Ph.D. Thesis

MULTIFUNCTIONAL PEPTIDE COMPLEXES FOR MIMICKING THE METAL ION BINDING PROTEINS

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I. Introduction and the aim of the work

Enzymes are biomolecules that catalyze chemical reactions by lowering the activation energy, thus dramatically increasing the rate of the reaction. However, enzymes do differ from most other catalysts by being highly specific. Most of enzymes are proteins, about 30% of which contain metal ions. Metalloenzymes (enzymes that contain one or more metal atoms as functional parts of their structures) play an important role in biochemical processes. Therefore the understanding of their structure and function is of significant importance.

The study of metal complexes as models for metalloenzymes may provide a deeper insight into the enzymatic processes, and it also may serve as the basis for the development of artificial enzymes. The advantage of using low molecular weight metal complexes as models is their easy syntheses and characterization. The transition metal ion coordination of synthetic enzyme models has been widely studied. There is, however, much less known about the catalytic behaviour of the peptide complexes, although peptides look as appropriate models for proteins, because of their similar composition. On the other hand, the application of short metal-binding peptides for modelling faces several difficulties. It has been recognized for a long time, that in short peptides in most cases the amide nitrogen coordination appears in copper(II) or nickel(II) containing systems, and the hydrolysis of the metal ion in the case of zinc(II) usually occurs at physiological pH. At the same time the metal ion coordination in metalloenzymes occurs mainly through the amino acid side-chains of the protein. Moreover proteins may form a stable conformation as the result of intramolecular interactions, creating a proper cavity for the metal ion, while this is not possible for small peptides. All these prevent the formation of suitable models.

The above problems might be avoided by increasing the number of efficient donor groups, using medium size oligopeptides. As an example, the side-chain type coordination may be achieved by properly designed histidine containing peptides. The amino acid sequence, thus the metal binding site can easily be modified by solid phase peptide synthesis. The other advantage of metal binding peptides (or proteins), as models, is that these molecules are not foreign to the living organism, but are naturally

occurring compounds. In addition they can routinely be prepared and/or modified by means of recombinant DNA technique.

It is well-known, that histidine containing peptides exert a great variety of coordination modes toward metal ions. Although copper(II) coordination to the peptides with histidine at different positions have extensively been investigated, there is still disagreement about the existence and the solution structure of some minor species in aqueous solutions. Our aim was to reveal the structural modalities of the complexes using circular dicroism (CD) spectroscopy, and to use our findings in initial predictions on the structure of more complicated systems. We have investigated the copper(II) complexes of the following tripeptides: **HisGlyGly, GlyHisGly, GlyGlyHis, HisAlaAla, AlaHisAla, AlaAlaHis, Ac-HisGlyGly-NH_2**.

However, if only one histidine amino acid is present in the peptide, it behaves as an anchor for the amide nitrogen deprotonation and coordination in the case of copper(II) oligopeptides and still the hydrolysis of the zinc(II) ions takes place. These processes can be prevented by multihistidine type peptides forming exclusively side-chain imidazole-nitrogen and (deprotonated) water molecule coordination around physiological pH. Such peptide complexes might therefore be of significant importance, and have recently been widely studied. The results, however, showed that even three or more histidine residues in the peptide sequence are not able to avoid the above mentioned problems. For the above reasons our main goal was to design, synthesize and investigate the copper(II), nickel(II) and zinc(II) complexes of appropriate multihistidine, N- and C-terminally protected peptide sequences, by inserting one or two proline residue(s), that serve(s) as “break-point(s)” in the consecutive amide nitrogen deprotonation. We have also inserted charged and polar amino acids in the peptide sequence, to increase the solubility of the complexes around the neutral pH. The sequences of the designed peptides were the following: **Ac-HisProHisHis-NH_2, Ac-HisProHisProHis-NH_2 and Ac-LysHisProHisProHisGln-NH_2**.

As highlighted above, the application of metal complexes of small peptides as enzyme models often faced difficulties. Both their coordination mode and the kinetic properties differ significantly from those of the native metalloenzymes, and usually the model complexes did not exert substrate specificity or selectivity. So it might prove to be useful to investigate complexes of larger peptides. Within this context we tried to design metal binding oligopeptides mimicking the active site of the purple acid phosphatases. Based on computational calculations a 20 and a 24 amino acid containing peptide, able to bind two metal ions, i.e. P20: **TyrLysAspProProThrAspHisLeuAspGln ArgValLeuAspLeuProHisHisAsn** and P24: **AspProProGlnValProHisLeuTyrGlyLeuPhe GlnIleAsnAspThrValHisGlyCysCysHisAsn** were selected for the further studies. Our aim was to synthesize and investigate the metal binding properties of these peptides. As a first step, molecular biology tools were used in order to facilitate the expression and purification of the above mentioned oligopeptides. By means of recombinant DNA technique the peptides were expressed as Glutatione S-transferase (GST) fusion proteins. To avoid the difficulties raised during the investigation of the fusion proteins, the N- and C- terminally protected peptides were also prepared by solid phase peptide synthesis, and the solution chemical properties of their metal complexes were assayed.

One of our main goals was to design appropriate functional models for nuclease enzymes. To find biomimetic molecules capable of catalyzing the cleavage of phospho(di)ester bond of a proper synthetic activated ester or of a macromolecular substrate (e.g. native circular DNA) we planned to investigate the hydrolytic activity of our copper(II) and zinc(II) multihistidine type peptide complexes. At the same time the investigated copper(II) complexes could be able to mimic copper containing oxidases (catecholase-like activity) and to act as efficient superoxide dismutase mimetics. Thus the redox activity of the investigated systems was also tested.

II. Experimental methods

The protonation constants of the peptides synthesized by solid phase peptide synthesis, and the stability constants of the copper(II), zinc(II) and nickel(II) complexes were determined by pH-potentiometric titrations in aqueous solutions. All measurements were carried out at constant temperature (298± 0.1 K) and ionic strength (I = 0.10 mol/dm^3), under inert argon atmosphere. Experimental data were evaluated by SUPERQUAD and PSEQUAD computer programs.

In copper(II) and nickel(II) containing systems the complex formation was followed *in situ* by Vis spectrophotometry in the 400 – 800 nm wavelength region. The evaluation of electronic absorption spectra gave use information about the number and quality of the coordinating donor atoms, as well as, about the geometry of the formed complexes. The individual spectra of formed species were calculated by PSEQUAD program.

Circular dichroism (CD) spectroscopy in the wavelength region of the visible light yielded information on the chiral perturbation of the *d-d* electronic transitions, observed when coordination of the metal ion to a chiral ligand occurs. Optical activity in transition metal ion complexes has been attributed to property of the donor atoms to transmit the optical activity of the chiral atoms in the ligand, and to conformational effects. The CD spectra were recorded in aqueous solutions, at room temperature, in the 300 – 800 nm wavelength region. For the conformational analysis of the fusion proteins, in the presence and absence of metal ions synchrotron radiation CD spectroscopy (SRCD) was performed.

Molecular biology tools, such as the recombinant DNA technique, were used for the synthesis of the fusion proteins. The DNA sequences encoding for the target peptide together with the gene of the glutathione S-transferase (GST), were inserted in a plasmid vector and expressed in bacteria, as fusion proteins, with the GST protein at the N-terminal part. The metal ion binding properties of the GST-P20 and GST-P24 fusion proteins were investigated by UV-CD spectroscopy, and metal ion affinity chromatography.

The redox and hydrolytic activity of metal complexes were assayed in different test reactions. For the determination of catecholase-like activity, the oxidation of 3,5-di-tert-butylcatechol to 3,5-di-tert-butyl-o-benzoquinone was monitored spectrophotometrically. The superoxide dismutase (SOD)-like activity was studied by an indirect method, where the superoxide anion was generated *in situ* by the xanthine/xanthine oxidase reaction and the inhibition of the reduction of NBT by the model systems was monitored spectrophotometrically. The phosphoesterase-like activity was examined by the ability of the peptide complexes to promote the hydrolytic cleavage of either the activated ester RNA-model 2-hydroxypropyl-4-nitrophenyl phosphate (hpnp), or the circular pUC18 DNA.

III. New scientific results

III./1. CD spectroscopic investigation of the HisGlyGly, GlyHisGly, GlyGlyHis, HisAlaAla, AlaHisAla, AlaAlaHis, Ac-HisGlyGly-NH_2 copper(II) complexes

1. Concerning the structure-spectral relationships, as the result of the investigation of copper(II) complexes by combined potentiometry, spectrophotometry, CD and EPR spectroscopy, we found that the chelate rings in the tripeptide complexes seem to be isolated from the point of view of their contribution to the chirality, thus, their contributions are additive.

2. Our results revealed that the coordinated histidine residue has a great contribution to the chirality, showing a significant positive Cotton effect, however the intensity of the CD band is strongly dependent on the position of histidine in the amino acid sequence of the peptide.

3. We found, that in the case of *bis* complexes, further conformational contribution to the optical activity arises, due to the interaction of two ligands in the coordination sphere of the copper(II) ion, which causes a chiral arrangement, i.e. the two imidazole rings are twisted from the coordination plane.

4. Our findings on the CD contributions allowed us to make initial predictions on the structure of more complicated peptide complexes from the molar CD spectra calculated for the individual species, but these alone are certainly not decisive.

III./2. Solution chemical properties of the multihistidine peptide complexes

5. Using pH-metric titrations we determined the protonation constants of the Ac-HisProHisHis-NH_2, Ac-HisProHisProHis-NH_2 and Ac-LysHisProHisProHisGln-NH_2 ligands, and their complex formation constants with different metal ions.

6. Our results showed, that in the copper(II)-Ac-HisProHisHis-NH_2 system, a {2×ImN, 1×N^-_{amide}, 1×H_2O} coordinated complex is formed around pH ~ 7, possessing one bound amide nitrogen. This structure was maintained up to pH ~ 10, with the deprotonation of a coordinated water molecule. In the nickel(II) complexes the metal promoted amide deprotonation shifted to above pH 8 in a slow process, therefore the {3×ImN} coordinated $[NiL]^{2+}$ was the dominant species in a wide pH range (6.5 – 8.5). In the zinc(II) containing system precipitation was observed above pH 7, suggesting that the {3×ImN} type coordination is not enough to prevent the hydrolysis of the metal ion.

7. In the copper(II)- and zinc(II)-Ac-HisProHisProHis-NH_2 systems precipitation was observed above pH 6.9, in spite of the higher stability of the parent complex compared to Ac-HisProHisHis-NH_2. We have determined the composition of the solid substance to be equal to the neutral complexes.

8. The copper(II), as well as, the zinc(II) complexes of the Ac-LysHisProHisProHisGln-NH_2 peptide were soluble in the whole investigated pH range (pH 2 – 11). Our results showed, that {3×ImN} coordinated mixed hydroxo complexes dominate around physiological pH, thus according to our knowledge this ligand provides the first example of a short peptide both preventing the amide nitrogen coordination in copper(II) and the hydrolysis in copper(II) and zinc(II) complexes.

III./3. Investigation of P20 and P24 systems

9. The metal ion binding ability of the GST-P20 and GST-P24 fusion proteins have been proven by CD spectroscopy. These results showed that at low metal ion to protein ratio a specific metal complex is formed, while at higher metal ion excess non-specific binding mode dominates, causing the denaturation of the protein.

10. We have also investigated the solution chemistry of the P20 peptide, synthesized by solid phase peptide synthesis. The complex stability constants with zinc(II) ions have been determined.

III./4. Catalytic activity assays

11. We demonstrated that the investigated peptide complexes possess versatile (oxidative and hydrolytic) catalytic activity, making up multifunctional models.

12. In the redox activity assay of the copper(II)-Ac-HisProHisHis-NH_2 system, the $[CuH_{-1}L(OH)]^0$ mononuclear complex showed an outstanding catecholase-like activity ($k_{cat} = 0.12\ s^{-1}$).

13. The SOD-like activity of the $[CuH_{-1}L]^+$ species, dominating around pH 7.4 in the copper(II)-Ac-HisProHisHis-NH_2 system can be characzterized by $IC_{50} = 0{,}26\times10^{-6}$ mol/dm^3. This is one of the most remarkable value reported so far.

14. Due to the hydrolytic activity of the $[ZnL]^{2+}$ ($[ZnHL(OH)]^{2+}$) species, dominating at pH 7.3 in the zinc(II)-Ac-LysHisProHisProHisGln-NH_2 system, an acceleration over 130-fold was found in the cleavage assay of the hpnp substrate, compared to the uncatalyzed cleavage. This value is remarkable for a monomeric zinc(II) peptide complex.

15. The investigation of the hydrolytic activity of the zinc(II)-Ac-LysHisProHisProHisGln-NH_2 peptide complexes toward the double stranded circular DNA showed a very high activity assigned to the $[ZnHL]^{3+}$ complexes. Consequently, this peptide sequence is promising for the preparation of artificial nuclease enzymes.

IV. Publications *

IV./1. Publications related to the subject of the dissertation

(1.) **I.N. Jakab**, B. Gyurcsik, T. Körtvélyesi, I. Vosekalna, J. Jensen, E. Larsen: "Design of histidine containing peptides for better understanding of their coordination mode toward copper(II) by CD spectroscopy"
Journal of Inorganic Biochemistry (2007) **101**, 1376-1385 IF: 3.663

(2.) **I.N. Jakab**, A. Jancsó, T. Gajda, B. Gyurcsik, A. Rockenbauer: "The coordiantion behaviour of N-acetyl-His-Pro-His-His-NH_2 peptide toward copper(II), nickel(II) and zinc(II) ions"
Journal of Inorganic Biochemistry (2008) **102**, 1438-1448 IF: 3.663

(3.) **I.N. Jakab**, O. Lőrincz, A. Jancsó, T. Gajda, B. Gyurcsik: "Approaching the minimal metal ion binding peptide for structural and functional metalloenzyme mimicking"
Dalton Transactions (2008) *accepted manuscript* IF: 3.212

(4.) **N.I. Jakab**, Zs. Jenei, B. Gyurcsik, T. Gajda, T. Körtvélyesi, A. Mikulová, L. Rulíšek, T. Raskó, A. Kiss: „Design, synthesis and metal ion binding properties of a peptide mimicking the active centre of purple acid phosphatases"
Achievements In Coordination, Bioinorganic And Applied Inorganic Chemistry (2007) **8**, 80-90 (Eds: M. Melník, J. Šima, M. Tatarko,) Slovak Technical University Press, Bratislava

ΣIF: 10.538

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IV./2. Other publications

(5.) A. Jancsó, Z. Paksi, **I.N. Jakab**, B. Gyurcsik, A. Rockenbauer, T. Gajda: "Solution chemical properties and catecholase-like activity of the copper(II)-Ac-His-His-Gly-His-OH system, a relevant functional model for copper containing oxidases"
Journal of the Chemical Society: Dalton Transactions (2005) 3187-3194 IF: 3.003

(6.) **I.N. Jakab**, K. Hernadi, D. Méhn, T. Kollár, I. Pálinkó: "Anchoring copper–amino acid complexes on silica or in montmorillonite"
Journal of Molecular Structure (2003) **651-653**, 109-114 IF: 1.122

(7.) I. Labádi, I. Szilágyi, **I.N. Jakab**, K. Hernádi, I. Pálinkó: "Metal complexes immobilised on porous matrices - possible enzyme mimics"
Materials Science (2003) **21**, 235-244 IF: 0.099

(8.) **I.N. Jakab**, K. Hernadi, J.T. Kiss, I. Palinko: "Covalent grafting of copper-amino acid complexes onto chloropropylated silica gel - an FT-IR study"
Journal of Molecular Structure (2005) **744-747**, 487-494 IF: 1.440

(9.) **I.N. Jakab**, É. Szabó, K. Hernádi, I. Pálinkó: "The synthesis and the catalytic (catalase and tyrosinase) activities of amino acid copper complexes covalently grafted onto silica gel"
Proc. 8th Pannonian Intern. Catal. Symp., Sampling Catalysis Research in the Pannonian Region (2006) 51-56 (Ed. I. Pálinkó), Hungarian Zeolite Association, Szeged, Hungary

ΣIF: 16.202

IV./2. Oral and poster presentations

(1) **I.N. Jakab,** K. Hernádi, D. Mehn, T. Kollar, I. Pálinkó: "Anchoring copper-amino acid complexes on silica or in montmorillonite – an FT-IR study" (oral); *26th European*

Congress on Molecular Spectroscopy (EUCMOS XXVI), 2002, September 1-6, Villeneuve d'Ascq, France

(2) **I.N. Jakab**, Z. Paksi, B. Gyurcsik, M. Győr, T. Gajda: "II-es típusú rézfehérjék szerkezeti és funkcionális modellezése multihisztidin peptidek segítségével" (oral); *XXXIX. Komplexkémiai Kollokvium*, 2004, May 26-28, Agárd-Gárdony, Hungary

(3) Z. Paksi, **I.N. Jakab**, B Gyurcsik, A. Rockenbauer, T. Gajda: "Multihisztidin peptidek réz(II)- és cink(II)komplexeinek oldatkémiai vizsgálata és DNS-sel való kölcsönhatása" (oral); *XXXIX. Komplexkémiai Kollokvium*, 2004, May 26-28, Agárd-Gárdony, Hungary

(4) **I.N. Jakab**, É. Szabó, K. Hernádi, I. Pálinkó: "The synthesis and catalytic activities of amino acid copper complexes covalently grafted onto silica gel" (poster); *13th International Congress on Catalysis*, 2004. July 11-16, Paris, France

(5) **I.N. Jakab**, K. Hernádi, J.T. Kiss, I. Pálinkó: "Covalent grafting of copper-amino acid complexes onto chloropropylated silica gel – an FT-IR study" (poster); *XXVII European Congress on Molecular Spectroscopy*, 2004, September 5-10, Kraków, Poland

(6) T. Gajda**, I.N. Jakab,** Z. Paksi, B. Gyurcsik**: "**Metallopeptides mimicking the structure and/or function of metalloenzymes" (oral); International Symposium on Metals, Environment and Health, 2004, June 24-27, Szklarska Poreba, Poland

(7) **I.N. Jakab,** T. Gajda, B. Gyurcsik, T. Raskó, A. Kiss, R. Lubomir: "Bíborsav-foszfatáz enzimek aktív centrumának modellezése" (oral), *XL. Komplexkémiai Kollokvium,* 2005, May 18-20, Dobogókő, Hungary

(8) **I.N. Jakab, B.** Gyurcsik, T. Gajda, T. Raskó, A. Kiss: "Fémion-kötő peptidek tervezése, előállítása és vizsgálata - a bíborsav foszfatáz enzim aktív centrumának

modellezése" (oral), *XLI. Komplexkémiai Kollokvium,* 2006, May 31- June 2, Mátrafüred, Hungary

(9) Z. Paksi**,** A. Jancsó, **I.N. Jakab**, B. Gyurcsik, A. Rockenbauer, T. Gajda: "Solution chemical properties and catecholase-like activity of the copper(II)-Ac-His-His-Gly-His-OH system, a relevant functional model for copper containing oxidases" (poster), *Eurobic8,* 2006, July 1-6, Aveiro, Portugal

(10) **I.N. Jakab,**, B. Gyurcsik, T. Gajda, A. Kiss: "Design, preparation and investigation of metal binding peptides – models for the active site of the purple acid phosphatases" (poster), *2nd International IMBG Meeting on Metals in Biocatalysis*, 2006, September 24-27, Autrans, France

(11) A. Jancsó, Z. Paksi, **I.N. Jakab**, B. Gyurcsik, A. Rockenbauer, T. Gajda: "Catecholase-like activity and solution chemical properties of the copper(II) complex of a multihistidine tetrapeptide, a functional model for copper containing oxidases" (poster), *2nd International IMBG Meeting on Metals in Biocatalysis*, 2006, September 24-27, Autrans, France

(12) B. Gyurcsik, **I.N. Jakab**, A. Kolozsi, A. Jancsó, T. Gajda: "Nukleáz hatású peptidkomplexek tervezése és vizsgálata" (oral), *XLII. Komplexkémiai Kollokvium,* 2007, May 23-25, Mátrafüred, Hungary

(13) B. Gyurcsik, **I.N. Jakab**, A. Kolozsi, A. Jancsó, T. Gajda: "Nukleáz hatású peptidkomplexek tervezése és vizsgálata" (oral), *Centenáriumi vegyészkonferencia,* 2007, May 29 - June 2, Sopron, Hungary

(14) **I.N. Jakab**, Zs. Jenei, B. Gyurcsik, T. Gajda, T. Körtvélyesi, L. Rulíšek, T. Raskó, A. Kiss: "Design, synthesis and metal ion binding properties of a peptide mimicking the active centre of purple acid phosphatases" (oral), *XXI. International Conference on*

Coordination and Bioinorganic Chemistry (ICCBIC), 2007, June 3-8, Smolenice, Slovakia

(15) **I.N. Jakab**, O. Lorincz, T. Gajda, B. Gyurcsik "Metalloenzyme mimicking His-Pro-rich peptide complexes" (poster), *Graduate School on Metal Ions in Biological systems (MIBS) – Characterization methods in biological systems*, 2007, 18-21 June, Søminestationen, Holbæk, Denmark

(16) **I.N. Jakab**, B. Gyurcsik, T. Gajda, T. Körtvélyesi: „Az L-hisztidil-glicin-Cu(II)komplexeinek konformációs analízise" (oral), Kemometria és Molekulamodellezés Munkabizottság – 7. KeMoMo-QSAR miniszimpózium, 2007, December 6 - 7, Szeged, Hungary