

**INTERACTION OF BIOINSPIRED
MULTIHISTIDINE LIGANDS WITH ZINC(II),
COPPER(II) AND NICKEL(II) IONS**

Abstract of the PhD thesis

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Introduction and the aims of the work

This thesis lies inside the domain of bioinorganic chemistry, a borderland that embraces the chemistry of metal-containing molecules within biological systems, since many metal ions are of vital importance for living creatures. This field is concerned with the control and use of metal ions in biochemical processes. To our knowledge up to now, approx. 30% of the enzymes catalysing biochemical processes contain metal ion(s). The effect of transition metals is exerted almost exclusively by binding to biomolecules, mostly to proteins. Learning about their role in proteins/enzymes is essential to understand the mechanisms of their actions. Modern bioinorganic chemistry has two important research directions; one is focusing on the more and more detailed exploration of the function and mechanisms of metalloproteins/enzymes, and the other is the development of artificial proteins/enzymes possessing the potential for future practical applications.

These kinds investigations can be classified into two parts supplementing each other, the study of (i) native proteins/enzymes and (ii) their small model complexes. Both approaches have advantages and disadvantages. The investigations of macromolecules are difficult. Many times the provided information are difficult to interpret because of the complexity of the system. Nevertheless, these studies are irreplaceable. On the other hand, even the best model complexes are only able to give a distorted picture of the metal binding site. However, they are much simpler than proteins, thus, they can be handled and examined easier. They present opportunity to gain knowledge on the role and features of some parts of the full processes and structural motifs. Often this is impossible for the native systems. Last but not least efficient model systems may help in developing proteins with therapeutic use, practical application and develop biomimetic catalysts/artificial enzymes.

Based on our present knowledge among the amino acids the side chain donors of histidine and cysteine form the strongest interaction with most transition metal ions. It is in line with the fact that the metal binding sites of the proteins are usually built from these amino acids. Since the imidazole ring plays a fundamental role in the metal binding sites of proteins, the metal complexes of many histidine-containing peptides have been studied in the previous decades. However, only few of these compounds could be considered as adequate models for the metal ion–protein interaction, since investigations of multihistidine peptides having 3–4 histidine units are very scarce. Precipitation occurred almost in all previously studied zinc(II)-containing model systems and amide-coordinated species are predominant in the physiological pH range for almost all previously studied peptides in the presence of copper(II) ion. That eliminates the structural and functional analogy between the proteins to be modelled and the peptides investigated. Probably, this is also a reason for the mentioned fact that metallopeptides have not yet been investigated as functional models for hydrolytic and oxidative enzymes. Another difficulty of model studies to be overcome is that the environment of the active centers has a fixed structure in most cases. Furthermore, the metal binding sites are often far away from each other in the amino acid sequence. These facts render the metalloenzyme mimicking by (small) peptides very difficult.

However, recent biochemical studies pointed out the presence of relatively short, histidine-rich subunits with no fixed structure in a large number of proteins and enzymes that operate more or less independently from other parts of the biomolecule, while having a great importance in view of the given function. These sequences have strong metal binding ability in most cases and the metal ion coordination – according to various biological studies – determines or contributes to the function of the protein/enzyme.

The main goal of our research was the investigation of the metal binding properties of such bioinspired, relatively short histidine-rich sequences,

including peptide fragments with potential therapeutic use, frequently appearing in proteins and enzymes.

The first main chapter is about the structural modelling of the metal binding site of the human histidine-rich glycoprotein (HRG) with the zinc(II) and copper(II) complexes of the tandem-repeat pentapeptide fragment **Ac-HHPHG-NH₂ (HP1)** and its 10-mer dimer **Ac-(HHPHG)₂-NH₂ (HP2)**. They are the minimum metal binding motifs of the histidine-rich region (HRR) and have been synthesized with protections at both termini. HRG is an abundant plasma protein having a central HRR with tandem sequence repetition (in humans (G)HHPH(G) is repeated 12 times). The His-rich domain of HRG has a remarkable ability to bind divalent metal ions, e.g. Zn(II) and Cu(II). HRG was found to regulate numerous biological processes, e.g. angiogenesis, cell adhesion and migration, fibrinolysis and coagulation. The metal ion (mostly Zn(II)) binding of the HRR region has probably a crucial importance in these functions, i.e. the coordination of Zn(II) to the HRR promotes the binding of HRG to other proteins and receptors. In order to get a deeper insight into the mechanism of the HRG functions and to clarify the role of metal ions, it is essential to characterize the metal binding properties of the macromolecule. This may be achieved by model studies on the metal complexes of specific peptide fragments of the HRR.

In the second part of the work we report on the solution chemical investigation of the zinc(II) and copper(II) complexes of **HSHRDFQPVLHL-NH₂** peptide, which is identical with the N-terminal fragment of human endostatine. Endostatine, a zinc-containing protein (approx. 20 kDa), being present also in humans, is a widely studied molecule due to its well documented antitumor activity provided without any side effects. The antitumor activity of the protein is caused by its antiangiogenic and antimigrating effects. The antitumor activity and mechanism of the action of the 25-amino-acid N-terminal fragment and the full protein are equivalent, which is of crucial importance for the sake of future therapeutic use. The presence of the metal ion is necessary to

exert the antitumor effect in both cases. The details of the zinc(II) ion interaction and the solution structure, especially for the N-terminal fragment possessing antitumor activity, is not known. Interestingly, endostatine also possesses an amino terminal Cu(II)- and Ni(II)-binding (ATCUN) motif, an efficient copper(II) binding site found in the N-terminus of many naturally occurring proteins.

The importance of endostatine and HRG lies behind their antitumor features. The antitumor activity of both proteins is assigned to their antiangiogenic properties, which is suggested to operate through their binding to the macromolecules participating in the control of angiogenesis. This binding is modulated by metal ions, such as zinc(II) or copper(II). It is worth noting at this point that a high angiogenic activity most often requires high levels of copper, and its reduction has recently been suggested as a therapeutic tool to control cancer growth. Multihistidine peptides can be ideal for the reduction of local copper(II) concentration.

The **HCDLPCG-NH₂** sequence is the N-terminal fragment of a recently discovered superoxide-dismutase (SOD) family. It contains only one histidine unit. The investigation of the metal-binding abilities of the sequence makes the third pillar of my thesis. Superoxide radical ($O_2^{\cdot -}$) is a toxic byproduct of aerobic respiration. Besides many free radicals are scavenged by dioxygen to form superoxide. Therefore, the SOD enzymes, catalyzing the disproportionation of superoxide, are the major regulators of free radical and reactive oxygen species balance in organisms. The aforementioned SOD family, occurring in *Streptomyces* bacteria, contains nickel and shows no homology with the SODs of higher organisms. The nickel center, responsible for the catalytic activity of the enzyme, is exclusively bound to the His1, Cys2 and Cys6 amino acids both in its oxidized (Ni^{III}) and reduced (Ni^{II}) forms. The well-conserved N-terminal sequence ¹HCDXPC– (X = G or L) provides almost all interactions critical for metal binding and catalysis. The investigation of Ni(II) complexes of the N-

terminal fragment forming the active center of these enzymes, and the comparison to the well-known Cu,Zn-SOD and Mn/Fe-SOD provide a possibility to explore the most efficient strategy of superoxide dismutation that may lead to the development of *e.g.* highly efficient antioxidant agents.

In spite of the short length of the above mentioned peptide fragments, other segments of the protein do not play a role in the metal binding, the amino acids forming the metal binding sites are close to each other. The histidine-rich metal binding sites, “developed” by nature, form an environment that is similar to the active centers of metalloproteins. A further common feature of endostatin and Ni-SOD is the lack of preorganized structures, since these peptides are located in N-terminal fragments. In case of HRG fragments the structural analogy was increased with protections of the amino and carboxylate groups at the termini. Based on these facts it may eliminate the serious, repeatedly mentioned disadvantage of short peptides as models *i.e.* the effects derived from the tertiary structure and conformation of the macromolecule can not prevail at the investigation of small molecules. Accordingly, the chosen bioinspired fragments without any modifications can be suitable to reach our goal, namely the binding of metal ions exclusively by side chain donor groups and therefore providing catalytic functions.

Beside the more and more detailed exploration of the functioning and mechanisms of proteins/metalloenzymes, bioinorganic chemistry has another important research direction focusing on the development of artificial proteins/enzymes having the potential for future practical applications. The functional and structural modelling of metallohydrolases with hydrolytic activity and type 2 and 3 copper-containing enzymes participating in the catalysis of the oxidation of various organic molecules attracted special attention in recent years. A number of model compounds of metallohydrolases or copper-containing oxidases are known from the literature. These compounds are, almost without exception, metal complexes of synthetic ligands. For the last few years,

metal ion–peptide systems have not been studied at all from this point of view, because amide-coordinated species were predominant in the physiological pH range in almost all previously studied copper(II)–peptide system. Hereby, the structural and functional analogy between the proteins to be modelled and the investigated peptides have been eliminated. The coordination of amide nitrogens significantly reduces the Lewis-acid character of the metal ion and stabilizes the +2 oxidation state of copper leading to the dramatic decrease of catalytic activity in both types of reactions. On the other hand research on metal complexes of histidine-containing peptides pointed out that the coordination mode of the ligand depends considerably on the number and position of the histidine subunits in the peptide sequence and the quality of the surrounding donor groups. In our research group we managed to prevent the coordination of amide nitrogen around neutral pH with a suitably chosen peptide sequence and created accurate functional and structural enzyme models.

In the fourth chapter of the thesis we follow the recent strategy and report the coordination chemical behavior of a novel type ligand in the presence of zinc(II) and copper(II) ions. Based on our previous experiences, we designed and prepared the **(His)₄-(Lys)₂-Lys-CONH₂** dendrimer type heptapeptide – consisting of three lysines and four histidines. Two lysines have been coupled to the α and ϵ amino groups of the C-terminal. Four histidines have been coupled to the 2-2 different amino groups of the second generation lysines. The branches of the dendrimer peptide are more flexible than peptides with straight chain, thus the density of the metal binding site might be higher. The ligand possesses eight primary nitrogen donors, thus this compound may form a binuclear metal complex that behaves as an efficient nuclease and/or oxidase enzyme model.

Experimental

Peptides were prepared by solid phase peptide synthesis. The (de)protonation constants of the ligands, the composition and formation constants of the zinc(II), copper(II) and nickel(II) complexes were calculated on the basis of experimental data of pH-potentiometric titrations using the PSEQUAD and SUPERQUAD computer programs. Titrations were performed mostly in aqueous solution (in case of the endostatin fragment in 80/20 (w/w%) dimethyl sulfoxide/water mixture, too) at temperature 298.0 ± 0.1 K, at ionic strength 0.1 mol/dm^3 under inert atmosphere. UV-visible and circular dichroism (CD) spectra were recorded in presence of copper(II) and nickel(II) ions to follow the complex formation and determine the solution structure of the developed species. The pH dependent UV-visible and CD spectra were evaluated by the computer program PSEQUAD, resulting the individual spectra of the different metal complexes. Synchrotron Radiation Circular Dichroism (SRCD) measurements were performed in the 180 – 260 nm wavelength range in the presence and absence of metal ions to get deeper insight into the geometrical change of the HRG peptides. With the aid of 1D and 2D $^1\text{H-NMR}$ measurements we could better describe the quality, the number of coordinating donor atoms and the environment of the metal center. In order to better clear up the structure of copper(II) complexes, pH dependent EPR spectra were recorded, too. The oxidative and hydrolytic activities of the potential enzyme mimicking species were tested in various assays. The SOD activity of the Ni-SOD model was determined by the inhibition of the reaction between nitroblue-tetrazolium-chloride and $\text{O}_2^{\bullet-}$, formed *in situ* during the reaction of xantine/xantine-oxidase. The pirocatechin-oxidase activity of the **(His)₄-(Lys)₂-Lys-CONH₂** – copper(II) system was determined by the oxidation of 3,5-ditertbuthyl-pirocatechin by spectrophotometry. The hydrolytic effect of the metal complexes of the dendrimer peptide were checked by the gel electrophoresis of circular DNA type pUC18.

New scientific results

The metal binding properties of Ac-HHPHG-NH₂ (HP1) and Ac-HHPHGHPHG-NH₂ (HP2) peptides related to the histidine-rich region of HRG

1 Both peptides show a SRCD pattern resembling to that of the polyproline II structure, similarly to that of the His-Pro-rich domain of the HRG protein. Exclusive coordination of the sidechain imidazoles of the peptides has been observed with both zinc(II) and copper(II) ions in the acidic and neutral pH range.

2. In alkaline solution precipitate occurred in the presence of zinc(II) and various amide-coordinated species formed in the copper(II) containing systems. On the basis of EPR measurements dimer complexes formed between pH 7-10 in the 1 to 1 ratio **HP1**-copper(II) and in the 1 to 2 ratio **HP2**-copper(II) systems.

3. In contrast to the pentapeptide, **HP2** provides a high affinity binding site for both metal ions around neutral pH, by the exclusive coordination of at least four side chain imidazole donors. The conformational change of **HP2** during the coordination of the first metal ion creates a favored binding site for the second metal ion, resulting in an important extra stabilization (Δ) for the $[M_2L]^{4+}$ complexes, as compared to the $[ML]^{2+}$ species of the shorter sequence: $\Delta_{Zn} = \log\beta_{Zn_2(HP2)} - 2 \times \log\beta_{Zn(HP1)} = 1.91$ and $\Delta_{Cu} = \log\beta_{Cu_2(HP2)} - 2 \times \log\beta_{Cu(HP1)} = 2.08$). Such conformational changes may explain the cooperative metal binding in our system, but whether similar changes occur in the native HRG protein upon metal binding is a question yet to be answered.

The metal binding properties of HSHRDFQPVLHL-NH₂ (L) peptide, identical with the N-terminus of endostatine

4. The **HSHRDFQPVLHL-NH₂** fragment of the anti-angiogenic human endostatin forms stable {NH₂,3N_{im},COO⁻} coordinated complex with zinc(II) in the neutral pH-range. This coordination mode is probably identical to that present in the zinc(II) complex of the N-terminal 25-mer peptide fragment of human endostatin, which has identical antitumor effect to the entire protein.
5. There is an albumin-like {NH₂,N⁻,N⁻,N_{im}} binding mode already in the [CuL]⁺ complex around pH ~ 5. An {NH₂,3N_{im},COO⁻} coordinated isomer species can also be revealed in small amount.
6. The peptide has extremely high copper(II) binding affinity (K_D = 2.6×10⁻¹⁵ M at pH = 7.4), close to those of copper-containing metalloenzymes, and forms albumin-like {NH₂,N⁻,N⁻,N_{im}} coordinated [CuH₂L]⁻ complex in the neutral pH-range. Since copper(II) acts as an essential co-factor in angiogenesis, this finding may suggest that copper(II) binding is involved in the biological activity of endostatine.
7. HSHRDFQPVLHL-NH₂ is able to bind a second copper(II) ion. In alkaline pH range its environment can be described with {N_{im},3N⁻} structure.

The structural and functional modelling of Ni-SOD enzymes

8. The **HCDLPCG-NH₂** heptapeptide identical with the N-terminal sequence of the lately discovered nickel containing superoxid-dismutases provides a unique possibility to mimick the nickel binding site. The square planar geometry – wich can be observed in the native enzyme already around pH ~ 4 – is present above pH ~ 6 as a binding isomers. But the {NH₂,N⁻,S⁻,S⁻} coordinated structure which is typical for the enzyme forms around pH ~ 9 in 100% in the equimolar solution of the ligand and nickel(II) ion. At pH 9 the metal binding strength of the peptide ($K_D = 2.0 \times 10^{-15}$ M) is commensurable with the enzyme's one.

9. The *cis* geometry of the Pro5 amide bond in the Ni-SOD enzymes evolve paralell with the binding of nickel and the formed H-bridges stabilize this structure. The NMR spectra of the peptide recorded in the presence of nickel suggest a *cis-trans* isomerism of proline amide bond, but in the lack of the stabilizing H-bridges the *cis* conformation is not exclusive.

10. The [NiL]⁻ and [NiH₁L]²⁻ species possess excellent SOD activity (IC₅₀ = 1,9×10⁻⁶ M) among the Ni-complexes, but their activity is less with two order of magnitudes than the native enzyme's one. Probably the reason for this phenomenom is lieing behind the different conformation and in the lack of the directing/stabilizing effect of Tyr9 that regulates the coupling of the substrate.

The metal binding properties of the dendrimer type

(His)₄-(Lys)₂-Lys-CONH₂ peptide

11. The novel branched peptide type ligand consisting of three lysines as branching units and four histidines as functional groups provides due to its flexible branches bis-histamine type coordination for both copper(II) and zinc(II) ions up to pH ~ 8.

12. While above pH ~ 8 the precipitation of a neutral complex was observed for both metal ions, in the copper(II) containing systems it dissolved in alkaline solutions (pH > 11.0). The resulting complex in equimolar system displays deprotonated amide-nitrogen coordination, with fused five-membered chelate rings around the metal ion in $[\text{CuH}_3\text{L}]^-$.

13. The ligand is able to bind two metal ions even in alkaline solutions. Only one copper(II) is able to coordinate in the same manner in the $[\text{Cu}_2\text{H}_5\text{L}]^-$ species. The second metal ion is probably surrounded by two amide nitrogens and two other donor groups either by amino or imidazole groups.

14. The mononuclear $[\text{CuL}]^{2+}$ and the binuclear $[\text{Cu}_2\text{L}]^{4+}$ complexes showed low activity in oxidation of 3,5-ditertbutyl-catechol.

15. The hydrolytic assays showed a promising picture. Based on our experiment we may state, that the $[\text{Cu}_2\text{L}]^{4+}$ species is moderately effective at pH = 7.1, but we can't be confident whether in oxidative or hydrolytic manner. The same experiments have been performed with the resin-linked ligand in the presence of metal ions. The gel electrophoresis demonstrated a significant effect of the zinc(II) containing system, where after 18 hours all of the superhelical DNA turned into open circular form.

PUBLICATIONS

Papers directly related to the thesis

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