DOI:10.1128/EC.00408-07.
- [259] M. E. Corkins, May, M., Ehrensberger, K. M., Hu, Y. M., Liu, Y. H., Bloor, S. D., Jenkins, B., Runge, K. W., Bird, A. J., Zinc finger protein Loz1 is required for zinc-responsive regulation of gene expression in fission yeast,, *Proceedings of the National Academy of Sciences of the United States of America* 2013, 110, 15371–15376, DOI:doi.org/10.1073/pnas..
- [260] Y. Fang, Sugiura, R., Ma, Y., Yada-Matsushima, T., Umeno, H., Kuno, T., Cation diffusion facilitator Cis4 is implicated in Golgi membrane trafficking via regulating zinc homeostasis in fission yeast, *Molecular biology of the cell*, 19, 1295–20081303,, DOI:10.1091/mbc.e07-08-0805.
- [261] E. Luk, Carroll, M., Baker, M., Culotta, V. C., Manganese activation of superoxide dismutase 2 in *Saccharomyces cerevisiae* requires MTM1, a member of the mitochondrial carrier family,, *Proceedings of the National Academy of Sciences of the United States of America* 2003, 100, 10353–10357, DOI:10.1073/pnas.1632471100.
- [262] J. Bian, Wang, L., Wu, J., Simth, N., Zhang, L., Wang, Y., Wu, X., MTM1 plays an important role in the regulation of zinc tolerance in *Saccharomyces cerevisiae*,, *Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements* 2021, 66, 126759, DOI:10.1016/j.jtemb.2021..
- [263] A. Mikhaylina, A. Z. Ksibe, D. J. Scanlan and C. A. Blindauer, Bacterial zinc uptake regulator proteins and their regulons, *Biochemical Society transactions* 2018, 46, 983–1001, DOI:10.1042/BST20170228.
- [264] D. A. Capdevila, J. Wang and D. P. Giedroc, Bacterial Strategies to Maintain Zinc Metallostasis at the Host-Pathogen Interface, *The Journal of biological chemistry* 2016, 291, 20858–20868, DOI:10.1074/jbc.R116.742023.
- [265] H. Reyes-Caballero, G. C. Campanello and D. P. Giedroc, Metalloregulatory proteins: metal selectivity and allosteric switching, *Biophysical chemistry* 2011, 156, 103–114 DOI:10.1016/j.bpc.2011.03.010.
- [266] F. E. Jacobsen, Kazmierczak, K. M., Lisher, J. P., Winkler, M. E., Giedroc, D. P., Interplay between manganese and zinc homeostasis in the human pathogen *Streptococcus pneumoniae*, *Metallomics : integrated biometal science* 2011, 3, 38–41, DOI:10.1039/c0mt00050g.
- [267] D. K. Blencowe and A. P. Morby, Zn(II) metabolism in prokaryotes, *FEMS microbiology reviews* 2003, 27(2-3), 291–311, DOI:10.1016/S0168-6445(03)00041-X.
- [268] C. PetitClerc and C. Fecteau, Mechanism of action of Zn²⁺ and Mg²⁺ on rat placenta alkaline phosphatase. II. Studies on membrane-bound phosphatase in tissue sections and in whole placenta, *Canadian journal of biochemistry* 1977, 55, 474–478, DOI:10.1139/o77-066.
- [269] M. Cerasi, Liu, J. Z., Ammendola, S., Poe, A. J., Petrarca, P., Pesciaroli, M., Pasquali, P., Raffatellu, M., Battistoni, A., The ZupT transporter plays an important role in zinc homeostasis and contributes to *Salmonella enterica* virulence,, *Metallomics : integrated biometal science* 2014, 6, 845–853, DOI:10.1039/c3mt00352c.

- [270] C. A. Blindauer, Advances in the molecular understanding of biological zinc transport, *Chemical Communications* 2015, 51, 4544-4563, DOI:10.1039/C4CC10174J.
- [271] K. Hantke, Bacterial zinc uptake and regulators, *Current opinion in microbiology* 2005, 8, 196–202, DOI:10.1016/j.mib.2005.02.001.
- [272] H. Nanamiya, Akanuma, G., Natori, Y., Murayama, R., Kosono, S., Kudo, T., Kobayashi, K., Ogasawara, N., Park, S. M., Ochi, K., Kawamura, F., Zinc is a key factor in controlling alternation of two types of L31 protein in the *Bacillus subtilis* ribosome,, *Molecular microbiology* 2004, 52, 273-283, DOI:10.1111/j.1365-2958.2003.03972.x.
- [273] S. H. Choi, K. L. Lee, J. H. Shin, Y. B. Cho, S. S. Cha and J. H. Roe, Zinc-dependent regulation of zinc import and export genes by zur, *Nature Communications* 2017, 8, 1-11, DOI:10.1038/ncomms15812.
- [274] H. Reyes-Caballero, Guerra, A. J., Jacobsen, F. E., Kazmierczak, K. M., Cowart, D., Koppolu, U. M., Scott, R. A., Winkler, M. E., Giedroc, D. P., The metalloregulatory zinc site in *Streptococcus pneumoniae* AdcR, a zinc-activated MarR family repressor,, *Journal of molecular biology* 2010, 403, 197–216, DOI:10.1016/j.jmb.2010.08.030.
- [275] J. M. Argüello, D. Raimunda and M. González-Guerrero, Metal transport across biomembranes: emerging models for a distinct chemistry, *The Journal of biological chemistry* 2012, 287, 13510–13517, DOI:10.1074/jbc.R111.319343.
- [276] J. S. Klein and O. Lewinson, Bacterial ATP-driven transporters of transition metals: physiological roles, mechanisms of action, and roles in bacterial virulence, *Metallomics : integrated biometal science* 2011, 3, 1098–1108, DOI:10.1039/c1mt00073j.
- [277] S. Bhubhanil, Sittipo, P., Chaoprasid, P., Nookabkaew, S., Sukchawalit, R., Mongkolsuk, S., Control of zinc homeostasis in *Agrobacterium tumefaciens* via zur and the zinc uptake genes znuABC and zinT, *Microbiology* 2014, 160, 2452–2463, DOI:10.1099/mic.0.082446-0.
- [278] M. Cerasi, S. Ammendola and A. Battistoni, Competition for zinc binding in the host-pathogen interaction, *Frontiers in cellular and infection microbiology* 2013, 3, 108, DOI:10.3389/fcimb.2013.00108.
- [279] K. Hollenstein, R. J. Dawson and K. P. Locher, Structure and mechanism of ABC transporter proteins, *Current opinion in structural biology* 2007, 17, 412–418, DOI:10.1016/j.sbi.2007.07.003.
- [280] C. F. Higgins, ABC transporters: from microorganisms to man, *Annual review of cell biology* 1992, 8, 67–113, DOI:10.1146/annurev.cb.08.110192.000435.
- [281] A. L. Davidson, E. Dassa, C. Orelle and J. Chen, Structure, function, and evolution of bacterial ATP-binding cassette systems, *Microbiology and molecular biology reviews : MMBR* 2008, 72, 317–364, DOI:10.1128/MMBR.00031-07.
- [282] D. C. Rees, E. Johnson and O. Lewinson, ABC transporters: the power to change, *Nature reviews. Molecular cell biology* 2009, 10, 218–227, DOI:10.1038/nrm2646.
- [283] I. B. Holland and M. A. Blight, ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans, *Journal of molecular biology* 1999, 293, 381–399, DOI:10.1006/jmbi.1999.2993.
- [284] E. Schneider and S. Hunke, ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolyzing subunits/domains, *FEMS microbiology reviews* 1998, 22, 1–20, DOI:10.1111/j.1574-6976.1998.tb00358.x.
- [285] C. F. Higgins, ABC transporters: physiology, structure and mechanism--an overview, *Research in microbiology* 2001, 152, 205–210, DOI:10.1016/s0923-2508(01)01193-7.

- [286] C. F. Higgins, I. D. Hiles, G. P. Salmond, D. R. Gill, J. A. Downie, I. J. Evans, I. B. Holland, L. Gray, S. D. Buckel and A. W. Bell, A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria, *Nature* 1986, 323, 448–450, DOI:10.1038/323448a0.
- [287] S. Silver and L. T. Phung, A bacterial view of the periodic table: genes and proteins for toxic inorganic ions, *Journal of industrial microbiology & biotechnology* 2005, 32, 587–605, DOI:10.1007/s10295-005-0019-6.
- [288] S. Wilkens, Structure and mechanism of ABC transporters, *F1000prime reports* 2015, 7, 14. DOI:10.12703/P7-14.
- [289] P. L. Pedersen, Transport ATPases into the year 2008: a brief overview related to types, structures, functions and roles in health and disease, *Journal of bioenergetics and biomembranes* 2007, 39, 349–355, DOI:10.1007/s10863-007-9123-9.
- [290] J. Stefková, R. Poledne and J. A. Hubáček, ATP-binding cassette (ABC) transporters in human metabolism and diseases, *Physiological research* 2004, 53, 235–243.
- [291] I. D. Hiles, M. P. Gallagher, D. J. Jamieson and C. F. Higgins, Molecular characterization of the oligopeptide permease of *Salmonella typhimurium*, *Journal of molecular biology* 1987, 195, 125–142, DOI:10.1016/0022-2836(87)90332-9.
- [292] M. Saraste, P. R. Sibbald and A. Wittinghofer, The P-loop--a common motif in ATP- and GTP-binding proteins, *Trends in biochemical sciences* 1990, 15, 430–434, DOI:10.1016/0968-0004(90)90281-f.
- [293] S. C. Hyde, Emsley, P., Hartshorn, M. J., Mimmack, M. M., Gileadi, U., Pearce, S. R., Gallagher, M. P., Gill, D. R., Hubbard, R. E., Higgins, C, Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport,, *Nature* 1990, 346, 362–365, DOI:10.1038/346362a0.
- [294] G. F. Ames, C. S. Mimura and V. Shyamala, Bacterial periplasmic permeases belong to a family of transport proteins operating from *Escherichia coli* to human: Traffic ATPases, *FEMS microbiology reviews* 1990, 6, 429–446, DOI:10.1111/j.1574-6968.1990.tb04110.x.
- [295] G. Aparicio, A. Buche, C. Méndez and J. A. Salas, Characterization of the ATPase activity of the N-terminal nucleotide binding domain of an ABC transporter involved in oleandomycin secretion by *Streptomyces antibioticus*, *FEMS microbiology letters* 1996, 141, 157–162, DOI:10.1111/j.1574-6968.19.
- [296] A. L. Davidson and H. Nikaido, Purification and characterization of the membrane-associated components of the maltose transport system from *Escherichia coli*, *The Journal of biological chemistry* 1991, 266, 8946–8951.
- [297] G. Obmolova, C. Ban, P. Hsieh and W. Yang, Crystal structures of mismatch repair protein MutS and its complex with a substrate DNA, *Nature* 2000, 407, 703–710, DOI:10.1038/35037509.
- [298] I. L. Urbatsch, M. K. al-Shawi and A. E. Senior, Characterization of the ATPase activity of purified Chinese hamster P-glycoprotein, *Biochemistry* 1994, 33, 7069–7076, DOI:10.1021/bi00189a008.
- [299] C. Randak, Neth, P., Auerswald, E. A., Eckerskorn, C., Assfalg-Machleidt, I., Machleidt, W., A recombinant polypeptide model of the second nucleotide-binding fold of the cystic fibrosis transmembrane conductance regulator functions as an active ATPase, GTPase ,, and adenylate kinase, *FEBS letters* 1997, 410, 180–186, DOI:10.1016/s0014-579.
- [300] S. Choi and A. J. Bird, Zinc'ing sensibly: controlling zinc homeostasis at the transcriptional level, *Metallomics*, 2014,6, 1198-1215, DOI:10.1039/C4MT00064A.
- [301] P. Petrarca, S. Ammendola, P. Pasquali and A. Battistoni, The Zur-regulated ZinT protein is an auxiliary component of the high-affinity ZnuABC zinc transporter that facilitates metal recruitment during severe zinc shortage, *Journal of bacteriology* 2010, 192, 1553–1564, DOI:10.1128/JB.01310-09.

- [302] S. I. Patzer and K. Hantke, The ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*, *Molecular microbiology* 1998, 28, 1199–1210, DOI:10.1046/j.1365-2958.1998.00883.x.
- [303] K. Hantke, Iron and metal regulation in bacteria, *Current opinion in microbiology* 2001 4, 172–177, DOI:10.1016/s1369-5274(00)00184-3.
- [304] M. Gopalani, Dhiman, A., Rahi, A., Kandari, D., Bhatnagar, R., Identification, Functional Characterization and Regulon Prediction of a Novel Two Component System Comprising BAS0540-BAS0541 of *Bacillus anthracis*, *PloS one* 2019, 11, e0158895,, DOI:10.1371/journal.pone.0158895.
- [305] A. G. Bobrov, Kirillina, O., Fetherston, J. D., Miller, M. C., Burlison, J. A., Perry, R. D., The *Yersinia pestis* siderophore, yersiniabactin, and the ZnuABC system both contribute to zinc acquisition and the development of lethal septicemic plague in mice,, *Molecular microbiology* 2014, 93, 759–775, DOI:10.1111/mmi.12693.
- [306] R. Gabbianelli, Scotti, R., Ammendola, S., Petrarca, P., Nicolini, L., Battistoni, A., Role of ZnuABC and ZinT in *Escherichia coli* O157:H7 zinc acquisition and interaction with epithelial cells, *BMC microbiology* 2011, 11, 36, DOI:10.1186/1471-2180-11-36.
- [307] A. I. Graham, Hunt, S., Stokes, S. L., Bramall, N., Bunch, J., Cox, A. G., McLeod, C. W., Poole, R. K., Severe zinc depletion of *Escherichia coli*: roles for high affinity zinc binding by ZinT, zinc transport and zinc-independent proteins,, *The Journal of biological chemistry* 2009, 284, 18377–18389, DOI:10.1074/jbc.M109.001503.
- [308] Z. Ma, F. E. Jacobsen and D. P. Giedroc, Coordination chemistry of bacterial metal transport and sensing, *Chemical reviews* 2009, 109, 4644–4681, DOI:10.1021/cr900077w.
- [309] M. F. Fillat, The FUR (ferric uptake regulator) superfamily: diversity and versatility of key transcriptional regulators, *Archives of biochemistry and biophysics* 2014, 546, 41–52, DOI:10.1016/j.abb.2014.01.029.
- [310] B. A. Gilston, Wang, S., Marcus, M. D., Canalizo-Hernández, M. A., Swindell, E. P., Xue, Y., Mondragón, A., O'Halloran, T. V., Structural and mechanistic basis of zinc regulation across the *E. coli* Zur regulon,, *PLoS biology* 2014, 12, e1001987, DOI:10.1371/journal.pbio.1001987.
- [311] D. Lucarelli, Russo, S., Garman, E., Milano, A., Meyer-Klaucke, W., Pohl, E., Crystal structure and function of the zinc uptake regulator FurB from *Mycobacterium tuberculosis*, *The Journal of biological chemistry* 2007, 282, 9914–9922, DOI:10.1074/jbc.M609974200.
- [312] L. Escolar, J. Pérez-Martín and V. de Lorenzo, Opening the iron box: transcriptional metalloregulation by the Fur protein, *Journal of bacteriology* 1999, 181, 6223–6229, DOI:10.1128/JB.181.20.6223-6229.1999.
- [313] B. E. Ahn, B. E., Cha, J., Lee, E. J., Han, A. R., Thompson, C. J., Roe, J. H., Nur, a nickel-responsive regulator of the Fur family, regulates superoxide dismutases and nickel transport in *Streptomyces coelicolor*, *Molecular microbiology* 2006, 59, 1848–1858,, DOI:10.1111/j.1365-2958.2006.05065.x.
- [314] E. Díaz-Mireles, Wexler, M., Sawers, G., Bellini, D., Todd, J. D., Johnston, A. W. B., The Fur-like protein Mur of *Rhizobium leguminosarum* is a Mn(2+)-responsive transcriptional regulator, *Microbiology* 2004, 150, 1447–1456, DOI:10.1099/mic.0.26961-0.
- [315] D. A. Traoré, El Ghazouani, A., Ilango, S., Dupuy, J., Jacquamet, L., Ferrer, J. L., Caux-Thang, C., Duarte, V., Latou J. M., Crystal structure of the apo-PerR-Zn protein from *Bacillus subtilis*, *Molecular microbiology* 2006, 61, 1211–1219,, DOI:10.1111/j.1365-2958.2006.05313.x.
- [316] L. Jacquamet, Traoré, D. A., Ferrer, J. L., Proux, O., Testemale, D., Hazemann, J. L., Nazarenko, E., El Ghazouani, A., Caux-Thang, C., Duarte, V., Latour, J. M., Structural characterization of the active form of PerR: insights into the, metal-

induced activation of PerR and Fur protei for DNA binding, *Molecular microbiology* 2009, 73, 20–31, DOI:10.1111/j.1365-2958.2009.06753.x.

- [317] C. M. Moore and J. D. Helmann, Metal ion homeostasis in *Bacillus subtilis*, *Current opinion in microbiology* 2005, 8, 188–195, DOI:10.1016/j.mib.2005.02.007.
- [318] D. W. Ellison and V. L. Miller, Regulation of virulence by members of the MarR/SlyA family, *Current opinion in microbiology* 2006, 9, 153–159, DOI:10.1016/j.mib.2006.02.003.
- [319] S. Shafeeq, O. P. Kuipers and T. G. Kloosterman, The role of zinc in the interplay between pathogenic streptococci and their hosts, *Molecular Microbiology*, 2013, 88, 1047–1057, DOI:10.1111/mmi.12256.
- [320] A. J. Guerra, C. E. Dann and D. P. Giedroc, Crystal structure of the zinc-dependent MarR family transcriptional regulator AdcR in the Zn(II)-bound state, *Journal of the American Chemical Society* 2011, 133, 19614–19617, DOI:10.1021/ja2080532.
- [321] M. Sanson, Makthal, N., Flores, A. R., Olsen, R. J., Musser, J. M., Kumaraswami, M., Adhesin competence repressor (AdcR) from *Streptococcus pyogenes* controls adaptive responses to zinc limitation and contributes to virulence,, *Nucleic acids research* 2015, 43, 418–432, DOI:10.1093/nar/gku1304.
- [322] C. V. Rosadini, Gawronski, J. D., Raimunda, D., Argüello, J. M., Akerley, B. J., A novel zinc binding system, ZevAB, is critical for survival of nontypeable *Haemophilus influenzae* in a murine lung infection model, *Infection and immunity* 2011, 79, 3366–3376,, DOI:10.1128/IAI.05135-11.
- [323] K. Dalet, E. Guoin, Y. Cenatiempo, P. Cossart and Y. Héchar, Characterisation of a new operon encoding a Zur-like protein and an associated ABC zinc permease in *Listeria monocytogenes*, *FEMS microbiology letters* 1999, 174, 111–116, DOI:10.1111/j.1574-6968.1999.tb13556.x.
- [324] D. Corbett, Wang, J., Schuler, S., Lopez-Castejon, G., Glenn, S., Brough, D., Andrew, P. W., Cavet, J. S., Roberts, I. S., Two zinc uptake systems contribute to the full virulence of *Listeria monocytogenes* during growth in vitro and in vivo,, *Infection and immunity* 2012, 80, 14–21, DOI:10.1128/IAI.05904-11.
- [325] M. L. Gueriot, The ZIP family of metal transporters, *Biochimica et biophysica acta* 2000, 1465, 190–198, DOI:10.1016/s0005-2736(00)00138-3.
- [326] D. Wang, O. Hosteen and C. A. Fierke, ZntR-mediated transcription of zntA responds to nanomolar intracellular free zinc, *Journal of inorganic biochemistry* 2012, 111, 173–181, DOI:10.1016/j.jinorgbio.2012.02.008.
- [327] N. E. Grossoehme, S. Akilesh, M. L. Gueriot and D. E. Wilcox, Metal-binding thermodynamics of the histidine-rich sequence from the metal-transport protein IRT1 of *Arabidopsis thaliana*, *Inorganic chemistry* 2006, 45, 8500–8508, DOI:10.1021/ic0606431.
- [328] D. J. Eide, The Zip Family of Zinc Transporters, *Molecular Biology Intelligence Unit* 2005, Chapter 35, 261–264, DOI:10.1007/0-387-27421-9_35.
- [329] T. P. Ajeesh Krishna, Maharajan, T., Victor Roch, G., Ignacimuthu, S., Antony Ceasar, S., Structure, Function, Regulation and Phylogenetic Relationship of ZIP Family Transporters of Plants, *Frontiers in plant science* 2020, 11, 662, DOI:10.3389/fpls.2020.00662.
- [330] J. Hu, Toward unzipping the ZIP metal transporters: structure, evolution, and implications on drug discovery against cancer, *The FEBS journal* 2021, 288, 5805–5825, DOI:10.1111/febs.15658.

- [331] C. A. Cotrim, R. J. Jarrott, J. L. Martin and D. Drew, A structural overview of the zinc transporters in the cation diffusion facilitator family, *Acta crystallographica. Section D, Structural biology* 2019, 75, 357–367, DOI:10.1107/S2059798319003814.
- [332] B. H. Eng, M. L. Guerinot, D. Eide and M. H. Saier Jr., Sequence Analyses and Phylogenetic Characterization of the ZIP Family of Metal Ion, *The Journal of Membrane Biology* 1998, 166, 1–7.
- [333] E. H. Kim, Nies, D. H., McEvoy, M. M., Rensing, C., Switch or funnel: how RND-type transport systems control periplasmic metal homeostasis, *Journal of bacteriology* 2011, 193, 2381–2387, DOI:10.1128/JB.01323-10.
- [334] M. Bublitz, J. P. Morth and P. Nissen, P-type ATPases at a glance, *Journal of cell science* 2016, 124(Pt 15), 2515–2519, DOI:10.1242/jcs.088716.
- [335] X. C. Zhang and H. Zhang, P-type ATPases use a domain-association mechanism to couple ATP hydrolysis to conformational change, *Biophysics Reports* 2019, 5, 167–175, DOI:10.1007/s41048-019-0087-1.
- [336] M. Palmgren, P-type ATPases: Many more enigmas left to solve, *The Journal of biological chemistry* 2021, 299, DOI:10.1016/j.jbc.2023.105352.
- [337] W. Kühlbrandt, Biology, structure and mechanism of P-type ATPases. *Nature reviews, Molecular cell biology* 2004, 5, 282–295, DOI:10.1038/nrm1354.
- [338] C. Toyoshima, M. Nakasako, H. Nomura and H. Ogawa, Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution, *Nature* 2000, 405, 647–655, DOI:10.1038/35015017.
- [339] M. Dyla, M. Kjærgaard, H. Poulsen and P. Nissen, Structure and Mechanism of P-Type ATPase Ion Pumps, *Annual review of biochemistry* 2020, 89, 583–603, DOI:10.1146/annurev-biochem-010611-112801.
- [340] O. Jardetzky, Simple allosteric model for membrane pumps, *Nature* 1966, 211, 969–970, DOI:10.1038/211969a0.
- [341] P. L. Pedersen and E. Carafoli, Ion motive ATPases. I. Ubiquity, properties, and significance to cell function, *Trends in Biochemical Sciences* 1987, 12, 146–150, DOI:10.1016/0968-0004(87)90071-5.
- [342] J. M. Argüello, E. Eren and M. González-Guerrero, The structure and function of heavy metal transport P1B-ATPases, *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* 2007, 20, 233–248, DOI:10.1007/s10534-006-9055-6.
- [343] M. Dyla, Terry, D. S., Kjaergaard, M., Sørensen, T. L., Lauwring Andersen, J., Andersen, J. P., Rohde Knudsen, C., Altman, R. B., Nissen, P., Blanchard, S. C. , Dynamics of P-type ATPase transport revealed by single-molecule FRET, *Nature* 2017, 551, 346–351, DOI:10.1038/nature24296.
- [344] P. L. Jorgensen, Purification and characterization of (Na⁺, K⁺)-ATPase. V. Conformational changes in the enzyme Transitions between the Na-form and the K-form studied with tryptic digestion as a tool, *Biochimica et biophysica acta* 1975, 401, 399–415, DOI:10.1016/0005-2736(75)90239-4.
- [345] T. L. Sørensen, J. V. Møller and P. Nissen, Phosphoryl transfer and calcium ion occlusion in the calcium pump, *Science* 2004, 304, 1672–1675, DOI:10.1126/science.1099366.
- [346] C. Toyoshima and T. Mizutani, Crystal structure of the calcium pump with a bound ATP analogue, *Nature* 2004, 430, 529–535, DOI:10.1038/nature02680.
- [347] C. Olesen, M. Picard, A. M. Winther, C. Gyrupe, J. P. Morth, C. Oxvig, J. V. Møller and P. Nissen, The structural basis of calcium transport by the calcium pump, *Nature* 2007, 450, 1036–1042, DOI:10.1038/nature06418.

- [348] B. Mitra and R. Sharma, The cysteine-rich amino-terminal domain of ZntA, a Pb(II)/Zn(II)/Cd(II)-translocating ATPase from *Escherichia coli*, is not essential for its function, *Biochemistry* 2001, 40(25), 7694–7699, DOI:10.1021/bi010576g.
- [349] D. Raimunda, P. Subramanian, T. Stemmler and J. M. Argüello, A tetrahedral coordination of Zinc during transmembrane transport by P-type Zn(2+)-ATPases, *Biochimica et biophysica acta* 2012, 1818, 1374–1377, DOI:10.1016/j.bbamem.2012.02.020.
- [350] G. Hu and J. W. Kronstad, A putative P-type ATPase, Apt1, is involved in stress tolerance and virulence in *Cryptococcus neoformans*, *Eukaryotic cell* 2010, 9, 74–83, DOI:10.1128/EC.00289-09.
- [351] J. L. Gustin, M. J. Zanis and D. E. Salt, Structure and evolution of the plant cation diffusion facilitator family of ion transporters, *BMC evolutionary biology* 2011, 11, 76, DOI:10.1186/1471-2148-11-76.
- [352] S. Barber-Zucker, A. Shahar, S. Kolusheva and R. Zarivach, The metal binding site composition of the cation diffusion facilitator protein MamM cytoplasmic domain impacts its metal responsivity, *Scientific reports* 2020, 10, 14022, DOI:10.1038/s41598-020-71036-4.
- [353] C. J. Haney, G. Grass, S. Franke and C. Rensing, New developments in the understanding of the cation diffusion facilitator family, *Journal of industrial microbiology & biotechnology* 2005, 32, 215–226, DOI:10.1007/s10295-005-0224-3.
- [354] D. H. Nies and S. Silver, Ion efflux systems involved in bacterial metal resistances, *Journal of industrial microbiology* 1995, 14, 186–199, DOI:10.1007/BF01569902.
- [355] C. Cubillas, P. Vinuesa, M. L. Tabche and A. García-de los Santos, Phylogenomic analysis of Cation Diffusion Facilitator proteins uncovers Ni²⁺/Co²⁺ transporters, *Metallomics : integrated biometal science* 2013, 5, 1634–1643, DOI:10.1039/c3mt00204g.
- [356] A. Anton, C. Grosse, J. Reissmann, T. Pribyl and D. H. Nies, CzcD is a heavy metal ion transporter involved in regulation of heavy metal resistance in *Ralstonia* sp. strain CH34, *Journal of bacteriology* 1999, 181, 6876–6881, DOI:10.1128/JB.181.22.6876-6881.1999.
- [357] T. Kambe, H. Narita, Y. Yamaguchi-Iwai, J. Hirose, T. Amano, N. Sugiura, R. Sasaki, K. Mori, T. Iwanaga and M. Nagao, Cloning and characterization of a novel mammalian zinc transporter, zinc transporter 5, abundantly expressed in pancreatic beta cells, *The Journal of biological chemistry* 2002, 277, 19049–19055, DOI:10.1074/jbc.M200910200.
- [358] M. Lu, J. Chai and D. Fu, Structural basis for autoregulation of the zinc transporter YiiP, *Nature structural & molecular biology* 2009, 16, 1063–1067, DOI:10.1038/nsmb.1662.
- [359] M. Lu and D. Fu, Structure of the zinc transporter YiiP, *Science* 2007, 317, 1746–1748, DOI:10.1126/science.1143748.
- [360] M. L. Lopez-Redondo, N. Coudray, Z. Zhang, J. Alexopoulos and D. L. Stokes, Structural basis for the alternating access mechanism of the cation diffusion facilitator YiiP, *Proceedings of the National Academy of Sciences of the United States of America* 2018, 115, 3042–3047, DOI:10.1073/pnas.1715051115.
- [361] T. Higuchi, M. Hattori, Y. Tanaka, R. Ishitani and O. Nureki, Crystal structure of the cytosolic domain of the cation diffusion facilitator family protein, *Proteins* 2009, 76, 768–771, DOI:10.1002/prot.22444.
- [362] H. T. Lei, J. R. Bolla, N. R. Bishop, C. C. Su and E. W. Yu, Crystal structures of CusC reveal conformational changes accompanying folding and transmembrane channel formation, *Journal of molecular biology* 2014, 426, 403–411, DOI:10.1016/j.jmb.2013.09.042.

- [363] M. Goldberg, T. Pribyl, S. Juhnke and D. H. Nies, Energetics and topology of CzcA, a cation/proton antiporter of the resistance-nodulation-cell division protein family, *The Journal of biological chemistry* 1999, 274, 26065–26070, DOI:10.1074/jbc.274.37.26065.
- [364] I. G. Auda, I. M. Ali Salman and J. G. Odah, Efflux pumps of Gram-negative bacteria in brief, *Gene Reports* 2020, 20, 100666, DOI:10.1016/j.genrep.2020.100666.
- [365] P. Hinchliffe, M. F. Symmons, C. Hughes and V. Koronakis, Structure and operation of bacterial tripartite pumps, *Annual review of microbiology* 2014, 67, 221–242, DOI:10.1146/annurev-micro-092412-155718.
- [366] H. Nikaido and Y. Takatsuka, Mechanisms of RND multidrug efflux pumps, *Biochimica et biophysica acta* 2009, 1794, 769–781, DOI:10.1016/j.bbapap.2008.10.004.
- [367] D. M. Fernando and A. Kumar, Resistance-Nodulation-Division Multidrug Efflux Pumps in Gram-Negative Bacteria: Role in Virulence, *Antibiotics* 2013, 2, 163–181, DOI:10.3390/antibiotics2010163.
- [368] L. Fernández and R. E. W. Hancock, Adaptive and Mutational Resistance: Role of Porins and Efflux Pumps in Drug Resistance, *Clinical Microbiology Reviews* 2012, 25, 163, DOI:10.1128/cmr.00043-12.
- [369] I. T. Paulsen, J. H. Park, P. S. Choi and M. H. J. Saier, A family of gram-negative bacterial outer membrane factors that function in the export of proteins, carbohydrates, drugs and heavy metals from gram-negative bacteria, *FEMS microbiology letters* 1997, 156, 1–8,, DOI:10.1111/j.1574-6968.1997.tb12697.x.
- [370] T. Dinh, I. T. Paulsen and M. H. J. Saier, A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of gram-negative bacteria, *Journal of bacteriology* 1994, 176, 3825–3831, DOI:10.1128/jb.176.13.3825-3831.1994.
- [371] X. Z. Li, P. Plésiat and H. Nikaido, The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria, *Clinical microbiology reviews* 2015, 28, 337–418, DOI:10.1128/CMR.00117-14.
- [372] H. I. Zgurskaya, G. Krishnamoorthy, A. Ntrel and S. Lu, Mechanism and Function of the Outer Membrane Channel TolC in Multidrug Resistance and Physiology of Enterobacteria, *Frontiers in microbiology* 2011, 2, 189, DOI:10.3389/fmicb.2011.00189.
- [373] H. Nikaido, RND transporters in the living world, *Research in microbiology* 2018, 169, 363–371, DOI:10.1016/j.resmic.2018.03.001.
- [374] D. Ma, D. A. Cook, M. Alberti, N. G. Pon, H. Nikaido and J. E. Hearst, Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*, *Journal of bacteriology* 1993, 175, 6299–6313, DOI:10.1128/jb.175.19.6299-6313.1993.
- [375] S. Murakami, R. Nakashima, E. Yamashita and A. Yamaguchi, Crystal structure of bacterial multidrug efflux transporter AcrB, *Nature* 2002, 419, 587–593, DOI:10.1038/nature01050.
- [376] F. Long, C. C. Su, M. T. Zimmermann, S. E. Boyken, K. R. Rajashankar, R. L. Jernigan and E. W. Yu, Crystal structures of the CusA efflux pump suggest methionine-mediated metal transport, *Nature* 2010, 467, 484–488, DOI:10.1038/nature09395.
- [377] A. Legatzki, G. Grass, A. Anton, C. Rensing and D. H. Nies, Interplay of the Czc system and two P-type ATPases in conferring metal resistance to *Ralstonia metallidurans*, *Journal of bacteriology* 2003, 185, 4354–4361, DOI:10.1128/JB.185.15.4354-4361.2003.

- [378] Z. Shen, X. Y. Pu and Q. Zhang, Salicylate functions as an efflux pump inducer and promotes the emergence of fluoroquinolone-resistant *Campylobacter jejuni* mutants, *Applied and environmental microbiology* 2011, 77, 7128–7133, DOI:10.1128/AEM.00763-11.
- [379] H. Wang, J. L. Dzink-Fox, M. Chen and S. B. Levy, Genetic characterization of highly fluoroquinolone-resistant clinical *Escherichia coli* strains from China: role of *acrR* mutations, *Antimicrobial agents and chemotherapy* 2001, 45, 1515–1521, DOI:10.1128/AAC.45.5.1515-1521.2001.
- [380] E. Y. Rosenberg, D. Bertenthal, M. L. Nilles, K. P. Bertrand and H. Nikaido, Bile salts and fatty acids induce the expression of *Escherichia coli* AcrAB multidrug efflux pump through their interaction with Rob regulatory protein, *Molecular microbiology* 2003, 48(6), 1609–1619, DOI:10.1046/j.1365-2958.2003.03531.x.
- [381] A. J. Worlock and R. L. Smith, ZntB is a novel Zn²⁺ transporter in *Salmonella enterica* serovar Typhimurium, *Journal of bacteriology* 2002, 184, 4369–4373, DOI:10.1128/JB.184.16.4369-4373.2002.
- [382] S. Leonhartsberger, A. Huber, F. Lottspeich and A. Böck, The *hydH/G* Genes from *Escherichia coli* code for a zinc and lead responsive two-component regulatory system, *Journal of molecular biology* 2001, 307, 93–105, DOI:10.1006/jmbi.2000.4451.
- [383] K. J. Kwon-Chung and J. A. Sugui, *Aspergillus fumigatus*—What makes the species a ubiquitous human fungal pathogen?, *PLOS Pathogens*, 2013, 9, e1003743, DOI:10.1371/journal.ppat.1003743.
- [384] G. Maschmeyer, A. Haas and O. A. Cornely, Invasive aspergillosis: Epidemiology, diagnosis and management in immunocompromised patients, *Drugs* 2007, 67, 1567–1601, DOI:10.2165/00003495-200767110-00004.
- [385] V. Balloy and M. Chignard, The innate immune response to *Aspergillus fumigatus*, *Microbes and Infection*, 2009, 11, 919–927, DOI:10.1016/j.micinf.2009.07.002.
- [386] A. McCormick, J. Loeffler and F. Ebel, *Aspergillus fumigatus*: Contours of an opportunistic human pathogen, *Cellular Microbiology*, 2010, 12, 1535–1543, DOI:10.1111/j.1462-5822.2010.01517.x.
- [387] A. Abad, J. V. Fernández-Molina, J. Bikandi, A. Ramírez, J. Margareto, J. Sendino, F. L. Hernando, J. Pontoñ, J. Garaizar and A. Rementeria, What makes *Aspergillus fumigatus* a successful pathogen?, *Revista Iberoamericana de Micología*, 2010, 27, 155–182, DOI:10.1016/j.riam.2010.10.003.
- [388] M. Kousha, R. Tadi and A. O. Soubani, Pulmonary aspergillosis: a clinical review, *European Respiratory Review*, 2011, 20, 156–174, DOI:10.1183/09059180.00001011.
- [389] S. Seyedmousavi, J. Guillot, P. Arne, G. S. de Hoog, J. W. Mouton, M. J. G. Melchers and P. E. Verweij, *Aspergillus* and aspergilloses in wild and domestic animals: a global health concern with parallels to human disease, *Medical Mycology*, 2015, 00, 1–33, DOI:10.1093/mmy/myv067.
- [390] S. J. Park and B. Mehrad, Innate immunity to *Aspergillus* species, *Clinical Microbiology Reviews*, 2009, 22, 535-551, DOI:10.1128/CMR.00014-09.
- [391] J. P. Latgé, *Aspergillus fumigatus* and aspergillosis, *Clinical Microbiology Reviews*, 1999, 12, 310–350, DOI:.
- [392] G. Greub and J. Bille, *Aspergillus* species isolated from clinical specimens: suggested clinical and microbiological criteria to determine significance, *Clinical Microbiology and Infection*, 1998, 4, 710-716, DOI:10.1111/j.1469-0691.1998.tb00656.x .
- [393] P. E. Verweij, S. J. Howard, W. J. G. Melchers and D. W. Denning, Azole-resistance in *Aspergillus*: proposed nomenclature and breakpoints, *Drug Resistance Updates*, 2009, 12, 141-147, DOI:10.1016/j.drug.2009.09.002.

- [394] T. Roemer and D. J. Krysan, Antifungal drug development: Challenges, unmet clinical needs, and new approaches, *Cold Spring Harbor Perspectives in Medicine*, 2014, 4, a019703, DOI:10.1101/cshperspect.a019703.
- [395] D. W. Denning and M. J. Bromley, Infectious Disease. How to bolster the antifungal pipeline, *Science* 2015, 347, 1414–1416, DOI:10.1126/science.aaa6097.
- [396] N. Robbins, T. Caplan and L. E. Cowen, Molecular Evolution of Antifungal Drug Resistance, *Annual Review of Microbiology*, 2017, 71, 753–775, DOI:10.1146/annurev-micro-030117-020345.
- [397] J. L. Xie, E. J. Polvi, T. Shekhar-Guturja and L. E. Cowen, Elucidating drug resistance in human fungal pathogens, *Future Microbiology*, 2014, 9, 523–542, DOI:10.2217/fmb.14.18.
- [398] E. Snelders, H. A. van der Lee, J. Kuijpers, A. J. Rijs, J. Varga, R. A. Samson, E. Mellado, A. R. T. Donders, W. J. G. Melchers and P. E. Verweij, Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism, *PLOS Medicine*, 2008, 5, e219, DOI:10.1371/journal.pmed.0050219.
- [399] S. F. Hurst, E. L. Berkow, K. L. Stevenson, A. P. Litvintseva and S. R. Lockhart, *Aspergillus fumigatus* from the environment in the south-eastern USA, *Journal of Antimicrobial Chemotherapy*, 2017, 72, 2443–2446, DOI:10.1093/jac/dkx168.
- [400] G. R. Magnuson, J. M. Puvathingal and W. J. Ray, The concentration of free Mg²⁺ and free Zn²⁺ in equine blood plasma, *Journal of Biological Chemistry*, 1987, 262, 11140–11148, DOI:.
- [401] T. R. Sandrin and R. M. Maier, Impact of metals on the biodegradation of organic pollutants, *Environmental Health Perspective*, 2003, 111, 1093–1101, DOI:10.1289/ehp.5840.
- [402] C. E. Martínez and H. L. Motto, Solubility of lead, zinc and copper added to mineral soils, *Environmental Pollution*, 2000, 107, 153–158, DOI:10.1016/S0269-7491(99)00111-6.
- [403] S. D. Willger , N. Grahl and R. A. Cramer Jr., *Aspergillus fumigatus* metabolism: clues to mechanisms of in vivo fungal growth and virulence, *Medical Mycology*, 2009, 47, 72–79, DOI:10.1080/13693780802455313.
- [404] N. T. Basta, J. A. Ryan and R. L. Chaney, Trace element chemistry in residual-treated soil: key concepts and metal bioavailability, *Journal of Environmental Quality*, 2005 34, 49–63, DOI:10.2134/jeq2005.0049dup.
- [405] J. W. Foote and H. T. Delves, Albumin bound and alpha 2-macroglobulin bound zinc concentrations in the sera of healthy adults, *Journal of Clinical Pathology*, 1984, 37, 1050–1054, DOI:10.1136/jcp.37.9.1050.
- [406] V. Iyengar and J. Woittiez, Trace elements in human clinical specimens: Evaluation of literature data to identify reference values, *Clinical Chemistry*, 1988, 34, 474–481, DOI:10.1093/clinchem/34.3.474.
- [407] J. W. Foote and H. T. Delves, Determination of nonprotein- bound zinc in human serum using ultrafiltration and atomic absorption spectrometry with electrothermal atomisation, *Analyst*, 1988, 113, 911–915, DOI:10.1039/an9881300911.
- [408] H. C. H. Hahne and W. Kroontje, Significance of pH and chloride concentration on behaviour of heavy metal pollutants: mercury (II), cadmium(II), zinc (II), and lead (II), *Journal of Environmental Quality*, 1973, 2, 444–450, DOI:.
- [409] D. Wilson, An evolutionary perspective on zinc uptake by human fungal pathogens, *Metallomics*, 2015, 7, 979–985, DOI:10.1039/c4mt00331d.
- [410] J. Amich, R. Vicentefranqueira, F. Leal and J. A. Calera, *Aspergillus fumigatus* survival in alkaline and extreme zinc-limiting environments relies on the induction of a zinc homeostasis system encoded by the *zrfC* and *aspf2* genes, *Eukaryot Cell*, 2010, 9, 424–437, DOI:10.1128/EC.00348-09.

- [411] M. Blatzer and J. P. Latgé, Metal-homeostasis in the pathobiology of the opportunistic human fungal pathogen *Aspergillus fumigatus*, *Current Opinion in Microbiology*, 2017, 40, 152–159, DOI:10.1016/j.mib.2017.11.015.
- [412] R. Vicentefranqueira, J. Amich, P. Laskaris, O. Ibrahim-Granet, J. P. Latgé, H. Toledo, F. Leal and J. A. Calera, Targeting zinc homeostasis to combat *Aspergillus fumigatus* infections, *Frontiers in Microbiology*. 2015, 6, 160, DOI:3389/fmicb.2015.00160.
- [413] G. von Heijne, Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule, *Journal of Molecular Biology*, 1992, 225, 487–494, DOI:10.1016/0022-2836(92)90934-c.
- [414] D. S. Auld, Zinc coordination sphere in biochemical zinc sites, *Biometals*, 2001, 14, 271–313, DOI:10.1023/a:1012976615056.
- [415] K. M. Taylor and R. I. Nicholson, The LZT proteins; the LIV-1 subfamily of zinc transporters, *Biochimica et Biophysica Acta*, 2003, 1611, 16-30, DOI:10.1016/s0005-2736(03)00048-8.
- [416] J. D. Thompson, T. J. Gibson, F. Plewniak, F. Jeanmougin and D. G. Higgins, The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, *Nucleic Acids Research*, 1997, 25, 4876–4882, DOI:10.1093/nar/25.24.4876.
- [417] A. M. Traynor, R. A. Owens, C. M. Coughlin, M. C. Holton, G. W. Jones, J. A. Calera and S. Doyle, At the metal-metabolite interface in *Aspergillus fumigatus*: Towards untangling the intersecting roles of zinc and gliotoxin, *Microbiology*, 2021, 167, 001106, DOI:10.1099/mic.0.001106.
- [418] B. Banerjee, P. A. Greenberger, J. N. Fink and V. P. Kurup, Immunological characterization of Asp2, a major allergen from *Aspergillus fumigatus* associated with allergic bronchopulmonary aspergillosis., *Infection and Immunity*, 1998, 66, 5175–5182, DOI:10.1128/iai.66.11.5175-5182.1998.
- [419] J. A. Calera, M. C. Ovejero, R. López-Medrano, M. Segurado, P. Puente and F. Leal, Characterization of the *Aspergillus nidulans* aspnd1 gene demonstrates that the ASPND1 antigen, which it encodes, and several *Aspergillus fumigatus* immunodominant antigens belong to the same family, *Infection and Immunity*, 1997, 65, 1335–1344, DOI:10.1128/iai.65.4.1335-1344.1997.
- [420] D. Wilson and G. S. J. Deepe, The intersection of host and fungus through the zinc lens, *Current Opinion in Microbiology*, 2019, 52, 35-40, DOI:10.1016/j.mib.2019.04.008.
- [421] P. Dasari, I. A. Shopova, M. Stroe, D. Wartenberg, H. Martin-Dahse, N. Beyersdorf, P. Hortschansky, S. Dietrich, Z. Cseresnyes, M. T. Figge, M. Westermann, C. Skerka, A. A. Brakhage and P. F. Zipfel, Asp2 from *Aspergillus fumigatus* recruits human immune regulators for immune evasion and cell damage, *Frontiers in Immunology*, 2018, 9, 1635, DOI:10.3389/fimmu.2018.01635.
- [422] R. Schneider de O, C. Diehl, F. M. dos Santos, A. C. Piffer, A. W. A. Garcia, M. I. R. Kulmann, A. Schrank, L. Kmetzsch, M. H. Vainstein and C. C. Staats, Effects of zinc transporters on *Cryptococcus gattii* virulence, *Scientific Reports*, 2015, 5, 10104, DOI: 10.1038/srep10104.
- [423] J. E. Stajich, T. Harris, B. P. Brunk, J. Brestelli, S. Fischer, O. S. Harb, J. C. Kissinger, W. Li, V. Nayak, D. F. Pinney, C. J. Stoeckert Jr. and D. S. Roos, FungiDB: an integrated functional genomics database for fungi, *Nucleic Acids Research*, 2012, 40, D675-681, DOI:10.1093/nar/gkr918.
- [424] J. Perlroth, B. Choi and B. Spellberg, Nosocomial fungal infections: epidemiology, diagnosis, and treatment., *Medical Mycology*, 2007, 45, 321–346, DOI:10.1080/13693780701218689.
- [425] D. Łoboda and M. Rowińska-Żyrek, Zinc binding sites in Pra1, a zincophore from *Candida albicans*, *Dalton Transactions*, 2017, 46, 13695, DOI:10.1039/c7dt01675a.

- [426] A. C. Crawford, L. E. Lehtovirta-Morley, O. Alamir, M. J. Niemiec, B. Alawfi, M. Alsarraf, V. Skrahina, A. C. B. P. Costa, A. Anderson, S. Yellagunda, E. R. Ballou, B. Hube, C. F. Urban and D. Wilson, Biphasic zinc compartmentalisation in a human fungal pathogen, *PLOS Pathogens*, 2018, 14, e1007013, DOI:10.1371/journal.ppat.1007013.
- [427] D. Łoboda and M. Rowińska-Żyrek, *Candida albicans* zincophore and zinc transporter interactions with Zn(ii) and Ni(ii), *Dalton Transactions*, 2018, 47, 2646–2654, DOI:10.1039/C7DT04403H.
- [428] D. P. Kelly, F. A. Rainey and A. P. Wood, The genus *Paracoccus*. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds) *The prokaryotes: Volume 5: Proteobacteria: alpha to Beta subclasses*, Springer New York 2006, 232–249.
- [429] D. P. Neupane, B. Jacquez, A. Sundararajan, T. Ramaraj, F. D. Schilkey and E. T. Yukl, Zinc-Dependent Transcriptional Regulation in *Paracoccus denitrificans*, *Frontiers in Microbiology* 2017, 8, 569, DOI:10.3389/fmicb.2017.00569.
- [430] K. Yoshida, M. Toyofuku, N. Obana and N. Nomura, Biofilm formation by *Paracoccus denitrificans* requires a type I secretion system-dependent adhesin BapA, *FEMS microbiology letters* 2017, 364, 10.1093/femsle/fnx029, DOI:10.1093/femsle/fnx029.
- [431] S. Bordel, D. Martín-González, T. Börner, R. Muñoz and F. Santos-Beneit, Genome-scale metabolic model of the versatile bacterium *Paracoccus denitrificans* Pd1222, *mSystems* 2024, 9, e01077-23, DOI:10.1128/msystems.01077-23.
- [432] J. Xia, L. Ye, H. Ren and X. X. Zhang, Microbial community structure and function in aerobic granular sludge, *Applied microbiology and biotechnology* 2018, 102, 3967–3979, DOI:10.1007/s00253-018-8905-9.
- [433] G. Quan, P. Xia, S. Lian, Y. Wu and G. Zhu, Zinc uptake system ZnuACB is essential for maintaining pathogenic phenotype of F4ac+ enterotoxigenic *E. coli* (ETEC) under a zinc restricted environment, *Veterinary Research* 2020, 51, 127, DOI:10.1186/s13567-020-00854-1.
- [434] D. P. Neupane, S. Kumar and E. T. Yukl, Two ABC Transporters and Periplasmic Metallochaperone Participate in Zinc Acquisition in *Paracoccus denitrificans*, *Biochemistry* 2019, 58, 126–136, DOI:10.1021/acs.biochem.8b00854.
- [435] D. Bellotti, M. Rowińska-Żyrek and M. Remelli, How Zinc-Binding Systems, Expressed by Human Pathogens, Acquire Zinc from the Colonized Host Environment: A Critical Review on Zincophores, *Current medicinal chemistry* 2021, 28(35), 7312–7338 DOI:10.2174/1389200222666210514012945.
- [436] O. Lewinson, A. T. Lee and D. C. Rees, A P-type ATPase importer that discriminates between essential and toxic transition metals, *Proceedings of the National Academy of Sciences* 2009, 106, 4677–4682, DOI:.
- [437] T. van der Heide and B. Poolman, ABC transporters: one, two or four extracytoplasmic substrate-binding sites?, *EMBO reports* 2002, 3, 938–943, DOI:10.1093/embo-reports/kvf201.
- [438] A. B. Meléndez, D. Valencia and E. T. Yukl, Specificity of Interactions between Components of Two Zinc ABC Transporters in *Paracoccus denitrificans*, *International journal of molecular sciences* 2020, 21, 9098, DOI:10.3390/ijms21239098.
- [439] E. Biemans-Oldehinkel, M. K. Doeven and B. Poolman, ABC transporter architecture and regulatory roles of accessory domains, *FEBS letters* 2006, 580, 1023–1035, DOI:10.1016/j.febslet.2005.11.079.
- [440] R. P. Berntsson, S. H. Smits, L. Schmitt, D. J. Slotboom and B. Poolman, A structural classification of substrate-binding proteins, *FEBS letters* 2010, 584, 2606–2617, DOI:10.1016/j.febslet.2010.04.043.

- [441] E. Loisel, L. Jacquamet, L. Serre, C. Bauvois, J. L. Ferrer, T. Vernet, A. M. Di Guilmi and C. Durmort, AdcAll, a new pneumococcal Zn-binding protein homologous with ABC transporters: biochemical and structural analysis, *Journal of molecular biology* 2008, 381, 594–606, DOI:10.1016/j.jmb.2008.05.068.
- [442] Y. Sheng, F. Fan, O. Jensen, Z. Zhong, B. Kan, H. Wang and J. Zhu, Dual Zinc Transporter Systems in *Vibrio cholerae* Promote Competitive Advantages over Gut Microbiome, *Infection and immunity* 2015, 83, 3902–3908, DOI:10.1128/IAI.00447-15.
- [443] A. Dintilhac, G. Alloing, C. Granadel and J. P. Claverys, Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases, *Molecular microbiology* 1997, 25, 727–739, DOI:10.1046/j.1365-2958.1997.5111879.x.
- [444] P. Chaoprasid, T. Dokpikul, J. Johnrod, S. Sirirakphaisarn, S. Nookabkaew, R. Sukchawalit and S. Mongkolsuk, *Agrobacterium tumefaciens* Zur Regulates the High-Affinity Zinc Uptake System TroCBA and the Putative Metal Chaperone YciC, along with ZinT and ZnuABC, for Survival under Zinc-Limiting Conditions, *Applied and environmental microbiology* 2016, 82, 3503–3514,, DOI:10.1128/AEM.00299-16.
- [445] J. P. Claverys, A new family of high-affinity ABC manganese and zinc permeases, *Research in Microbiology* 2001, 152, 231-243, DOI:10.1016/S0923-2508(01)01195-0.
- [446] D. P. Neupane, S. H. Fullam, K. N. Chacón and E. T. Yukl, Crystal structures of AztD provide mechanistic insights into direct zinc transfer between proteins, *Communications biology* 2019, 2, 308, DOI:10.1038/s42003-019-0542-z.
- [447] M. Handali, H. Roychowdhury, D. P. Neupane and E. T. Yukl, AztD, a Periplasmic Zinc Metallochaperone to an ATP-binding Cassette (ABC) Transporter System in *Paracoccus denitrificans*, *The Journal of biological chemistry* 2015, 290, 29984–29992, DOI:10.1074/jbc.M115.684506.
- [448] D. P. Neupane, D. Avalos, S. Fullam, H. Roychowdhury and E. T. Yukl, Mechanisms of zinc binding to the solute-binding protein AztC and transfer from the metallochaperone AztD, *The Journal of biological chemistry* 2017, 292, 17496–17505, DOI:10.1074/jbc.M117.804799.
- [449] A. Hecel, A. Kola, D. Valensin, H. Kozłowski and M. Rowińska-Żyrek, Metal Complexes of Two Specific Regions of ZnuA, a Periplasmic Zinc(II) Transporter from *Escherichia coli*, *Inorganic chemistry* 2020, 59, 1947–1958, DOI:10.1021/acs.inorgchem.9b03298.
- [450] D. Bellotti, M. Rowińska-Żyrek and M. Remelli, Novel insights into the metal binding ability of ZinT periplasmic protein from *Escherichia coli* and *Salmonella enterica*, *Dalton Transactions* 2020, 49, 9393-9403,, DOI:10.1039/D0DT01626H.
- [451] L. A. Yatsunyk, J. A. Easton, L. R. Kim, S. A. Sugarbaker, B. Bennett, R. M. Breece, I. I. Vorontsov, D. L. Tierney, M. W. Crowder and A. C. Rosenzweig, Structure and metal binding properties of ZnuA, a periplasmic zinc transporter from *Escherichia coli*, *Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry* 2008, 13, 271–288,, DOI:10.1007/s00775-007-0320-0.
- [452] S. Banerjee, B. Wei, M. Bhattacharyya-Pakrasi, H. B. Pakrasi and T. J. Smith, Structural determinants of metal specificity in the zinc transport protein ZnuA from *synechocystis* 6803, *Journal of molecular biology* 2003, 333, 1061–1069, DOI:10.1016/j.jmb.2003.09.008.
- [453] S. Castelli, L. Stella, P. Petrarca, A. Battistoni, A. Desideri and M. Falconi, Zinc ion coordination as a modulating factor of the ZnuA histidine-rich loop flexibility: A molecular modeling and fluorescence spectroscopy study, *Biochemical and Biophysical Research Communications* 2013, 430, 769-773, DOI:10.1016/j.bbrc.2012.11.073.
- [454] S. Lory, The Family Streptococcaceae, *The Prokaryotes – Firmicutes and Tenericutes*, 2014, 4, 367-370, DOI 10.1007/978-3-642-30120-9_345.

- [455] D. Ajdic', W. M. McShan, R. E. McLaughlin, G. Savic', J. Chang, M. C. Carson, C. Primeaux, R. Tian, S. Kenton, H. Jia, S. Lin, Y. Qian, S. Li, H. Zhu, F. Najar, H. Lai, J. White, B. A. Roe and J. J. Ferretti, Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen, *The Proceedings of the National Academy of Sciences*, 2002, 99, 14434–14439, DOI:10.1073/pnas.172501299.
- [456] W. H. Bowen, R. A. Burne, H. Wu and H. Koo, Oral Biofilms: Pathogens, matrix, and polymicrobial interactions in microenvironments. *Trends in Microbiology*, 2018, 26, 229–242, DOI:10.1016/j.tim.2017.09.008.
- [457] J. A. Lemos, S. R. Palmer, L. Zeng, Z. T. Wen, J. K. Kajfasz, I. A. Freires, J. Abranches and L. J. Brady, The Biology of *Streptococcus mutans*, *Microbiology Spectrum*, 2019, 7, 1-18, DOI:10.1128/microbiolspec.GPP3-0051-2018.
- [458] S. Pant, N. J. Patel, A. Deshmukh, H. Golwala, N. Patel, A. Badheka, G. A. Hirsch and J. L. Mehta, Trends in infective endocarditis incidence, microbiology, and valve replacement in the United States from 2000 to 2011. *Journal of the American College of Cardiology*, 2015, 65, 2070–2076, DOI:10.1016/j.jacc.2015.03.518.
- [459] E. Hajishengallis, Y. Parsaei, M. I. Klein and H. Koo, Advances in the microbial etiology and pathogenesis of early childhood caries, *Molecular Oral Microbiology*, 2017, 32, 24-34, DOI:10.1111/omi.12152.
- [460] Y. Pan, Y. Chen, J. Chen, Q. Ma, T. Gong, S. Yu, Q. Zhang, J. Zou and Y. Li, The *Adc* regulon mediates zinc homeostasis in *Streptococcus mutans*, *Molecular Oral Microbiology*, 2021, 36, 278–290, DOI:10.1111/omi.12350.
- [461] G. Ferrazzano, T. Cantile, M. Coda, B. Alcidi, G. Sangianantoni, A. Ingenito, M. Di Stasio and M. Volpe, In vivo release kinetics and antibacterial activity of novel polyphenols-enriched chewing gums, *Molecules*, 2016, 21, 1008, DOI:10.3390/molecules21081008.
- [462] L. J. Rich and M. Seshadri, Photoacoustic imaging of salivary glands, *Biomedical Optics Express*, 2005, 6, 3157–3162, DOI:10.1364.
- [463] A. M. Uwitonze, N. Ojeh, J. Murererehe, A. Atfi and M. S. Razzaque, Zinc adequacy is essential for the maintenance of optimal oral health, *Nutrients*, 2020, 12, 949, DOI:10.3390/nu12040949.
- [464] J. M. Berg and Y. Shi, The galvanization of biology: a growing appreciation for the roles of zinc, *Science*, 1996, 271, 1081–1085, DOI:10.1126/science.271.5252.1081.
- [465] P. Whittaker, Iron and zinc interactions in humans, *The American Journal of Clinical Nutrition*, 1998, 68, 442S–446S, DOI:10.1093/ajcn/68.2.442S.
- [466] C. Andreini and I. Bertini, A bioinformatics view of zinc enzymes, *Journal of Inorganic Biochemistry*, 2012, 111, 150–156, DOI:10.1016/j.jinorgbio.2011.11.020.
- [467] D. Corbett, J. Wang, S. Schuler, G. Lopez-Castejon, S. Glenn, D. Brough, P. W. Andrew, J. S. Cavet and I. S. Roberts, Two zinc uptake systems contribute to the full virulence of *Listeria monocytogenes* during growth in vitro and in vivo, *Infection and Immunity*, 2012, 80, 14–21, DOI:10.1128/iai.05904-11.
- [468] M. I. Hood, B. L. Mortensen, J. L. Moore, Y. Zhang, T. E. Kehl-Fie, N. Sugitani, W. J. Chazin, R. M. Caprioli and E. P. Skaar, Identification of an *Acinetobacter baumannii* zinc acquisition system that facilitates resistance to calprotectin-mediated zinc sequestration, *PLoS Pathogens*, 2012, 8, e1003068, DOI:10.1371/journal.ppat.1003068.
- [469] L. D. Palmer and E. P. Skaar, Transition metals and virulence in bacteria. *Annual Review of Genetics*, 2016, 50, 67–91, DOI:10.1146/annurev-genet-120215-035146.
- [470] M. T. Rahman, A. Hossain, C. H. Pin and N. A. Yahya, Zinc and metallothionein in the development and progression of dental caries, *Biological Trace Element Research*, 2019, 187, 51–58, DOI:10.1007/s12011-018-1369-z.

- [471] R. J. Lynch, Zinc in the mouth, its interactions with dental enamel and possible effects on caries; a review of the literature, *International Dental Journal*, 2011, 61, 46–54, DOI:10.1111/j.1875-595X.2011.00049.x.
- [472] T. Ganguly, A. M. Peterson, J. K. Kajfasz, J. Abranches and J. A. Lemos, Zinc Import Mediated by AdcABC is Critical for Colonization of the Dental Biofilm by *Streptococcus mutans* in an Animal Model, *Molecular Oral Microbiology* 2021, 36, 214–224, DOI:10.1111/omi.12337.
- [473] L. Bayle, S. Chimalapati, G. Schoehn, J. Brown, T. Vernet and C. Durmort, Zinc uptake by *Streptococcus pneumoniae* depends on both AdcA and AdcAll and is essential for normal bacterial morphology and virulence, *Molecular Microbiology* 2011, 82, 904–916, DOI:10.1111/j..
- [474] M. S. Akbari, K. S. Doran and L. R. Burcham, Metal Homeostasis in Pathogenic *Streptococci*, *Microorganisms* 2022, 10, 1501, DOI:10.3390/microorganisms10081501.
- [475] L. Bayle, S. Chimalapati, G. Schoehn, J. Brown, T. Vernet and C. Durmort, Zinc uptake by *Streptococcus pneumoniae* depends on both AdcA and AdcAll and is essential for normal bacterial morphology and virulence, *Molecular Microbiology* 2011, 82, 904–916, DOI:10.1111/j..
- [476] L. R. Burcham, B. Y. Le, J. N. Radin, B. L. Spencer, L. Deng, A. Hiron, M. R. Ransom, d. C. Mendonca, A. T. Belew, N. M. El-Sayed, K. S. McIver, T. E. Kehl-Fie and K. S. Doran, Identification of zinc-dependent mechanisms used by Group B *Streptococcus* to overcome calprotectin-mediated stress. *mBio*, 2020, 11, e02302-20, DOI:10.1128/mBio.02302-20.
- [477] C. Y. Loo, K. Mittrakul, I. B. Voss, C. V. Hughes and N. Ganeshkumar, Involvement of the adc operon and manganese homeostasis in *Streptococcus gordonii* biofilm formation, *Journal of Bacteriology* 2003, 185, 2887–2900, DOI:10.1128/jb.185.9.2887-2900.2003.
- [478] C. Y. Ong, O. Berking, M. J. Walker and A. G. McEwan, New insights into the role of zinc acquisition and zinc tolerance in Group A *Streptococcal* infection, *Infection and Immunity* 2018, 86, e00048-18, DOI:10.1128/IAI.00048-18.
- [479] H. Reyes-Caballero, A. J. Guerra, E. F. Jacobsen, K. M. Kazmierczak, D. Cowart, U. M. Koppolu, R. A. Scott, M. E. Winkler and D. P. Giedroc, The metalloregulatory zinc site in *Streptococcus pneumoniae* AdcR, a zinc-activated MarR family repressor, *Journal of Molecular Biology* 2010, 403, 197–216, DOI:10.1016/j.jmb.2010.08.030.
- [480] Y. Pan, Y. Chen, J. Chen, Q. Ma, T. Gong, S. Yu, Q. Zhang, J. Zou and Y. Li, The Adc regulon mediates zinc homeostasis in *Streptococcus mutans*, *Molecular Oral Microbiology* 2021, 36, 278–290, DOI:10.1111/omi.12350.
- [481] T. Ganguly, A. M. Peterson, J. K. Kajfasz, J. Abranches and J. A. Lemos, Zinc Import Mediated by AdcABC is Critical for Colonization of the Dental Biofilm by *Streptococcus mutans* in an Animal Model, *Molecular Oral Microbiology* 2021, 36, 214–224, DOI:10.1111/omi.12337.
- [482] T. Ganguly, A. Peterson, M. Burkholder, J. K. Kajfasz, J. Abranches and J. A. Lemos, ZccE is a Novel P-type ATPase That Protects *Streptococcus mutans* against Zinc Intoxication, *PLOS Pathogens* 2022, 18, e1010477, DOI:10.1101/2022.03.29.486188.
- [483] B. Bersch, C. Bougault, L. Roux, A. Favier, T. Vernet and C. Durmort, New insights into histidine triad proteins: solution structure of a *Streptococcus pneumoniae* PhtD domain and zinc transfer to AdcAll, *PLoS One* 2013, 8, e81168, DOI:10.1371/journal.pone.0081168.
- [484] B. Zheng, Q. Zhang, J. GaO, H. Han, M. Li, J. Zhang, J. Qi, J. Yan and G. F. Gao, Insight into the interaction of metal ions with TroA from *Streptococcus suis*, *PLoS One* 2011, 6, e19510, DOI:10.1371/journal.pone.0019510.
- [485] C. D. Plumtre, A. D. Ogunniyi and J. C. Paton, Polyhistidine triad proteins of pathogenic streptococci, *Trends in Microbiology* 2012, 20, 485-493, DOI:10.1016/j.tim.2012.06.004.

- [486] M. S. Akbari, K. S. Doran and L. R. Burcham, Metal Homeostasis in Pathogenic Streptococci, *Microorganisms* 2022 10, 1501, DOI:10.3390/microorganisms10081501.
- [487] M. B. Brophy, J. A. Hayden and E. M. Nolan, Calcium ion gradients modulate the zinc affinity and antibacterial activity of human calprotectin, *Journal of the American Chemical Society* 2012, 134, 18089–18100, DOI:10.1021/ja307974e.
- [488] T. G. Nakashige, B. Zhang, C. Krebs and E. M. Nolan, Human calprotectin is an iron-sequestering host-defense protein, *Nature Chemical Biology* 2015, 11, 765–771, DOI:10.1038/nchembio.1891.
- [489] J. P. Zackular, W. J. Chazin and E. P. Skaar, Nutritional Immunity: S100 Proteins at the Host-Pathogen Interface, *The Journal of biological chemistry* 2015, 290, 18991–18998, DOI:10.1074/jbc.R115.645085.
- [490] K. Cao, N. Li, H. Wang, X. Cao, J. He, B. Zhang, Q. Y. He, G. Zhang and X. Sun, Two zinc-binding domains in the transporter AdcA from *Streptococcus pyogenes* facilitate high-affinity binding and fast transport of zinc, *Journal of Biological Chemistry* 2018, 293, 6075–6089, DOI:10.1074/jbc.M117.818997.
- [491] K. Garstka, D. Bellotti, J. Wątyły, H. Kozłowski, M. Remelli and M. Rowińska-Żyrek, Metal coordination to solute binding proteins – exciting chemistry with potential biological meaning, *Dalton Transactions*, 2023, 52, 16140-16150, DOI:10.1039/d3dt02417b.
- [492] K. Garstka, A. Hecel, H. Kozłowski and M. Rowińska-Żyrek, Specific Zn(II)-binding site in the C-terminus of AspF2, a zincophore from *Aspergillus fumigatus*, *Metallomics* 2022, 14, mfac042, DOI:10.1093/mtomcs/mfac042.
- [493] K. Garstka, G. Potoczniak, H. Kozłowski and M. Rowińska-Żyrek, *Aspergillus fumigatus* ZrFC Zn(II) transporter scavengers zincophore-bound Zn(II), *Dalton Transactions*, 2024, 53, 2848-2858, DOI:10.1039/d3dt04083f.
- [494] F. Habashi, Zinc, Physical and Chemical Properties. In: Kretsinger, R.H., Uversky, V.N., Permyakov, E.A. *Encyclopedia of Metalloproteins* 2013, DOI:10.1007/978-1-4614-1533-6_179.
- [495] T. A. Saiyed, J. O. Adeyemi and D. C. Onwudiwe, The structural chemistry of zinc(II) and nickel(II) dithiocarbamate complexes, *Open Chemistry* 2021, 19, 974-986, DOI:10.1515/chem-2021-0080.
- [496] R. Jackson, D. Baird and S. Els, The effect of the heavy metals lead (Pb2+) and zinc (Zn2+) on, *Water SA*. 2005, 31, 107–116, DOI:10.4314/wsa.v31i1.5127.
- [497] I. Stojiljkovic and D. Perkins-Balding, Processing of heme and heme-containing proteins by bacteria, *DNA and cell biology* 2002, 21, 281–295 DOI:10.1089/104454902753759708.
- [498] M. Franks, A. Gadzhieva, L. Ghandhi, D. Murrell, A. J. Blake, E. S. Davies, W. Lewis, F. Moro, J. McMaster and M. Schröder, Five coordinate M(II)-diphenolate [M = Zn(II), Ni(II), and Cu(II)] Schiff base complexes exhibiting metal- and ligand-based redox chemistry, *Inorganic chemistry* 2013, 52, 660–670, DOI:10.1021/ic301731w.
- [499] Z. Q. Liu, Y. M. Ng, P. J. Tiong, R. A. Abu Talip, N. Jasin, V. Y. Mian Jong and M. G. Tay, Five-Coordinate Zinc(II) Complex: Synthesis, Characterization, Molecular Structure, and Antibacterial Activities of Bis-[(E)-2-hydroxy-N'-(1-(4-methoxyphenyl)ethylidene)benzohydrazido]dimethylsulfoxidezinc(II) Complex,, *International Journal of Inorganic Chemistry* 2017, 1-8, DOI:10.1155/2017/7520640.
- [500] S. J. Archibald, *Comprehensive Coordination Chemistry II* 2003, 6, 1147-1251, DOI:10.1016/B0-08-043748-6/05130-6.
- [501] T. Dudev and C. Lim, Tetrahedral vs Octahedral Zinc Complexes with Ligands of Biological Interest: A DFT/CDM Study, *Journal of the American Chemical Society* 2000, 22, 45, 11146–11153 DOI:10.1021/ja0010296.
- [502] J. J. Ellison, K. Ruhlandt-Senge, H. H. Hope and P. P. Power, Synthesis and Characterization of the New Selenolate Ligand -SeC6H3-2,6-Mes2 (Mes = C6H2-2,4,6-Me3) and Its Two-Coordinate Zinc and Manganese Derivatives: Factors

Affecting Bending in Two-Coordinate Metal Complexes with Aryl-Substituted Ligands, *Inorganic Chemistry* 1995, 34, 1, 49–54, DOI:10.1021/ic00105a012.

- [503] J. J. Ellison and P. P. Power, Synthesis and Characterization of New Thiolato Derivatives of Lithium, Magnesium, and Zinc: Examples of Two-Coordinate Lithium and Zinc Species Ligated by Sulfur,, *Inorganic Chemistry* 1994, 33, 4231-4234.
- [504] P. D. Beer, M. G. B. Drew, P. B. Leeson and M. I. Ogden, Versatile cation complexation by a calix[4]arene tetraamide (L). Synthesis and crystal structure of [ML][ClO₄]₂·nMeCN (M = FeII, NiII, CuII, ZnII or PbII),, *Journal of the Chemical Society, Dalton Transactions* 1995, 8, 1273-1283, DOI:10.1039/dt9950001273.
- [505] E. S. Gruff and S. A. Koch, Trigonal planar [Zn (SR) ₃] ¹-complex. A possible new coordination mode for zinc-cysteine centers,, *Journal of the American Chemical Society* 1989, 111, 8762–8763.
- [506] M. Ray, B. S. Hammes, G. P. A. Yap, A. L. Rheingold and A. S. Borovik, Structure and Physical Properties of Trigonal Monopyramidal Iron(II), Cobalt(II), Nickel(II), and Zinc(II) Complexes,, *Inorganic Chemistry* 1998, 37, 7, 1527–1532, DOI:10.1021/ic970831e.
- [507] M. H. Chisholm, J. C. Gallucci, H. Zhen and J. C. Huffman, Three-coordinate zinc amide and phenoxide complexes supported by a bulky Schiff base ligand, *Inorganic chemistry* 2001, 40, 5051–5054, DOI:10.1021/ic010560e.
- [508] B. Modéc, Crystal chemistry of zinc quinaldinate complexes with pyridine-based ligands, *Crystals* 2018, 8, 7–14, DOI:10.3390/cryst8010052.
- [509] B. L. Vallee and D. S. Auld, Active-site zinc ligands and activated H₂O of zinc enzymes, *Proceedings of the National Academy of Sciences of the United States of America* 1990, 87, 220–224, DOI:10.1073/pnas.87.1.220.
- [510] M. Stefanidou, C. Maravelias, A. Dona and C. Spiliopoulou, Zinc: a multipurpose trace element, *Archives of toxicology* 2006, 80, 1–9, DOI:10.1007/s00204-005-0009-5.
- [511] P. Sharma, P. K. Reddy and B. Kumar, Trace Element Zinc, a Nature’s Gift to Fight Unprecedented Global Pandemic COVID-19, *Biological Trace Element Research* 2021, 199, 3213–3221, DOI:10.1007/s12011-020-02462-8.
- [512] R. Seth, Corniola, R. S., Gower-Winter, S. D., Morgan, T. J., Jr, Bishop, B., Levenson, C. W., Zinc deficiency induces apoptosis via mitochondrial p53- and caspase-dependent pathways in human neuronal precursor cells,, *Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements* 2015, 30, 59–65, DOI:10.1016/j.jtemb.2014.10.010.
- [513] D. G. Barceloux, Zinc, *Journal of toxicology. Clinical toxicology* 1999, 37, 279–292, DOI:10.1081/clt-100102426.
- [514] E. Mocchegiani, M. Muzzioli and R. Giacconi, Zinc and immunoresistance to infection in aging: new biological tools, *Trends in pharmacological sciences* 2000, 21, 205–208, DOI:10.1016/s0165-6147(00)01476-0.
- [515] A. Q. Truong-Tran, J. Carter, R. E. Ruffin and P. D. Zalewski, The role of zinc in caspase activation and apoptotic cell death, *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* 2001, 14, 315–330, DOI:10.1023/a:1012993017026.
- [516] W. A. Pryor, *Free Radicals*, New York: McGraw-Hill, 1966.
- [517] A. Klug and D. Rhodes, Zinc fingers: a novel protein fold for nucleic acid recognition, *Cold Spring Harbor symposia on quantitative biology* 1987, 52, 473–482, DOI:10.1101/sqb.1987.052.01.054.
- [518] L. S. Reid, H. B. Gray, C. Dalvit, P. E. Wright and P. Saltman, Electron transfer from cytochrome b₅ to iron and copper complexes, *Biochemistry* 1987, 26, 7102–7107, DOI:10.1021/bi00396a035.

- [519] SCF, Opinion of the Scientific Committee on Food on the tolerable upper intake level of zinc, European Commission 2003.
- [520] R. J. Cousins, Systemic Transport of Zinc, Zinc in Human Biology 1989, 79–83.
- [521] J. De Kok, C. Van Der Schoot, M. Veldhuizen and H. T. Wolterbeek, The uptake of zinc by erythrocytes under near-physiological conditions, Biological trace element research 1993, 38, 13–26, DOI:10.1007/BF02783978.
- [522] C. H. Cho, L. Y. Fong, P. C. Ma and C. W. Ogle, Zinc deficiency: its role in gastric secretion and stress-induced gastric ulceration in rats, Pharmacology, biochemistry, and behavior 1987, 26, 293–297, DOI:10.1016/0091-3057(87)90121-3.
- [523] B. L. Vallee and K. H. Falchuk, The biochemical basis of zinc physiology, Physiological reviews 1993, 73, 79–118, DOI:10.1152/physrev.1993.73.1.79.
- [524] T. Filippini, S. Cilloni, M. Malavolti, F. Violi, C. Malagoli, M. Tesaro, I. Bottecchi, A. Ferrari, L. Vescovi and M. Vinceti, Dietary intake of cadmium, chromium, copper, manganese, selenium and zinc in a Northern Italy community, Journal of Trace Elements in Medicine and Biology 2018, 50, 508–517, DOI:10.1016/j.jtemb.2018.03.001.
- [525] K. H. Falchuk, The molecular basis for the role of zinc in developmental biology, Molecular and cellular biochemistry 1998, 188, 41–48.
- [526] G. Parkin, The bioinorganic chemistry of zinc: synthetic analogues of zinc enzymes that feature tripodal ligands, Chemical Communications 2000, 1971–1985, DOI:10.1039/B004816J.
- [527] A. G. Estévez, Crow, J. P., Sampson, J. B., Reiter, C., Zhuang, Y., Richardson, G. J., Tarpey, M. M., Barbeito, L., Beckman, J. S., Induction of nitric oxide-dependent apoptosis in motor neurons by zinc-deficient superoxide dismutase, Science 1999, 286, 2498–2500,, DOI:10.1126/science.286.5449.2498.
- [528] W. Ahmad, W. F. U. Haque, V. Brancolini, H. C. Tsou, S. U. Haque, H. Lam, V. M. Aita, J. Owen, M. de Blaquiere, J. Frank, P. B. Cserhalmi-Friedman, A. Leask, J. A. McGrath, M. Peacocke, M. Ahmad, J. Ott and A. M. Christiano, Alopecia Universalis Associated with a Mutation in the Human hairless Gene, Science 1998, 279, 720–724, DOI:10.1126/science.279.5351.720.
- [529] L. M. Gielda and V. J. DiRita, Zinc competition among the intestinal microbiota, mBio 2012, 3, e00171-12, DOI:10.1128/mBio.00171-12.
- [530] F. Habashi, Nickel, Physical and Chemical Properties, Encyclopedia of Metalloproteins 2013, 1520, DOI:10.1007/978-1-4614-1533-6_338.
- [531] D. R. Eaton and K. Zaw, Geometry of nickel(II) complexes, Journal of the American Chemical Society 1972, 94, 4394–4395, DOI:10.1021/ja00767a087.
- [532] P. D. Desai, Thermodynamic properties of nickel, International Journal of Thermophysics 1987, 8, 763–780, DOI:10.1007/BF00500793.
- [533] R. Vafazadeh and M. Bagheri, Kinetics and Mechanism of the Ligand Exchange Reaction Between Tetradentate Schiff Base N,N'-ethylen-bis (salicylaldehyde) and Ni(N,N'-propylen-bis(salicylaldehyde)),, South African Journal of Chemistry 2015, 68, 21–6, DOI:10.17159/0379-4350/2015/v68a4.
- [534] M. Rowińska-Żyrek, J. Zakrzewska-Czerwińska, A. Zawilak-Pawlik and H. Kozłowski, Ni²⁺ chemistry in pathogens – a possible target for eradication, Dalton Transactions 2014, 43, 8976, DOI:10.1039/c4dt00421c.
- [535] R. B. Martin, M. Chamberlin and J. T. Edsall, The Association of Nickel(II) Ion with Peptides, Journal of the American Chemical Society 1960, 82, 495, DOI:10.1021/ja01487a064.

- [536] H. Sigel and B. Martin, Coordinating properties of the amide bond, Stability and structure of metal ion complexes of peptides and related ligands, *Chemical Reviews* 1982, 82, 4, 385–426, DOI:10.1021/cr00050a003.
- [537] H. Kozłowski, W. Bał, M. Dyba and T. Kowalik-Jankowska, Specific structure–stability relations in metallopeptides, *Coordination Chemistry Reviews* 1999, 184, 319–346, DOI:10.1016/S0010-8545(98)00261-6.
- [538] T. Kowalik-Jankowska, H. Kozłowski, E. Farkas and I. Sóvágó, Nickel Ion Complexes of Amino Acids and Peptides, Nickel and Its Surprising Impact in Nature 2007, 2, 63–108, DOI:10.1002/9780470028131.ch3.
- [539] R. J. Maier and S. L. Benoit, Role of Nickel in Microbial Pathogenesis, *Inorganics* 2019, 7, 80, DOI:10.3390/inorganics7070080.
- [540] G. Camporesi, A. Minzoni, L. Morasso, S. Ciurli and F. Musiani, Nickel import and export in the human pathogen *Helicobacter pylori*, perspectives from molecular modelling, *Metallomics : integrated biometal science* 2012, 13, mfab066, DOI:10.1093/mtomcs/mfab066.
- [541] M. Alfano and C. Cavazza, Structure, function, and biosynthesis of nickel-dependent enzymes, *Protein science : a publication of the Protein Society* 2020, 29, 1071–1089, DOI:10.1002/pro.3836.
- [542] K. Yokoi, E. O. Uthus, J. G. Penland and F. H. Nielsen, Effect of dietary nickel deprivation on vision, olfaction, and taste in rats, *Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements* 2013, 28, 436–440, DOI:10.1016/j.jtemb.2014.07.014.
- [543] J. L. Boer, S. B. Mulrooney and R. P. Hausinger, Nickel-dependent metalloenzymes, *Archives of biochemistry and biophysics* 2014, 544, 142–152, DOI:10.1016/j.abb.2013.09.002.
- [544] M. J. Maroney and S. Ciurli, Nonredox nickel enzymes, *Chemical reviews* 2014, 114, 4206–4228, DOI:10.1021/cr4004488.
- [545] G. V. Iyengar, H. J. M. Bowen and W. E. Kollmer, The elemental composition of human tissues and body fluids : a compilation of values for adults, Verlag Chemie, Weinheim, 1978.
- [546] B. Zambelli and S. Ciurli, Nickel and human health, *Metal ions in life sciences* 2013, 13, 321–357, DOI:10.1007/978-94-007-7500-8_10.
- [547] W. Maret, Metalloproteomics, metalloproteomes, and the annotation of metalloproteins, *Metallomics : integrated biometal science* 2010, 2, 117–125, DOI:10.1039/b915804a.
- [548] K. Schauer, B. Gouget, M. Carrière, A. Labigne and H. de Reuse, Novel nickel transport mechanism across the bacterial outer membrane energized by the TonB/ExbB/ExbD machinery, *Molecular microbiology* 2007, 63, 1054–1068, DOI:10.1111/j.1365-2958.2006.05578.x.
- [549] H. de Reuse, Nickel and Virulence in Bacterial Pathogens, *The Biological Chemistry of Nickel, Metallobiology* 2017, 339–356, DOI:10.1039/9781788010580-00339.
- [550] R. J. Maier, S. L. Benoit and S. Seshadri, Nickel-binding and accessory proteins facilitating Ni-enzyme maturation in *Helicobacter pylori*, *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* 2007, 20, 655–664, DOI:10.1007/s10534-006-906.
- [551] S. L. Benoit and R. J. Maier, Hydrogen and nickel metabolism in *helicobacter* species, *Annals of the New York Academy of Sciences* 2008, 1125, 242–251, DOI:10.1196/annals.1419.014.

- [552] K. C. Ryan, A. I. Guce, O. E. Johnson, T. C. Brunold, D. E. Cabelli, S. C. Garman and M. J. Maroney, Nickel superoxide dismutase: structural and functional roles of His1 and its H-bonding network, *Biochemistry* 2015, 54, 1016–1027, DOI:10.1021/bi501258u.
- [553] L. Mazzei, F. Musiani and S. Ciurli, Urease, *The Biological Chemistry of Nickel*, *Metallobiology* 2017, 60-97, DOI:10.1039/9781788010580-00060.
- [554] W. Lubitz, H. Ogata, O. Rüdiger and E. Reijerse, Hydrogenases, *Chemical reviews* 2014, 114, 4081–4148, DOI:10.1021/cr4005814.
- [555] S. L. Benoit, E. F. Miller and R. J. Maier, *Helicobacter pylori* stores nickel to aid its host colonization, *Infection and immunity* 2013, 81, 580–584, DOI:10.1128/IAI.00858-12.
- [556] J. W. Olson and R. J. Maier, Molecular hydrogen as an energy source for *Helicobacter pylori*, *Science* 2012, 298, 1788–1790, DOI:10.1126/science.1077123.
- [557] Y. Dai, P. C. Wensink and R. H. Abeles, One protein, two enzymes, *The Journal of biological chemistry* 1999, 274, 1193–1195, DOI:10.1074/jbc.274.3.1193.
- [558] A. R. Deshpande, T. C. Pochapsky and D. Ringe, The Metal Drives the Chemistry: Dual Functions of Acireductone Dioxygenase, *Chemical reviews* 2017, 117, 10474–10501, DOI:10.1021/acs.chemrev.7b00117.
- [559] P. N. Evans, Boyd, J. A., Leu, A. O., Woodcroft, B. J., Parks, D. H., Hugenholtz, P., Tyson, G. W., An evolving view of methane metabolism in the Archaea,, *Nature reviews. Microbiology* 2019, 17, 219–232, DOI:10.1038/s41579-018-0136-7.
- [560] A. H. van Vliet, Stoof, J., Vlasblom, R., Wainwright, S. A., Hughes, N. J., Kelly, D. J., Bereswill, S., Bijlsma, J. J., Hoogenboezem, T., Vandenbroucke-Grauls, C. M., Kist, M., Kuipers, E. J., Kusters, J. G., The role of the Ferric Uptake Regulator (Fur) in regulation, of *Helicobacter pylori* iron uptake, *Helicobacter* 2002, 7, 237–244, DOI:10.1046/j..
- [561] K. Postle, TonB system, in vivo assays and characterization, *Methods in enzymology* 2007, 422, 245–269, DOI:10.1016/S0076-6879(06)22012-3.
- [562] H. de Reuse, D. Vinella and C. Cavazza, Common themes and unique proteins for the uptake and trafficking of nickel, a metal essential for the virulence of *Helicobacter pylori*, *Frontiers in cellular and infection microbiology* 2013, 3, 94, DOI:10.3389/fcimb.2013.00094.
- [563] P. T. Chivers, Nickel recognition by bacterial importer proteins, *Metallomics* 2015, 7, 590–595, DOI:10.1039/C4MT00310A.
- [564] D. A. Rodionov, P. Hebbeln, M. S. Gelfand and T. Eitinger, Comparative and functional genomic analysis of prokaryotic nickel and cobalt uptake transporters: evidence for a novel group of ATP-binding cassette transporters, *Journal of bacteriology* 2006, 188, 317–327, DOI:10.1128/JB.188.1.317-327.2006.
- [565] F. Fischer, Robbe-Saule, M., Turlin, E., Mancuso, F., Michel, V., Richaud, P., Veyrier, F. J., De Reuse, H., Vinella, D.Characterization in *Helicobacter pylori* of a Nickel Transporter Essential for Colonization, That Was Acquired during Evolution by Gastric *Helicobacter* Species, *PLoS pathogens* 2016, 12, e1006018, DOI:10.1371/journal.ppat.1006018.
- [566] K. P. Locher, Mechanistic diversity in ATP-binding cassette (ABC) transporters, *Nature structural & molecular biology* 2016, 23, 487–493, DOI:10.1038/nsmb.3216.
- [567] C. J. Zeer-Wanklyn and D. B. Zamble, Microbial nickel: cellular uptake and delivery to enzyme centers, *Current opinion in chemical biology* 2017, 37, 80–88, DOI:10.1016/j.cbpa.2017.01.014.

- [568] D. Vinella, F. Fischer, E. Vorontsov, J. Gallaud, C. Malosse, V. Michel, C. Cavazza, M. Robbe-Saule, P. Richaud, J. Chamot-Rooke, C. Brochier-Armanet and H. De Reuse, Evolution of *Helicobacter*: Acquisition by Gastric Species of Two Histidine-Rich Proteins Essential for Colonization, *PLoS pathogens* 2015, 11, e1005312, DOI:10.1371/journal.ppat.1005312.
- [569] B. Boeckmann, J. Bairoch, A. Apweiler, R. Blatter, M. C., Estreicher, A., Gasteiger, E., Martin, M. J., Michoud, K., O'Donovan, C., Phan, I., Pilbout, S., Schneider, M., The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003,, *Nucleic acids research* 2003, 31, 365–370, DOI:10.1093/nar/gkg095.
- [570] R. Ge, R. M. Watt, X. Sun, J. A. Tanner, Q. Y. He, J. D. Huang and H. Sun, Expression and characterization of a histidine-rich protein, Hpn: potential for Ni²⁺ storage in *Helicobacter pylori*, *The Biochemical journal* 2006, 393, 285–293, DOI:10.1042/BJ20051160.
- [571] Y. B. Zeng, D. M. Zhang, H. Li and H. Sun, Binding of Ni²⁺ to a histidine- and glutamine-rich protein, Hpn-like, *Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry* 2008, 13, 1121–1131, DOI:10.1007/s00775-008-0397-0.
- [572] B. Zambelli, F. Musiani, S. Benini and S. Ciurli, Chemistry of Ni²⁺ in urease: sensing, trafficking, and catalysis, *Accounts of chemical research* 2011, 44, 520–530, DOI:10.1021/ar200041k.
- [573] M. Contreras, J. M. Thiberge, M. A. Mandrand-Berthelot and A. Labigne, Characterization of the roles of NikR, a nickel-responsive pleiotropic autoregulator of *Helicobacter pylori*, *Molecular microbiology* 2003, 49, 947–963, DOI:10.1046/j.1365-2958.2003.03621.x.
- [574] S. L. Benoit and R. J. Maier, Mua (HP0868) is a nickel-binding protein that modulates urease activity in *Helicobacter pylori*, *mBio* 2011, 2, e00039–e11, DOI:10.1128/mBio.00039-11.
- [575] D. Koch, D. H. Nies and G. Grass, The RcnRA (YohLM) system of *Escherichia coli*: a connection between nickel, cobalt and iron homeostasis, *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* 2007, 20, 759–771, DOI:10.1007/s10534-006-9039-6.
- [576] R. P. Hausinger, Nickel Utilization by Microorganisms, *Microbiological Reviews* 1978, 51, 22.
- [577] V. E. Diaz-Ochoa, S. Jellbauer, S. Klaus and M. Raffatellu, Transition metal ions at the crossroads of mucosal immunity and microbial pathogenesis, *Frontiers in cellular and infection microbiology* 2014, 4, 2, DOI:10.3389/fcimb.2014.00002.
- [578] R. J. Maier, Availability and use of molecular hydrogen as an energy substrate for *Helicobacter* species, *Microbes and infection* 2003, 5, 1159–1163, DOI:10.1016/j.micinf.2003.08.002.
- [579] D. Mora and S. Arioli, Microbial urease in health and disease, *PLoS pathogens* 2014, 10, e1004472, DOI:10.1371/journal.ppat.1004472.
- [580] K. Garstka, V. Dzyhovskyi, J. Wątyły, K. Stokowa-Sołtys, J. Świątek-Kozłowska, H. Kozłowski, M. Barceló-Oliver, D. Bellotti and M. Rowińska-Żyrek, CH vs. HC—Promiscuous Metal Sponges in Antimicrobial Peptides and Metallophores, *Molecules*, 2023, 28, 3985/1-3985/14, DOI:10.3390/molecules28103985.
- [581] M. Vasák and D. W. Hasler, Metallothioneins: new functional and structural insights, *Current opinion in chemical biology* 2000, 4, 177–183, DOI:10.1016/s1367-5931(00)00082-x.
- [582] M. D. Michelitsch and J. S. Weissman, A census of glutamine/asparagine-rich regions: implications for their conserved function and the prediction of novel prions, *Proceedings of the National Academy of Sciences of the United States of America* 2000, 97, 11910–11915,, DOI:10.1073/pnas.97.22.11910.

- [583] T. Cheng, W. Xia, P. Wang, F. Huang, J. Wang and H. Sun, Histidine-rich proteins in prokaryotes: metal homeostasis and environmental habitat-related occurrence, *Metallomics : integrated biometal science* 2013, 5, 1423–1429, DOI:10.1039/c3mt00059a.
- [584] K. Duncan, Metallothioneins and Related Chelators, *Metal Ions in Life Sciences Vol. 5*, Edited by Astrid Sigel, Helmut Sigel and Roland K. O. Sigel, *Angewandte Chemie* 2009, 48, 7966–7967,, DOI:10.1002/anie.200903833.
- [585] T. Fukada, S. Yamasaki, K. Nishida, M. Murakami and T. Hirano, Zinc homeostasis and signaling in health and diseases: Zinc signaling, *Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry* 2011, 16, 1123–1134, DOI:10.1007/s00775-011-0797-4.
- [586] K. Smidt, N. Jessen, A. B. Petersen, A. Larsen, N. E. Magnusson, J. B. Jeppesen, M. Stoltenberg, J. G. Culvenor, A. Tsatsanis, B. Brock, O. Schmitz, L. Wogensen, A. I. Bush and J. Rungby, SLC30A3 responds to glucose- and zinc variations in beta-cells and is critical for insulin production and in vivo glucose-metabolism during beta-cell stress, *PLoS One* 2009, 4, 1932–6203, DOI:10.1371/journal.pone.0005684.
- [587] S. Overbeck, L. Rink and H. Haase, Modulating the immune response by oral zinc supplementation: a single approach for multiple diseases, *Archivum immunologiae et therapiae experimentalis* 2008, 56, 15–30 DOI:10.1007/s00005-008-0003-8.
- [588] C. Andreini, L. Banci, I. Bertini and A. Rosato, Counting the zinc-proteins encoded in the human genome, *Journal of Proteome Research*, 2006, 5, 196–201, DOI:10.1021/pr050361j.
- [589] E. Loisel, L. Jacquamet, L. Serre, C. Bauvois, J. L. Ferrer, T. Vernet, A. M. Di Guilmi and C. Durmort, AdcAll, a new pneumococcal Zn-binding protein homologous with ABC transporters: biochemical and structural analysis, *Journal of Molecular Biology*, 381, 594–606, DOI:10.1016/j.jmb.2008.05.068.
- [590] J. D. Van Horn, G. Bulaj, D. P. Goldenberg and C. J. Burrows, The Cys-Xaa-His metal-binding motif: [N] versus [S] coordination and nickel-mediated formation of cysteinyl sulfinic acid. *Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry* 2003,, 8, 601–610, DOI:10.1007/s00775-003-0454-7.
- [591] H. Kozłowski, A. Lebki, C. O. Onindo, L. D. Pettit and J. F. Gale, The influence of aspartic or glutamic acid residues in tetrapeptides on the formation of complexes with nickel(II) and zinc(II), *Polyhedron* 1995, 14, 211–218, DOI:10.1016/0277-5387(94)00239-B.
- [592] T. Kowalik-Jankowska, H. Kozłowski, E. Farkas and I. Sóvágó, Nickel Ion Complexes of Amino Acids and Peptides, *Impact in Nature* 2007, 2, 63–107, DOI:10.1002/9780470028131.ch3.
- [593] L. A. Kelley, S. Mezulis, C. M. Yates, M. N. Wass and M. J. Sternberg, The Phyre2 web portal for protein modeling, prediction and analysis, *Nature protocols* 2015, 10, 845–858, DOI:10.1038/nprot.2015.053.
- [594] L. A. Basile and J. E. Coleman, Optical activity associated with the sulfur to metal charge transfer bands of Zn and Cd GAL4, *Protein science : a publication of the Protein Society* 1992, 1, 617–624, DOI:10.1002/pro.5560010508.
- [595] The PyMOL Molecular Graphics System, Version 1.8, Schrödinger, LLC.
- [596] K. Cherifi, B. Decock-Le Reverend, K. Varnagy, T. Kiss, I. Sovago, C. Locheux and H. Kozłowski, Transition metal complexes of L-cysteine containing di- and tripeptides, *Journal of inorganic biochemistry* 1990, 38, 69–80, DOI:10.1016/0162-0134(90)85008-k.
- [597] Q. Y. Zhang, Z. B. Yan, Y. M. Meng, X. Y. Hong, G. Shao, J. J. Ma, X. R. Cheng, J. Liu, J. Kang and C. Y. Fu, Antimicrobial peptides: mechanism of action, activity and clinical potential, *Military Medical Research* 2021, 8, 48, DOI:10.1186/s40779-021-00343-2.

- [598] K. Kluska, J. Adamczyk and A. Krężel, Metal binding properties of zinc fingers with a naturally altered metal binding site, *Metallomics : integrated biometal science* 2018, 10, 248–263, DOI:10.1039/c7mt00256d.
- [599] H. Sigel and D. B. McCormick, Discriminating behavior of metal ions and ligands with regard to their biological significance, *Accounts of Chemical Research* 1970, 3, 6, 201–208, DOI:10.1021/ar50030a004.
- [600] A. A. Fedotova, A. N. Bonchuk, V. A. Mogila and P. G. Georgiev, C2H2 Zinc Finger Proteins: The Largest but Poorly Explored Family of Higher Eukaryotic Transcription Factors, *Acta naturae* 2017, 9, 47–58.
- [601] A. Janicka-Kłos, E. Porciatti, D. Valensin, C. Conato, M. Remelli, S. Oldziej, G. Valensin and H. Kozłowski, The unusual stabilization of the Ni²⁺ and Cu²⁺ complexes with NSF_{RY}, *Dalton Transactions* 2013, 42, 448–458, DOI:10.1039/C2DT31959D.
- [602] S. Iuchi, Three classes of C₂H₂ zinc finger proteins, *Cellular and Molecular Life Sciences CMLS* 2001, 58, 625–635.
- [603] O. McCubbin Stepanic, J. Ward, J. E. Penner-Hahn, A. Deb, U. Bergmann and S. DeBeer, Probing a Silent Metal: A Combined X-ray Absorption and Emission Spectroscopic Study of Biologically Relevant Zinc Complexes, *Inorganic Chemistry* 2020, 59, 13551–13560, DOI:10.1021/acs.inorgchem.0c01931.
- [604] K. Garstka, V. Dzyhovskyi, J. Wątyły, K. Stokowa-Sołtys, J. Świątek-Kozłowska, H. Kozłowski, M. Barceló-Oliver, D. Bellotti and M. Rowińska-Żyrek, CH vs. HC—Promiscuous Metal Sponges in Antimicrobial Peptides and Metallophores, *Molecules*, 2023, 28, 3985/1–3985/14, DOI:10.3390/molecules28103985.
- [605] G. Wang, X. Li and Z. Wang, APD3: the antimicrobial peptide database as a tool for research and education, *Nucleic acids research* 2016, 44, D1087–D1093, DOI:10.1093/nar/gkv1278.
- [606] M. Vasák and D. W. Hasler, Metallothioneins: new functional and structural insights, *Current opinion in chemical biology* 2000, 4, 177–183, DOI:10.1016/s1367-5931(00)00082-x.
- [607] M. D. Michelitsch and J. S. Weissman, A census of glutamine/asparagine-rich regions: implications for their conserved function and the prediction of novel prions, *Proceedings of the National Academy of Sciences of the United States of America* 2000, 97, 11910–11915, DOI:10.1073/pnas.97.22.11910.
- [608] T. Cheng, W. Xia, P. Wang, F. Huang, J. Wang and H. Sun, Histidine-rich proteins in prokaryotes: metal homeostasis and environmental habitat-related occurrence, *Metallomics : integrated biometal science* 2013, 5, 1423–1429, DOI:10.1039/c3mt00059a.
- [609] K. Duncan, Metallothioneins and Related Chelators, *Metal Ions in Life Sciences Vol. 5*, Edited by Astrid Sigel, Helmut Sigel and Roland K. O. Sigel, *Angewandte Chemie* 2009, 48, 7966–7967, DOI:10.1002/anie.200903833.
- [610] K. Kulon, D. Valensin, W. Kamysz, R. Nadolny, E. Gaggelli, G. Valensin and H. Kozłowski, Binding of Ni²⁺ and Cu²⁺ ions to peptides with a Cys–His motif, *Dalton Transactions* 2008, 5323–5330, DOI:10.1039/b806851h.
- [611] J. Wątyły, A. Hecel, M. Rowińska-Żyrek and H. Kozłowski, Impact of histidine spacing on modified polyhistidine tag – Metal ion interactions, *Inorganica Chimica Acta*, 2018, 472, 119–126, DOI:10.1016/j.ica.2017.06.053.
- [612] A. Hecel, J. Wątyły, M. Rowińska-Żyrek, J. Świątek-Kozłowska and H. Kozłowski, Histidine tracts in human transcription factors: insight into metal ion coordination ability, *JBIC Journal of Biological Inorganic Chemistry* 2018, 23, 81–90.
- [613] A. Janicka-Kłos, E. Porciatti, D. Valensin, C. Conato, M. Remelli, S. Oldziej, G. Valensin and H. Kozłowski, The unusual stabilization of the Ni²⁺ and Cu²⁺, *Dalton Transactions* 2013, 42, 448–458.

- [614] H. Hong, S. Park, R. H. Flores Jiménez, D. Rinehart and L. K. Tamm, Role of Aromatic Side Chains in the Folding and Thermodynamic Stability of Integral Membrane Proteins, *Journal of the American Chemical Society* 2007, 129, 26, 8320–8327.
- [615] A. Miller, A. Matera-Witkiewicz, A. Mikołajczyk, R. Wieczorek and M. Rowińska-Żyrek, Chemical “Butterfly Effect” Explaining the Coordination Chemistry and Antimicrobial Properties of Clavanin Complexes, *Inorganic Chemistry* 2021, 60, 17, 12730–12734.
- [616] K. Garstka, A. Hecel, H. Kozłowski and M. Rowińska-Żyrek, AdcA lipoprotein involved in Zn(II) transport in *Streptococcus mutans* – is it as metal-specific as expected?, under review.
- [617] R. Fors and M. Persson, „Nickel in dental plaque and saliva in patients with and without orthodontic appliances, *European Journal of Orthodontics*, 2006, 28, 292–297, DOI:10.1093/ejo/cji091”.
- [618] R. Eckert, R. Sullivan and W. Shi, Targeted antimicrobial treatment to re-establish a healthy microbial flora for long-term protection, *Advances in dental research* 2012, 24, 94–97, DOI:10.1177/0022034512453725.
- [619] J. Mai, X. Tian, J. W. Gallant, N. Merkley, Z. Biswas, R. Syvitski, S. E. Douglas, J. Ling and J. Li, A Novel Target-Specific, Salt-Resistant Antimicrobial Peptide against the Cariogenic Pathogen *Streptococcus mutans*, *Antimicrobial Agents and Chemotherapy* 2011, 55, 5205 - 5213, DOI:10.1128/aac.05175-11.
- [620] P. Sarma, S. Mahendiratta, A. Prakash and B. Medhi, Specifically targeted antimicrobial peptides: A new and promising avenue in selective antimicrobial therapy, *Indian journal of pharmacology* 2018, 50, 1-3, DOI:10.4103/ijp.IJP_218_18.
- [621] J. Huan, Q. Kong, H. Mou and H. Yi, Antimicrobial Peptides: Classification, Design, Application and Research Progress in Multiple Fields, *Frontiers in Microbiology* 2020, 11, 582779, DOI:10.3389/fmicb.2020.582779.
- [622] J. Lei, L. Sun, S. Huang, C. Zhu, P. Li, J. He, V. Mackey, D. H. Coy and Q. He, The antimicrobial peptides and their potential clinical applications, *American journal of translational research* 2019, 11, 3919–3931.
- [623] X. Tian, F. Sun, X. Zhou, S. Luo and L. Chen, Role of peptide self-assembly in antimicrobial peptides, *Journal of Peptide Science* 2014, 21, 530-539, DOI:10.1002/psc.2788.
- [624] D. Łoboda, H. Kozłowski and M. Rowińska-Żyrek, Antimicrobial peptide–metal ion interactions – a potential way of activity enhancement, *New Journal of Chemistry* 2018, 42, 7560-7568, DOI:10.1039/C7NJ04709F.
- [625] C. Di Natale, I. De Benedictis, A. De Benedictis and D. Marasco, Metal-Peptide Complexes as Promising Antibiotics to Fight Emerging Drug Resistance: New Perspectives in Tuberculosis, *Antibiotics* 2020, 9, 337, DOI:10.3390/antibiotics9060337.
- [626] R. Rathinakumar, W. F. Walkenhorst and W. C. Wimley, Broad-Spectrum Antimicrobial Peptides by Rational Combinatorial Design and High-Throughput Screening: The Importance of Interfacial Activity, *Journal of the American Chemical Society* 2009, 131, 22, 7609–7617, DOI:10.1021/ja8093247.

X. LIST OF TABLES

Table 1 Mutations of Zn(II) transporters and the diseases they cause

Table 2 Catalytic reactions for Ni(II)-dependent enzymes

Tab 3 Amino acid sequences of unstructured fragments of zincophores and proteins involved in Zn(II) transport in *Aspergillus fumigatus*, *Paracoccus denitrificans* and *Streptococcus mutans* and model sequences

Table 4 Charge transfer transitions in Ni(II) complexes (CD spectra)

Table 5 Characteristic parameters of d-d transitions in Ni(II) complexes depending on their geometry

Table 6 Potentiometric data for the proton, Zn(II) and Ni(II) complexes of the, Ac-ARHAKAH-NH₂, Ac-MHRLYHVP-NH₂, Ac-MHRLYHVPVGGQGWVDHFAD-NH₂ and Ac-PNCHTHEGGQLHCT in a water solution of 4 mM HClO₄ with I = 0.1 M NaClO₄, T = 298 K for Ac-ARHAKAH-NH₂ and Ac-PNCHTHEGGQLHCT peptides or 4 mM HClO₄ with I = 0.04 M SDS, T = 298 K for Ac-MHRLYHVP-NH₂ and Ac-MHRLYHVPVGGQGWVDHFAD-NH₂ peptides. Standard deviations are presented in parentheses

Table 7 Potentiometric data for the proton, Zn(II) and Ni(II) complexes of the Ac-TGCHSHGS-NH₂, Ac-MNCHFAGVEHCIGAGESESSQ-NH₂ in a water solution of 4 mM HClO₄ with I = 0.1 M NaClO₄, T = 298 K. Standard deviations are presented in parentheses

Table 8 Potentiometric data for the proton, Zn(II) and Ni(II) complexes of the Ac-AHCA-NH₂ and Ac-ACHA-NH₂ in a water solution of 4 mM HClO₄ with I = 0.1 M NaClO₄, T = 298 K. Standard deviations are presented in parentheses

Table 9 Potentiometric data for the proton, Zn(II) and Ni(II) complexes of the Ac-HEHEHEHEHEHEHEHEH-NH₂, Ac-GGEHEHEHEHEHEHEHEHDGHHGAEEQAHHDDHDSG-NH₂ and Ac-GGGHYHYIDGKAVFHAG-NH₂ in a water solution of 4 mM HClO₄ with I = 0.1 M NaClO₄, T = 298 K. Standard deviations are presented in parentheses

Table 10 Potentiometric data for the proton, Zn(II) and Ni(II) complexes of the Ac-EGHGHKGHHHA-NH₂, Ac-HGKSQKAEHFH-NH₂ in a water solution of 4 mM HClO₄ with I = 0.1 M NaClO₄, T = 298 K. Standard deviations are presented in parentheses

Table 11 Summary of proposed Zn(II)- and Ni(II)-binding models for unstructured fragments of Asp2 zincophore, ZrfC transporter, ZnuA solute binding protein, AztC solute binding protein, AdcA lipoprotein at physiological pH (pH = 7.4) with their geometrical shapes

XI. LIST OF FIGURES

Figure 1 The timeline shows the introduction of antibiotics into treatment and the most important dates (at the bottom of the timeline). The antibiotics are colored according to their source: blue- bacteria; green- fungi; red- synthetic antibiotics

Figure 2 Schematic model of Zn(II) distribution in the human body. Dietary Zn(II) is absorbed in the small intestine (duodenum and jejunum) and then distributed to peripheral tissues. Most of the serum Zn(II) is stored in skeletal muscles, bones, liver and skin. The remaining amount of Zn(II) is distributed to other tissues, like the brain, kidney or pancreas

Figure 3 Zn(II) is absorbed throughout the whole small intestine, as shown in Figure 2. The losses of Zn(II) include fecal Zn(II) excretions and excretions with urine, sweat, loss of hairs and nails, exfoliation of the skin or menstrual flow, and semen for adults

Figure 4 The predicted topologies of ZIP transporters. They form homodimers with eight transmembrane helices

Figure 5 The predicted topologies of ZnT transporters. They form dimers with six transmembrane helices

Figure 6 The schematic model of the defined zincophore systems

Figure 7 Proposed model of Zn(II) acquisition in fungi

Figure 8 Mechanism of Zn(II) transport in *Candida albicans* based on peptide zincophores

Figure 9 Topology of vacuolar Zn(II) importers

Figure 10 Main proteins involved in bacterial Zn(II) homeostasis. From left: transporters (ATPase, CDF and RND), periplasmic proteins (ZraP and ZraSR) and sensors (SmtB, ZntR) involved in Zn(II) homeostasis in the case of Zn(II) excess; from right: different molecules and systems involved in Zn(II) uptake in Zn(II) deficiency

Figure 11 The inward-facing type I transporter binds to the substrate binding protein and two molecules of ATP. NBDs dimerize and result in an outward-facing conformation. The substrate leaves the binding protein and binds to the TMDs mid-membrane. In the last step, the ATP is hydrolyzed and the product is released, together with the dissociation of the NBD. The outward-facing type II importer binds to the substrate binding protein and two molecules of ATP. The dimerization of the NBDs results in an occluded conformation, with the substrate confined to a sealed cavity mid-membrane. The ATP hydrolysis and NBD dissociation allows the substrate to escape into the cytoplasm

Figure 12 The schematic mechanism of Zur under Zn(II) excess and Zn(II) deficiency conditions

Figure 13 A schematic topological model of ZIP proteins for most bacterial ZIP proteins

Figure 14 The Post-Albers (E_1 to E_2) reaction cycle of Zn(II)-transporting P-type ATPase. A high-affinity state (E_1), which is open to the intracellular space, where it binds Zn(II) and then enters an occluded state and phosphorylation. After that, (E_1P) triggers the release of the Zn(II) and goes into a low-affinity state (E_2P). In the next step, the inorganic phosphate (P_i) is released, which yields the fully dephosphorylated conformation (E_2). After the E_2 state, conformation is restored to the inward-facing conformation (E_1), which ends this reaction cycle and initiates a new one.

Figure 15 Proposed CDF mechanisms of action from *Escherichia coli*

Figure 16 An early schematic view of the tripartite pump complex in Gram-negative bacteria

Figure 17 Topological model of ZrfC transporter insertion in the plasma membrane

Figure 18 Schematic model of *Aspergillus fumigatus* Zn(II) scavenging from host cells. After invasion of the host cell, Aspf2 is expressed and secreted. It binds Zn(II), either in the form of free Zn(II) or from Zn(II)-binding proteins of the host. Reassociation with the *Aspergillus fumigatus* cell surface and Zn(II) transportation into the cell occurs via a Aspf2-ZrfC interaction

Figure 19 Schematic model of the AztC loop from *Paracoccus denitrificans*

Figure 20 Schematic model of the ZnuA loop from *Paracoccus denitrificans*

Figure 21 A simplified model for zinc acquisition through ABC transporters in *Paracoccus denitrificans*

Figure 22 A summary diagram of all known types of transporters for Zn(II) in Streptococci with arrows indicating metal import or export: histidine triad proteins (Pht), ABC transporter, CDF pump: CzcD. The ZccE (P-type ATPase) is only found in *S. mutants*

Figure 23 The model of Zn(II) uptake in *Streptococcus* via AdcA in a Zn(II)-deficient environment

Figure 24 Typical geometries for 4, 5 and 6 coordinated Zn(II)

Figure 25 Typical geometries for 4 and 6 coordinated Ni(II)

Figure 26 The scheme of Ni(II) homeostasis in bacteria

Figure 27 The schematic representation of the ligands investigated in this work

Figure 28 Amino acid sequence of Aspf2 zincophore; probable Zn(II) binding sites are highlighted in different colors

Figure 29 ESI-MS spectrum of A) Zn(II)-Ac-ARHAKAH-NH₂ and B) Ni(II)-Ac-ARHAKAH-NH₂

Figure 30 ESI-MS spectrum of A) Zn(II)-Ac-MHRLYHVP-NH₂ and B) Ni(II)-Ac-MHRLYHVP-NH₂

Figure 31 ESI-MS spectrum of A) Zn(II)-Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂ and B) Ni(II)-Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂

Figure 32 ESI-MS spectrum of A) Zn(II)-Ac-PNCHTHEGGQLHCT and B) Ni(II)-Ac-PNCHTHEGGQLHCT

Figure 33 Species distribution diagram for the formation of Zn(II) complex with the Ac-ARHAKAH-NH₂ Aspf2 fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

Figure 34 Species distribution diagram for the formation of Ni(II) complex with the Ac-ARHAKAH-NH₂ Aspf2 fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 35 CD spectra of Ni(II) complex with the Ac-ARHAKAH-NH₂ fragment from Aspf2 protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 36 UV-Vis spectra of Ni(II) complex with the Ac-ARHAKAH-NH₂ fragment from Aspf2 protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 37 Species distribution diagram for the formation of Zn(II) complex with the Ac-MHRLYHVP-NH₂ Aspf2 fragment, T = 298 K, I = 0.04 M SDS, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

Figure 38 Species distribution diagram for the formation of Ni(II) complex with the Ac-MHRLYHVP-NH₂ Aspf2 fragment, T = 298 K, I = 0.04 M SDS, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 39 UV-Vis spectra of Ni(II) complex with the Ac-MHRLYHVP-NH₂ fragment from Aspf2 protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 40 CD spectra of Ni(II) complex with the Ac-MHRLYHVP-NH₂ fragment from Aspf2 protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 41 Species distribution diagram for the formation of Zn(II) complex with the Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂ Aspf2 fragment, T = 298 K, I = 0.04 M SDS, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

Figure 42 Species distribution diagram for the formation of Ni(II) complex with the Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂ Aspf2 fragment, T = 298 K, I = 0.04 M SDS, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 43 UV-Vis spectra of Ni(II) complex with the Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂ fragment from Aspf2 protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 44 CD spectra of Ni(II) complex with the Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂ fragment from Aspf2 protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 45 Species distribution diagram for the formation of Zn(II) complex with the Ac-PNCHTHEGGQLHCT Aspf2 fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

Figure 46 Proposed binding mode for Zn(II)-Ac-PNCHTHEGGQLHCT fragment of Aspf2 protein at pH 7.4 (form - {2N_{im}, S'} binding mode). The Aspf2 structure is based on a Phyre2 simulation [593] and the figure was generated by PyMOL [595]

Figure 47 UV-Vis spectra of Zn(II) complexes of the Aspf2 protein fragment (Ac-PNCHTHEGGQLHCT) at the pH 7.4, titrated with Zn(II) ions at a step of 0.2 molar equivalents. Final conditions: [Zn(II)] : [Ac-PNCHTHEGGQLHCT] = 1 : 1. Spectra were recorded at 298 K, in the range 220- 240 nm and with the optical path 0.1 cm

Figure 48 TOCSY spectra of 3 mM Ac-PNCHTHEGGQLHCT Aspf2 fragment at pH 7.4, in the absence (gray) and presence of 1 Zn(II) equivalent (green)

Figure 49 Species distribution diagram for the formation of Ni(II) complex with the Ac-PNCHTHEGGQLHCT Aspf2 fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 50 CD spectra of Ni(II) complex with the Ac-PNCHTHEGGQLHCT fragment from Aspf2 protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 51 TOCSY spectra of 3 mM Ac-PNCHTHEGGQLHCT Aspf2 fragment at pH 7.4, in the absence (gray) and presence of 0.1 Ni(II) equivalent (orange)

Figure 52 UV-Vis spectra of Ni(II) complex with the Ac-PNCHTHEGGQLHCT fragment from Aspf2 protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 53 Proposed binding mode for Ni(II)-Ac-PNCHTHEGGQLHCT fragment of Aspf2 protein at pH 7.4 (form - {2N_{im}, S⁻} binding mode). The Aspf2 structure is based on a Phyre2 simulation [593] and the figure was generated by PyMOL [595]

Figure 54 CD spectra of A) Ac-PNCHTHEGGQLHCT and its complexes with B) Zn(II) and C) Ni(II), PPII- polyproline II helix; optical path 0.1 mm, Zn(II) and Ni(II)/L molar ratio=1:1.

Figure 55 A competition plot between Aspf2 fragments: Ac-ARHAKAH-NH₂, Ac-MHRLYHVP-NH₂, Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂, Ac-PNCHTHEGGQLHCT, and Zn(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 6. Conditions: 298 K, I = 0.1 M NaClO₄ or I = 0.04 M SDS, [Zn(II)] = [Ac-ARHAKAH-NH₂] = [Ac-MHRLYHVP-NH₂] = [Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂] = [Ac-PNCHTHEGGQLHCT] = 0.001 M.

Figure 56 A competition plot between Aspf2 fragment: Ac-PNCHTHEGGQLHCT, Pra1 fragment: Ac-SHCHTHADGEVHC and Zn(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 6 and [426]. Conditions: 298 K, I = 0.1 M NaClO₄, [Zn(II)] = [Ac-PNCHTHEGGQLHCT] = [Ac-SHCHTHADGEVHC] = 0.001 M.

Figure 57 A competition plot between Aspf2 fragments: Ac-ARHAKAH-NH₂, Ac-MHRLYHVP-NH₂, Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂, Ac-PNCHTHEGGQLHCT, and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 6. Conditions: 298 K, I = 0.1 M NaClO₄ or I = 0.04 M SDS, [Ni(II)] = [Ac-ARHAKAH-NH₂] = [Ac-MHRLYHVP-NH₂] = [Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂] = [Ac-PNCHTHEGGQLHCT] = 0.001 M.

Figure 58 A competition plot between Aspf2 fragment Ac-PNCHTHEGGQLHCT, Zn(II) and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of the three reagents are mixed. Calculations are based on binding constants from Table 6

Figure 59 CD spectra of Ni(II) complexes of the Aspf2 protein fragment (Ac-PNCHTHEGGQLHCT) at the pH 7.4, titrated with Zn(II) ions at a step of 0.1 molar equivalents. Final conditions: [Zn(II)] : [Ni(II)] : [Ac-PNCHTHEGGQLHCT] = 1 : 1 : 1. Spectra were recorded at 298 K, in the range 240 - 650 nm and with the optical path 1 cm

Figure 60 Competition plot between Aspf2 fragments; where: L1- Ac-ARHAKAH-NH₂; L2- Ac-MHRLYHVP-NH₂; L3- Ac-MHRLYHVPVAVGQGWVDHFAD-NH₂; L4- Ac-PNCHTHEGGQLHCT; Zn(II) and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 6

Figure 61 Amino acid sequence of ZrfC transporter; probable Zn(II) binding sites are highlighted in different colors

Figure 62 ESI-MS spectrum of A) Zn(II)-Ac-TGCHSHGS-NH₂ and B) Ni(II)-Ac-TGCHSHGS-NH₂

Figure 63 ESI-MS spectrum of A) Zn(II)-Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ and B) Ni(II)-Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂

Figure 64 Species distribution diagram for the formation of Zn(II) complex with the Ac-TGCHSHGS-NH₂ ZrFC fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

Figure 65 Proposed binding mode for Zn(II)-Ac-TGCHSHGS-NH₂ fragment of ZrFC protein at pH 7.4 (ZnL form - {2N_{im}, S⁻} binding mode). The ZrFC structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

Figure 66 Species distribution diagram for the formation of Ni(II) complex with the Ac-TGCHSHGS-NH₂ ZrFC fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 67 CD spectra of Ni(II) complex with the Ac-TGCHSHGS-NH₂ fragment from ZrFC protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 68 UV-Vis spectra of Ni(II) complex with the Ac-TGCHSHGS-NH₂ fragment from ZrFC protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 69 Proposed binding mode for Ni(II)-Ac-TGCHSHGS-NH₂ fragment of ZrFC protein at pH 7.4 (NiL form - {2N_{im}, S⁻} binding mode). The ZrFC structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

Figure 70 Species distribution diagram for the formation of Zn(II) complex with the Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ ZrFC fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

Figure 71 Proposed binding mode for Zn(II)-Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ fragment of ZrFC protein at pH 7.4 (ZnL form - {2N_{im}, 2S⁻} binding mode). The ZrFC structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

Figure 72 Species distribution diagram for the formation of Ni(II) complex with the Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ ZrFC fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 73 CD spectra of Ni(II) complex with the Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ fragment from ZrFC protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 74 UV-Vis spectra of Ni(II) complex with the Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ fragment from ZrFC protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 75 Proposed binding mode for Ni(II)-Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ fragment of ZrFC protein at pH 7.4 (from the left: NiHL form – {3N_{im}, S⁻} binding mode and NiH₁L form – {1N_{im}, 2S⁻, N⁻} binding mode). The ZrFC structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

Figure 76 CD spectra of A) Ac-TGCHSHGS-NH₂ and its complexes with b) Zn(II) and C) Ni(II); optical path 0.1 mm, Ni(II)/L molar ratio = 1 : 1

Figure 77 CD spectra of A) Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ and its complexes with b) Zn(II) and C) Ni(II); optical path 0.1 mm, Ni(II)/L molar ratio = 1 : 1

Figure 78 A competition plot between ZrFC fragments: Ac-TGCHSHGS-NH₂, Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂ and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 7. Conditions: T=298 K, I=0.1 M NaClO₄, [Ni(II)] = [Ac-TGCHSHGS-NH₂] = [Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂] = 0.001 M

Figure 79 A competition plot between two most efficient Zn(II) binding sites in the ZrFC transporter (Ac-MNCHFAGVEHCIGAGESESGSSQ-NH₂), in the AspF2 zincophore (Ac-PNCHTHEGGQLHCT) and Zn(II); it describes

complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 7 and [493]. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂] = [Ac-PNCHTHEGGQLHCT] = 0.001 M

Figure 80 A competition plot between ZrFC fragments: Ac-TGCHSHGS-NH₂, Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂ and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 7. Conditions: T=298 K, I=0.1 M NaClO₄, [Ni(II)] = [Ac-TGCHSHGS-NH₂] = [Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂] = 0.001 M

Figure 81 A competition plot between ZrFC fragment: Ac-TGCHSHGS-NH₂, Zn(II) and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 7. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ni(II)] = [Ac-TGCHSHGS-NH₂] = 0.001 M

Figure 82 A competition plot between ZrFC fragment: MNCHFAGVEHCIGAGESEGSSQ-NH₂, Zn(II) and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 7. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ni(II)] = [Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂] = 0.001 M

Figure 83 CD spectra of Ni(II) complexes of the ZrFC transporter fragment (Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂) at the pH 7.4, titrated with Zn(II) ions at a step of 0.1 molar equivalents. Final conditions: [Zn(II)] : [Ni(II)] : [Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂] = 1 : 1 : 1. Spectra were recorded at 298 K, in the range 250-800 nm and with the optical path 1 cm

Figure 84 CD spectra of Zn(II) complexes of the ZrFC transporter fragment (Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂) at the pH 7.4, titrated with Ni(II) ions at a step of 0.1 molar equivalents. Final conditions: [Ni(II)] : [Zn(II)] : [Ac-MNCHFAGVEHCIGAGESEGSSQ-NH₂] = 1 : 1 : 1. Spectra were recorded at 298 K, in the range 250-800 nm and with the optical path 1 cm. Finally, the pH value was raised to pH 10.5 at a step of 0.5

Figure 85 Amino acid sequences of model ligands

Figure 86 Species distribution diagram for the formation of Zn(II) complex with the Ac-AHCA-NH₂, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

Figure 87 Proposed binding mode for Zn(II)-Ac-AHCA-NH₂

Figure 88 Species distribution diagram for the formation of Ni(II) complex with the Ac-AHCA-NH₂, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 89 CD spectra of Ni(II) complex with the Ac-AHCA-NH₂ in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 90 Proposed binding mode for Ni(II)-Ac-AHCA-NH₂

Figure 91 UV-Vis spectra of Ni(II) complex with the Ac-AHCA-NH₂ in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 92 Species distribution diagram for the formation of Zn(II) complex with the Ac-ACHA-NH₂, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

Figure 93 Proposed binding mode for Zn(II)-Ac-ACHA-NH₂

Figure 94 Species distribution diagram for the formation of Ni(II) complex with the Ac-ACHA-NH₂, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 95 Proposed binding mode for Ni(II)-Ac-ACHA-NH₂

Figure 96 CD spectra of Ni(II) complex with the Ac-ACHA-NH₂ in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 97 UV-Vis spectra of Ni(II) complex with the Ac-ACHA-NH₂ in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 98 A competition plot between ligands: Ac-AHCA-NH₂, Ac-ACHA-NH₂ and Zn(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 8. Conditions: 298 K, I = 0.1 M NaClO₄, [Zn(II)] = [Ac-AHCA-NH₂] = [Ac-ACHA-NH₂] = 0.001 M

Figure 99 A competition plot between ligands: Ac-AHCA-NH₂, Ac-ACHA-NH₂ and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 8. Conditions: 298 K, I = 0.1 M NaClO₄, [Ni(II)] = [Ac-AHCA-NH₂] = [Ac-ACHA-NH₂] = 0.001 M

Figure 100 A competition plot between ligands: Ac-AHCA-NH₂, Ac-ACHA-NH₂, Ac-CC-NH₂, Ac-GSCCHTGNHD-NH₂, Ac-EEGCCHGHHE-NH₂, Ac-CCSTSDSHHQ-NH₂ and Zn(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 8 and [187]. Conditions: 298 K, I = 0.1 M NaClO₄, [Zn(II)] = [Ac-AHCA-NH₂] = [Ac-ACHA-NH₂] = [Ac-CC-NH₂] = [Ac-GSCCHTGNHD-NH₂] = [Ac-CCSTSDSHHQ-NH₂] = 0.001 M

Figure 101 A competition plot between ligands: Ac-AHCA-NH₂, Ac-ACHA-NH₂, Ac-GHEITHG-NH₂, Ac-GHTIEHG-NH₂ and Zn(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 8 and [591]. Conditions: 298 K, I = 0.1 M NaClO₄, [Zn(II)] = [Ac-AHCA-NH₂] = [Ac-ACHA-NH₂] = [Ac-GHEITHG-NH₂] = [Ac-GHTIEHG-NH₂] = 0.001 M

Figure 102 A competition plot between ligands: Ac-AHCA-NH₂, Ac-ACHA-NH₂, Ac-CC-NH₂, Ac-GSCCHTGNHD-NH₂, Ac-EEGCCHGHHE-NH₂, Ac-CCSTSDSHHQ-NH₂ and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 8 and [187]. Conditions: 298 K, I = 0.1 M NaClO₄, [Ni(II)] = [Ac-AHCA-NH₂] = [Ac-ACHA-NH₂] = [Ac-CC-NH₂] = [Ac-GSCCHTGNHD-NH₂] = [Ac-CCSTSDSHHQ-NH₂] = 0.001 M

Figure 103 A competition plot between ligands: Ac-AHCA-NH₂, Ac-ACHA-NH₂, Ac-GGKPDLRPCHP-NH₂, Ac-PCHYIPRPKPR-NH₂, Ac-PCHPPCHYIPR-NH₂ and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 8 and [610]. Conditions: 298 K, I = 0.1 M NaClO₄, [Ni(II)] = [Ac-AHCA-NH₂] = [Ac-ACHA-NH₂] = [Ac-GGKPDLRPCHP-NH₂] = [Ac-PCHYIPRPKPR-NH₂] = [Ac-PCHPPCHYIPR-NH₂] = 0.001 M

Figure 104 A competition plot between the Ac-AHCA-NH₂ peptide, Zn(II) and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 8. Conditions: 298 K, I = 0.1 M NaClO₄, [Zn(II)] = [Ni(II)] = [Ac-AHCA-NH₂] = 0.001 M

Figure 104 A competition plot between the Ac-ACHA-NH₂ peptide, Zn(II) and Ni(II), describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 8. Conditions: 298 K, I = 0.1 M NaClO₄, [Zn(II)] = [Ni(II)] = [Ac-ACHA-NH₂] = 0.001 M

Figure 105 Amino acid sequence of ZnuA transporter; probable Zn(II) binding sites are highlighted in different colors

Figure 106 Amino acid sequence of AztC transporter; probable Zn(II) binding sites are highlighted in different colors

Figure 107 ESI-MS spectrum of A) Zn(II)-Ac-HEHEHEHEHEHEHEHEH-NH₂ and B) Ni(II)-Ac-HEHEHEHEHEHEHEHEH-NH₂

Figure 108 ESI-MS spectrum of A) Zn(II)-Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDS-NH₂ and B) Ni(II)-Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDS-NH₂

Figure 109 ESI-MS spectrum of A) Zn(II)-Ac-GGGHYHYIDGKAVFHAG-NH₂ and B) Ni(II)-Ac-GGGHYHYIDGKAVFHAG-NH₂

Figure 110 Species distribution diagram for the formation of Zn(II) complex with the Ac-HEHEHEHEHEHEHEH-NH₂ ZnuA fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

Figure 111 Proposed binding mode for Zn(II)-Ac-HEHEHEHEHEHEHEH-NH₂ fragment of ZnuA protein at pH 7.4 (ZnHL form - {4N_{im}} binding mode). The ZnuA structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

Figure 112 Species distribution diagram for the formation of Ni(II) complex with the Ac-HEHEHEHEHEHEHEH-NH₂ ZnuA fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 113 Proposed binding mode for Ni(II)-Ac-HEHEHEHEHEHEHEH-NH₂ fragment of ZnuA protein at pH 7.4 (NiL form - {6N_{im}} binding mode). The ZnuA structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

Figure 114 CD spectra of Ni(II) complex with the Ac-HEHEHEHEHEHEHEH-NH₂ fragment from ZnuA protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 115 UV-Vis spectra of Ni(II) complex with the Ac-HEHEHEHEHEHEHEH-NH₂ fragment from ZnuA protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 116 Species distribution diagram for the formation of Zn(II) complex with the Ac-GGEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ ZnuA fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 117 Proposed binding mode for Zn(II)-Ac-GGEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ fragment of ZnuA protein at pH 7.4 (ZnH₂L form - {4N_{im}} binding mode). The ZnuA structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

Figure 118 Species distribution diagram for the formation of Ni(II) complex with the Ac-GGEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ ZnuA fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 119 Proposed binding mode for Ni(II)-Ac-GGEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ fragment of ZnuA protein at pH 7.4 (NiH₂L form - {3N_{im}, N⁻} binding mode). The ZnuA structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

Figure 120 CD spectra of Ni(II) complex with the Ac-GGEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ fragment from ZnuA protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 121 UV-Vis spectra of Ni(II) complex with the Ac-GGEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ fragment from ZnuA protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 122 Species distribution diagram for the formation of Zn(II) complex with the Ac-GGGHYHYIDGKAVFHAG-NH₂ AztC fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

Figure 123 Proposed binding mode for Zn(II)-Ac-GGGHYHYIDGKAVFHAG-NH₂ fragment of AztC protein at pH 7.4 (ZnH₂L form - {3N_{im}, H₂O} binding mode). The AztC structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

Figure 124 Species distribution diagram for the formation of Ni(II) complex with the Ac-GGGHYHYIDGKAVFHAG-NH₂ AztC fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 125 Proposed binding mode for Ni(II)-Ac-GGGHYHYIDGKAVFHAG-NH₂ fragment of AztC protein at pH 7.4 (NiH₃L form - {3N_{im}} binding mode). The AztC structure is based on a Phyre2 simulation [593], and the figure was generated by PyMOL [595]

Figure 126 CD spectra of Ni(II) complex with the Ac-GGGHYHYIDGKAVFHAG-NH₂ fragment from AztC protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 127 UV-Vis spectra of Ni(II) complex with the Ac-GGGHYHYIDGKAVFHAG-NH₂ fragment from AztC protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 128 CD spectra of A) Ac-HEHEHEHEHEHEHEHEH-NH₂ and its complexes with b) Zn(II) and C) Ni(II); optical path 0.1 mm, Ni(II)/L molar ratio = 1 : 1

Figure 129 CD spectra of A) Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂ and its complexes with b) Zn(II) and C) Ni(II); optical path 0.1 mm, Ni(II)/L molar ratio = 1 : 1

Figure 130 CD spectra of A) Ac-GGGHYHYIDGKAVFHAG-NH₂ and its complexes with b) Zn(II) and C) Ni(II); optical path 0.1 mm, Ni(II)/L molar ratio = 1 : 1

Figure 131 A competition plot between ZnuA fragments: Ac-HEHEHEHEHEHEHEHEH-NH₂, Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂, AztC fragment: Ac-GGGHYHYIDGKAVFHAG-NH₂ and Zn(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 9. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ac-HEHEHEHEHEHEHEHEH-NH₂] = [Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂] = [Ac-GGGHYHYIDGKAVFHAG-NH₂] = 0.001 M

Figure 132 A competition plot between ZnuA fragments: Ac-HEHEHEHEHEHEHEHEH-NH₂, Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂, AztC fragment: Ac-GGGHYHYIDGKAVFHAG-NH₂, and Ni(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 9. Conditions: T=298 K, I=0.1 M NaClO₄, [Ni(II)] = [Ac-HEHEHEHEHEHEHEHEH-NH₂] = [Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂] = [Ac-GGGHYHYIDGKAVFHAG-NH₂] = 0.001 M

Figure 133 A competition plot between ZnuA fragments: Ac-HEHEHEHEHEHEHEHEH-NH₂, Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂, AztC fragment: Ac-GGGHYHYIDGKAVFHAG-NH₂, AspF2 fragment: Ac-PNCHTHEGGQLHCT and Zn(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 9 and [493]. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ac-HEHEHEHEHEHEHEHEH-NH₂] = [Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂] = [Ac-GGGHYHYIDGKAVFHAG-NH₂] = [Ac-PNCHTHEGGQLHCT] = 0.001 M

Figure 134 A competition plot between ZnuA fragments: Ac-HEHEHEHEHEHEHEHEH-NH₂, Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂, AztC fragment: Ac-GGGHYHYIDGKAVFHAG-NH₂, clavanin A: VFQFLGKIIHHVGNFVHGFSHFV-COOH, clavanin B: VFQFLGRIIHHVGNFVHGFSHFV-COOH, clavanin C: VFHLLGKIIHHVGNFVYGFSHVF-COOH, clavanin D: AFKLLGRIIHHVGNFVYGFSHVF-COOH, clavanin E: LFKLLGKIIHHVGNFVHGFSHFV-COO and Zn(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 9 and [615]. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ac-HEHEHEHEHEHEHEHEH-NH₂] = [Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂] = [Ac-GGGHYHYIDGKAVFHAG-NH₂] = [VFQFLGKIIHHVGNFVHGFSHFV-COOH] = [VFQFLGRIIHHVGNFVHGFSHFV-COOH] = [VFHLLGKIIHHVGNFVYGFSHVF-COOH] = [AFKLLGRIIHHVGNFVYGFSHVF-COOH] = [LFKLLGKIIHHVGNFVHGFSHFV-COOH]

Figure 135 A competition plot between ZnuA fragments: A) Ac-HEHEHEHEHEHEHEHEH-NH₂, B) Ac-GGEHEHEHEHEHEHEHEHDGHHGHAEEQAHHDDHDSG-NH₂, C) AztC fragment: Ac-GGGHYHYIDGKAVFHAG-NH₂ and Zn(II) describes complex formation at different pH values in a hypothetical situation in which equimolar

amounts of all reagents are mixed. Calculations are based on binding constants from Table 9. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ac-HEHEHEHEHEHEHEH-NH₂] = [Ac-GGEHEHEHEHEHEHEHEHDGHGHAEEQAHHDDHDSG-NH₂] = [Ac-GGGHYHYIDGKAVFHAG-NH₂] = 0.001 M

Figure 136 Amino acid sequence of AdcA transporter; probable Zn(II) binding sites are highlighted in different colors

Figure 137 Species distribution diagram for the formation of Zn(II) complex with the Ac-EGHGHKGHHHA-NH₂ AdcA fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

Figure 138 Species distribution diagram for the formation of Ni(II) complex with the Ac-EGHGHKGHHHA-NH₂ AdcA fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 139 CD spectra of Ni(II) complex with the Ac-EGHGHKGHHHA-NH₂ fragment from AdcA protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 140 UV-Vis spectra of Ni(II) complex with the Ac-EGHGHKGHHHA-NH₂ fragment from AdcA protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 141 Species distribution diagram for the formation of Zn(II) complex with the Ac-HGIKSQKAEHFH-NH₂ AdcA fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Zn(II)/L molar ratio = 1 : 1

Figure 142 Species distribution diagram for the formation of Ni(II) complex with the Ac-HGIKSQKAEHFH-NH₂ AdcA fragment, T = 298 K, I = 0.1 M NaClO₄, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 143 CD spectra of Ni(II) complex with the Ac-HGIKSQKAEHFH-NH₂ fragment from AdcA protein in the range 240 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 144 UV-Vis spectra of Ni(II) complex with the Ac-HGIKSQKAEHFH-NH₂ fragment from AdcA protein in the range 200 – 800 nm an pH range 2.5 – 10.5, T = 298 K, optical path = 1 cm, [L] = 0.0005 M, Ni(II)/L molar ratio = 1 : 1

Figure 145 CD spectra of A) Ac-GIKSQAHEFH-NH₂ and its complexes with b) Zn(II) and C) Ni(II); optical path 0.1 mm, Ni(II)/L molar ratio = 1 : 1

Figure 146 CD spectra of A) Ac-HGIKSQKAEHFH-NH₂ and its complexes with b) Zn(II) and C) Ni(II); optical path 0.1 mm, Ni(II)/L molar ratio = 1 : 1

Figure 147 A competition plot between AdcA fragments: Ac-EGHGHKGHHHA-NH₂, Ac-HGIKSQKAEHFH-NH₂ and Zn(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 10. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ac-EGHGHKGHHHA-NH₂] = [Ac-HGIKSQKAEHFH-NH₂] = 0.001 M

Figure 148 A competition plot between AdcA fragments: Ac-EGHGHKGHHHA-NH₂, Ac-HGIKSQKAEHFH-NH₂ and Ni(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 10. Conditions: T=298 K, I=0.1 M NaClO₄, [Ni(II)] = [Ac-EGHGHKGHHHA-NH₂] = [Ac-HGIKSQKAEHFH-NH₂] = 0.001 M

Figure 149 A competition plot between AdcA fragment: Ac-EGHGHKGHHHA-NH₂, Zn(II) and Ni(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 10. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ni(II)] = [Ac-EGHGHKGHHHA-NH₂] = 0.001 M

Figure 150 A competition plot between AdcA fragment: Ac-HGIKSQKAEHFH-NH₂, Zn(II) and Ni(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 10. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Ni(II)] = [Ac-HGIKSQKAEHFH-NH₂] = 0.001 M

Figure 151 A competition plot between AdcA fragment: Ac-EGHGHKGHHHA-NH₂, Zn(II) and Ni(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 10; hypothetical metal concentrations are taken from the literature [472], [617] and equal: [Zn(II)] = 0.25 µg/ml, [Ni(II)] = 0.0019 µg/ml (in people without orthodontic treatment). Conditions: T=298 K, I=0.1 M NaClO₄

Figure 152 A competition plot between AdcA fragment: Ac-HGIKSQKAEHFH-NH₂, Zn(II) and Ni(II) describes complex formation at different pH values in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 10; hypothetical metal concentrations are taken from the literature [472], [617] and equal: [Zn(II)] = 0.25 µg/ml, [Ni(II)] = 0.0019 µg/ml (in people without orthodontic treatment). Conditions: T=298 K, I=0.1 M NaClO₄

Figure 153 A competition plot between Zn(II) and Aspf2, ZrfC, ZnuA, AztC and AdcA fragments, describes complex formation at pH 7.4 in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 6, Table 7, Table 9 and Table 10. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Aspf2, ZrfC, ZnuA, AztC and AdcA fragments] = 0.001 M

Figure 154 Enlargement of the competition plot between Zn(II) and Aspf2, ZrfC, ZnuA, AztC and AdcA fragments, describes complex formation at pH 7.4 in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 6, Table 7, Table 9 and Table 10. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Aspf2, ZrfC, ZnuA, AztC and AdcA fragments] = 0.001 M

Figure 155 A competition plot between Ni(II) and Aspf2, ZrfC, ZnuA, AztC and AdcA fragments, describes complex formation at pH 7.4 in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 6, Table 7, Table 9 and Table 10. Conditions: T=298 K, I=0.1 M NaClO₄, [Ni(II)] = [Aspf2, ZrfC, ZnuA, AztC and AdcA fragments] = 0.001 M

Figure 156 Enlargement of the competition plot between Ni(II) and Aspf2, ZrfC, ZnuA, AztC and AdcA fragments, describes complex formation at pH 7.4 in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 6, Table 7, Table 9 and Table 10. Conditions: T=298 K, I=0.1 M NaClO₄, [Ni(II)] = [Aspf2, ZrfC, ZnuA, AztC and AdcA fragments] = 0.001 M

Figure 157 Enlargement of the competition plot between Zn(II) and Aspf2, ZrfC, ZnuA, AztC and AdcA fragments and model sequences, describes complex formation at pH 7.4 in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 6, Table 7, Table 8, Table 9 and Table 10. Conditions: T=298 K, I=0.1 M NaClO₄, [Zn(II)] = [Aspf2, ZrfC, ZnuA, AztC and AdcA fragments] = [Ac-ACHA-NH₂ and Ac-AHCA-NH₂] = 0.001 M

Figure 158 Enlargement of the competition plot between Ni(II) and Aspf2, ZrfC, ZnuA, AztC and AdcA fragments and model sequences, describes complex formation at pH 7.4 in a hypothetical situation in which equimolar amounts of all reagents are mixed. Calculations are based on binding constants from Table 6, Table 7, Table 8, Table 9 and Table 10. Conditions: T=298 K, I=0.1 M NaClO₄, [Ni(II)] = [Aspf2, ZrfC, ZnuA, AztC and AdcA fragments] = [Ac-ACHA-NH₂ and Ac-AHCA-NH₂] = 0.001 M

Figure 159 Schematic model of drugs based on STAMP technology. STAMPs are composed of two domains: AMP domain and a targeting domain.

Figure 160 The STAMP-based drugs A) do not interact with commensal cells, but B) accumulate around the targeted pathogenic cells and kill them.

Scheme 1 Schematic model of zincophore-based transport mechanism in *Aspergillus fumigatus*

Scheme 2 Schematic model of Zn(II) transport mechanism in *Paracoccus denitrificans*

Scheme 3 Schematic model of Zn(II) transport mechanism in *Streptococcus mutans*

XII. LIST OF PUBLICATIONS

PUBLICATIONS RELATED TO THE DOCTORAL THESIS:

1. Garstka Kinga, Potoczniak Gabriela, Kozłowski Henryk, Rowińska-Żyrek Magdalena, *Aspergillus fumigatus* ZrfC Zn(II) transporter scavengers zincophore-bound Zn(II), *Dalton Transactions* **2024**, 53, 2848-2858, DOI:10.1039/d3dt04083f
2. Garstka Kinga, Bellotti Denise, Wąty Joanna, Kozłowski Henryk, Remelli Maurizio, Rowińska-Żyrek Magdalena, Metal coordination to solute binding proteins – exciting chemistry with potential biological meaning, *Dalton Transactions* **2023**, 52, 16140-16150, DOI:10.1039/d3dt02417b
3. Garstka Kinga, Dzyhovskiy Valentyn, Wąty Joanna, Stokowa-Sołtys Kamila, Świątek-Kozłowska Jolanta, Kozłowski Henryk, Barceló-Oliver Miquel, Bellotti Denise, Rowińska-Żyrek Magdalena, CH vs. HC Promiscuous Metal Sponges in Antimicrobial Peptides and Metallophores, *Molecules* **2023**, 28, DOI:10.3390/molecules28103985
4. Garstka Kinga, Hecel Aleksandra, Kozłowski Henryk, Rowińska-Żyrek Magdalena, Specific Zn(II)-binding site in the C-terminus of Aspf2, a zincophore from *Aspergillus fumigatus*, *Metallomics* **2022**, 14, mfac042, DOI:10.1093/mtomcs/mfac042

PUBLICATIONS- ACCEPTED PUBLICATION- RELATED TO THE DOCTORAL THESIS:

5. Garstka Kinga, Hecel Aleksandra, Kozłowski Henryk, Rowińska-Żyrek Magdalena, AdcA lipoprotein involved in Zn(II) transport in *Streptococcus mutans* – not as metal-specific as expected?

PUBLICATIONS NOT RELATED TO THE DOCTORAL THESIS:

6. Hecel Aleksandra, Garstka Kinga, Kozłowski Henryk, Rowińska-Żyrek Magdalena, –HH and –HAAAH motifs act as fishing nets for biologically relevant metal ions in metallopeptides, *Journal of Inorganic Biochemistry* **2024**, 252, DOI:10.1016/j.jinorgbio.2023.112456
7. Dudek Dorota, Garstka Kinga, Miller Adriana, Dzień Emilia, Wąty Joanna, Hecel Aleksandra, Kozłowski Henryk, Rowińska-Żyrek Magdalena, Oddziaływanie przeciwdrobnoustrojowych peptydów z jonami metali- Relacja między chemią koordynacyjną, strukturą, termodynamiką a sposobem działania = The interaction of antimicrobial peptides with metal ions- The relationship between coordination chemistry, structure, thermodynamics and mode of action, *Wiadomości Chemiczne* **2022**, 76, 365-391, DOI:10.53584/wiadchem.2022.5.7

PUBLICATIONS- ACCEPTED PUBLICATION- NOT RELATED TO THE DOCTORAL THESIS:

8. Miller Adriana, Mikolajczyk Aleksandra, Bellotti Denise, Garstka Kinga, Watly Joanna, Hecel Aleksandra, Wieczorek Robert, Matera-Witkiewicz Agnieszka, Rowińska-Żyrek Magdalena, Zn(II) and Cu(II) coordination enhances the antimicrobial activity of piscidin 3, but not that of piscidin 1 and 2, *Inorganic Chemistry* **2024**

PUBLICATIONS- UNDER REVIEW- NOT RELATED TO THE DOCTORAL THESIS:

9. Leveraro Silvia, Garstka Kinga, Śliwka Paulina, Janek Tomasz, Rowińska-Żyrek Magdalena, Remelli Maurizio, Bellotti Denise, Metal coordination governs the antimicrobial efficacy of calcitermin derivatives
10. Leveraro Silvia, Dzyhovskiy Valentyn, Garstka Kinga, Bellotti Denise, Stokowa-Softys Kamila, Remelli Maurizio, Rowińska-Żyrek Magdalena, Metal-induced amide deprotonation and binding – typical for Cu(II), not possible for Zn(II) and Fe(II)

XIII. SCIENTIFIC ACTIVITY

INTERNATIONAL CONFERENCES:

1. Participation in the scientific conference: 45th International Conference on Coordination Chemistry **ICCC 2024**, 28.07-03.08.2024 Port Collins, **Poster**: „Zincophore-based Zn(II) transport mechanism in *Aspergillus fumigatus*”
2. Participation in the scientific conference: International Conference on Metal Binding Peptides: Methodologies and Applications **MBP 2024**, 10.06-12.06.2024 Toulouse, **Presentation**: „*Aspergillus fumigatus* zincophore and zinc transporter interactions with Zn(II) and Ni(II)”
3. Participation in the scientific conference: International Symposium on Thermodynamics of Metal Complexes **ISMEC 2024**, 10.07-12.07.2024 Nice, **Presentation**: „Interactions of the AspF2 zincophore from *Aspergillus fumigatus* with its zinc transporter, ZrFC”
4. Participation in the scientific conference: **CanBIC-8**, 23.05-27.05.2023 Parry Sound, **Presentation**: „The C-terminal fragment of the *Aspergillus fumigatus* AspF2 zincophore is its most probable Zn(II) binding site”
5. Participation in the scientific conference: International Symposium on Metal Complexes **ISMEC 2022**, 05.06-08.06.2022 Valencia, **Poster**: „Specific Zn(II) binding sites in AspF2, a zincophore from *Aspergillus fumigatus*”
6. Participation in the scientific conference: International Conference on Metal Binding Peptides: Methodologies and Applications **MBP 2022**, 05.07-08.07.2022 Nancy,

Poster: „Specific Zn(II) binding sites in C- terminus of Aspf2, a zincophore from *Aspergillus fumigatus*”

7. Participation in the scientific conference: International Symposium on Thermodynamics of Metal Complexes **ISMEC 2021**, 16.06-18.06.2022 Białystok,
Poster: „Zinc binding sites in Aspf2, a zincophore from *Aspergillus fumigatus*”

NATIONAL CONFERENCES:

8. Participation in the scientific conference: 19th National Seminar of Doctoral Students and Students- „**On the Border of Chemistry and Biology 2023**”, 04.06-07.06.2023 Trzebieszowice, **Presentation:** „Coordination of metal ions to unstructured protein fragments involved in Zn(II) transport and its potential biological applications”
9. Participation in the scientific conference: **WrocChem2023**, 12.06.2023 Wrocław, **Presentation:** „Potential biological applications of unstructured protein fragments involved in Zn(II) transport”
10. Participation in the scientific conference: 65th Scientific Congress of the Polish Chemical Society **PTChem2023**, 18-22.09.2023 Toruń,
Presentation: „Methods of coordinating metal ions to unstructured protein fragments involved in Zn(II) transport in pathogens”

SCIENTIFIC PROJECTS:

1. 10.2020- present – **NCN Maestro**: Metalofory- zrozumienie (i wizualizacja) transportu metali w patogenach, by pomóc gospodarzowi oszukać najeźdźców rola,
Project Coordinator: **prof. Henryk Kozłowski**
2. 01.01.2020- present – **NCN Sonata Bis**: Oddziaływanie przeciwdrobnoustrojowych peptydów z jonami metali- zrozumienie relacji między chemią koordynacyjną, strukturą, termodynamiką a sposobem działania, Project Coordinator: **dr hab. Magdalena Rowińska-Żyrek, prof. UWr**

SCHOLARSHIPS AND INTERNSHIPS:

1. CA18202- Network for Equilibria and Chemical Thermodynamics Advanced Research
01.09- 31.09.2021 Ferrara
2. 1st ISMEC – NECTAR Training School on the Determination, Analysis and Use of Thermodynamic Data SOLvE – Advances in SOLution Equilibria
26.07-27.07.2021

3. 2nd ISMEC – NECTAR Training School on the Determination, Analysis and Use of Thermodynamic Data SOLvE – Advances in SOLution Equilibria
25.07-27.07.2022
4. Certified completion of AFM and FTIR imaging workshops, 24.01.2024 Wrocław
5. Certified completion of chromatography training in Design of Experiments (DoE), Analytical Quality by Design (AQbD) and LabSolutions Method Development software, 08.05.2023 Wrocław
6. Certified completion of training in the operation of the SHIMADZU LC-40 series HPLC chromatograph and LabSolutions software, 06.10.2022 Wrocław

COMPETITIONS AND PRIZES:

1. Young Researcher Grant to cover the costs of active participation in the international scientific conference: 45th International Conference on Coordination Chemistry, ICCC 2024
2. Short-Term Scientific Mission GRANT COST Action CA18202, 11.09-10.10.2021 Ferrara, University of Ferrara, Italy
3. Award for the best oral presentation: WrocChem2023, 12.06.2023 Wrocław
4. Award for the best poster presentation: International Symposium on Metal Complexes ISMEC 2022, 05.06-08.06.2022 Valencia

OTHER:

1. 11.05.2024, 18.05.2024 Wrocław, Children's University
Lecture: „How the soap like soap washes away dirt?”
2. 28.01.2024 Żórawina, Grand Finale- Great Orchestra of Christmas Charity Foundation
Chemical shows
3. 16.11.2023 Wrocław, Wrocław Science and Technology Days
Lecture: „How to overcome antibiotic resistance?”

09.09.2023 Wrocław, Children's University
Lecture: „How to create smoke, flame and explosive mixture?”