

Short thesis for the degree of doctor of philosophy (PhD)

**The Role of Side Chains in the Complex
Formation Properties of CuZnSOD-Enzyme-
Mimicking Peptides**

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I. INTRODUCTION AND OBJECTIVES

Cu,Zn-superoxide dismutase (CuZnSOD), occurring in the most advanced biological organisms, is a metalloenzyme that contains copper(II) and zinc(II) ions in its active site. The Cu(II) ion is coordinated to three imidazole nitrogens, whereas the Zn(II) ion is stabilised by coordination to two imidazole nitrogens and the carboxylate group of an aspartic acid residue. In addition, the two metal ions are bridged by an imidazolate ligand. The copper(II) ion functions as a redox-active centre, enabling the conversion of the superoxide radical anion, while the zinc(II) ion plays a structural role. Malfunction or decreased activity of CuZnSOD has been associated with several neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS). Therefore, modelling and investigating this metalloenzyme is of significant biological and medical relevance.

One of the prominent areas of bioinorganic chemistry is the mimicking of the function of naturally occurring enzymes, that is, the development of mimetics. The aim of CuZnSOD mimetics is to effectively replace the enzyme in biological systems, particularly in cases where its activity is impaired. The application of oligopeptide metal complexes offers an opportunity to mimic the structural and functional properties of the enzyme, while also enabling the development of more stable models or even those with enhanced catalytic activity.

Our research group has previously investigated peptides designed to model the Cu(II) binding site (Ac-HVH-NH₂ tripeptide) and the Zn(II) binding site (Ac-HVGD-NH₂ tetrapeptide) of the CuZnSOD active centre. Subsequently, multihistidine peptides were synthesised, in which histidine was incorporated in different positions and environments, and the side chains were systematically varied in the sequence to understand the specific effects of different types of side chains. My PhD work was integrated into these studies. The work can be divided into two main parts: i) continuing the previously initiated systematic investigations by studying peptides containing

aspartic acid, which has a negatively charged side chain, and ii) using the results obtained so far for the synthesis and investigation of CuZnSOD model peptides.

The aim of the present doctoral work is to model the function of the CuZnSOD enzyme with oligopeptides and to investigate these in aqueous medium with various metal ions. The following objectives were formulated for the research:

- Design and synthesis of terminally protected multihistidine peptides, potentially suitable for modelling the active site of the CuZnSOD enzyme and for investigation in aqueous medium.
- Determination of the composition, stability, and structure of Cu(II), Zn(II), and Ni(II) complexes of the multihistidine ligands.
- Electrochemical investigation of Cu(II) complexes of the model peptides.
- Determination of the SOD activity of the Cu(II) complexes.

By achieving the above objectives, we can contribute to the design and understanding of new CuZnSOD mimetics and promote their application in the field of bioinorganic chemistry.

II. EXPERIMENTAL CONDITIONS AND METHODS OF INVESTIGATION

The ligands under study (Figures 1 and 2) were synthesised by solid-phase peptide synthesis using a microwave-assisted Liberty 1TM peptide synthesiser (CEM, Matthews, NC). Among the peptides investigated, Ac-HADH-NH₂, Ac-HDAH-NH₂, Ac-HAHDH-NH₂, Ac-HDHAH-NH₂, Ac-HGFHVH-NH₂, and Ac-HDHAHDH-NH₂ were synthesised by Dr. Gizella Csire and undergraduate students working on their theses. As a solid support, we used Rink Amide AM resin containing an amino group, thereby obtaining C-terminally amidated derivatives. In all peptide syntheses, the orthogonal protecting group (Fmoc/tBu) strategy was applied, while activation of the carboxyl group of the amino acids to be coupled was performed according to the TBTU/HOBt/DIPEA strategy. The protection of the N-terminal amino group was achieved by acetylation. The purity of the peptides was confirmed by reversed-phase HPLC, ESI-MS and/or MALDI-TOF, as well as by pH-potentiometric techniques. Based on chromatographic procedures, all ligands examined proved to be single-component compounds.

According to **pH-potentiometric** measurements, the active ingredient content of the synthesised ligands was found to be between 90% and 95%. This technique also provided further important information on the complex formation processes occurring in solution. The measurements were performed in aqueous solution at 298 K. The metal ion-to-ligand ratio was varied between 1:3 and 2:1, and a constant ionic strength of 0.20 M was maintained by the addition of KCl. From the fitting of the titration curves, the exact concentration of the ligand stock solution and the deprotonation constants of the functional groups containing dissociable protons were determined using the SUPERQUAD computer program. Evaluation of the systems containing one or two metal ions, as well as determination of the stoichiometry and stability of the complexes formed, was carried out with the PSEQUAD program. The concentration distribution

curves characteristic of the given system were constructed using the MEDUSA computer program, based on the protonation constants of the ligands, the composition and stability constants of the complexes, and the concentrations of the components.

Additional information on the formed metal complexes was obtained by various structural investigation methods.

UV-visible spectrophotometry provided information on the number and type of donor atoms coordinated to the metal ion. The absorption spectra of the samples with different metal ion-to-ligand ratios were recorded in the wavelength range of 200-900 nm, in cuvettes with a path length of 1.000 cm, at different pH values, using a Perkin Elmer Lambda 25 double-beam and a VWR UV-1600PC single-beam spectrophotometer.

Since the peptide complexes formed with copper(II) and nickel(II) ions are optically active, **circular dichroism (CD) spectroscopy** is a suitable technique for elucidating their structure. CD measurements were carried out at the Department of Organic Chemistry, University of Debrecen, on a JASCO-810 instrument. The spectra were recorded at different metal ion-to-ligand ratios and various pH values, in the wavelength range of 200-800 nm. The cuvettes used for the measurements had path lengths of 0.100 cm and 1.000 cm.

The redox properties of the metal complexes were studied by **cyclic voltammetry (CV)**. The measurements were performed using a Methrom 746 VA Trace Analyzer and a Basi Epsilon Eclipse instrument. In a three-electrode system (Ag/AgCl reference electrode, platinum auxiliary electrode, and carbon and platinum working electrodes), voltammograms were recorded at different scan rates, in the potential range from 800 to -800 mV, with a limiting current of 140 μ A. The parameters required for evaluation were obtained with the eL-Chem Viewer program.

One of the most widely used indirect methods for the determination of **superoxide dismutase (SOD) activity** is the spectrophotometric monitoring of the reaction between the superoxide radical anion generated in situ by the xanthine/xanthine oxidase

system and nitroblue tetrazolium chloride (NBT). For carrying out the measurements, phosphate buffer (pH = 6.8) was used. The amount of xanthine oxidase was adjusted so that the absorbance change at 560 nm fell within the range of 0.020-0.025 min⁻¹. The reaction was monitored at room temperature in a cuvette with a 1.00 cm path length, using an Agilent Cary 8454 diode array spectrophotometer.

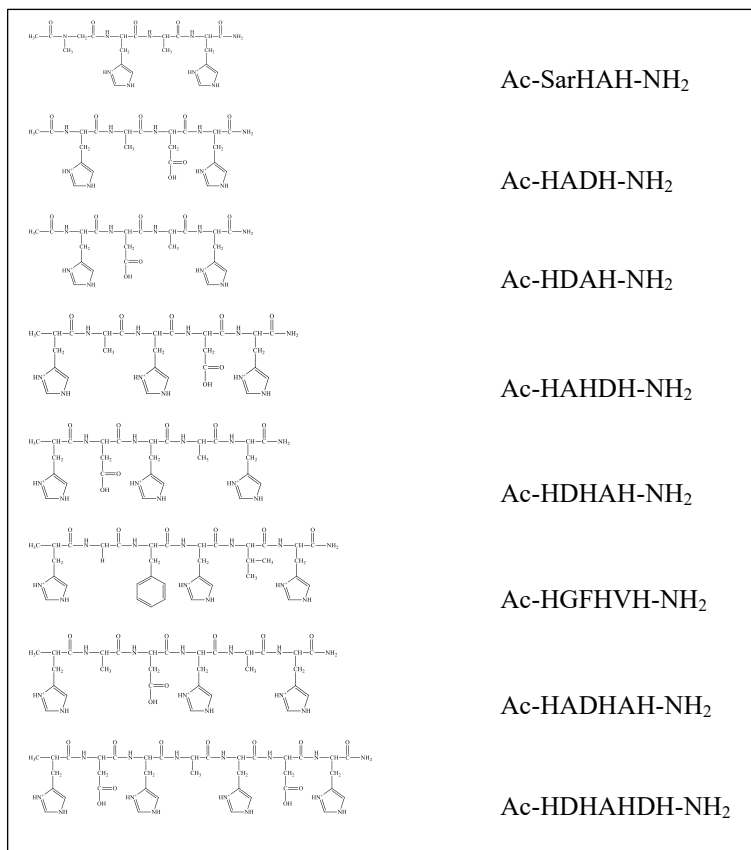


Figure 1. Structural formulas of the ligands investigated, containing different donor groups in the side chain



investigated

III. NEM SCIENTIFIC ACHIEVEMENTS

3.1. Based on the summary and comparison of the results obtained for the Cu(II), Ni(II), and Zn(II) complexes of the terminally protected peptides containing two or three histidine and aspartic acid residues (Ac-SarHAH-NH₂, Ac-HADHAH-NH₂, Ac-HDHAHDH-NH₂, Ac-HADH-NH₂, Ac-HDAH-NH₂, Ac-HAHDH-NH₂, Ac-HDHAH-NH₂, and Ac-HGFHVH-NH₂), which were previously studied by myself and by our research group, I concluded that the presence and position of the aspartic acid side chain increase the stability of the Cu(II), Ni(II), and Zn(II) complexes of multihistidine peptides, particularly in the case of imidazole nitrogen-coordinated complexes.

- The side chain of aspartic acid has the greatest effect compared to other amino acid side chains present in the peptide chain (alanine, valine, phenylalanine, lysine).
- The presence of a negatively charged side chain and the weak axial coordination of the carboxylate group increase the stability of imidazole-coordinated complexes formed with all three examined metal ions (Figure 3).

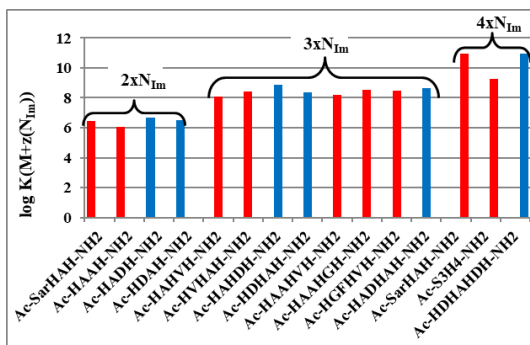
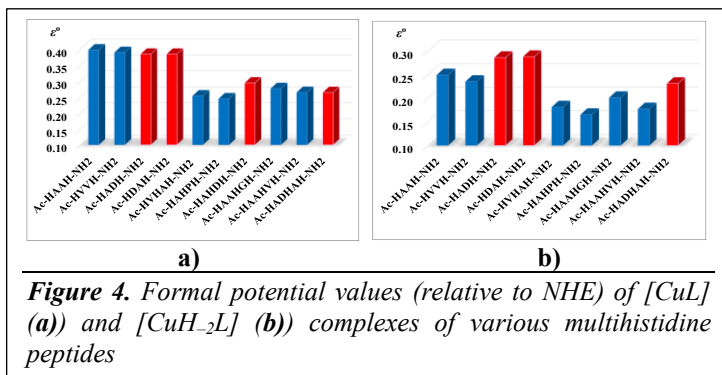


Figure 3. Derived equilibrium constants of imidazole nitrogen-coordinated Cu(II) complexes, with the values corresponding to the aspartic acid-containing peptides indicated in blue

- In the case of penta- and heptapeptides containing aspartic acid, coordination isomers of $[\text{CuH}_x\text{L}]$ ($x = 1-3$) complexes are formed, the ratio of which is influenced by the position of the aspartic acid residue.
- Due to the axial interaction of the carboxylate group of aspartic acid, in the Ac-HADHAH-NH₂ hexapeptide the binding of the Cu(II) ion to the intermediate histidine is favoured, whereas in the heptapeptide (Ac-HDHAHDH-NH₂) the –HDH– motifs are the main metal-binding sites.
- The carboxylate group of the aspartic acid side chain, similar to that observed for Cu(II) ions, slightly increases the stability of imidazole-coordinated complexes of Ni(II) over a wide pH range (pH = 4–8).
- For all peptides studied, above pH 9 the mononuclear $[\text{NiH}_3\text{L}]$ complex is the dominant species. The deprotonation of amide nitrogens is accompanied by the appearance of an intense band around 435 nm, the intensity of which increases in parallel with the formation of $[\text{NiH}_2\text{L}]$ and $[\text{NiH}_3\text{L}]$ complexes, corresponding to the deprotonation of two and three amide nitrogens, respectively.
- Based on the CD spectra of the Ni(II) complexes, in the case of small peptides, coordination starts from the C-termini, while in larger membered peptides isomeric structures appear, and the intermediate histidine is the main metal-binding site.
- The presence of aspartic acid has a significant effect on the complex formation processes occurring in the presence of Zn(II) ions. For peptides containing the –HADH– and –HDAH– sequences, coordination of the carboxylate group increases the stability of the Zn(II) complexes in the physiological pH range and promotes Zn(II)-induced deprotonation and coordination of amide nitrogens.

3.2. Based on the electrochemical investigations, I established that the redox potential values of Cu(II) complexes are primarily determined by the number of coordinated donor atoms, although the presence of aspartic acid slightly increases the redox potential of amide nitrogen-coordinated complexes.

- The redox potential of imidazole nitrogen-coordinated Cu(II) complexes decreases with the increasing number of imidazole nitrogens in the coordination sphere and with the increasing stability of the [CuL] complexes.
- Since the formal potential values of imidazole nitrogen-coordinated complexes are practically identical to those of peptides containing aliphatic side chains previously investigated, the presence of aspartic acid does not significantly affect the redox properties of imidazole nitrogen-coordinated complexes in the sequence (Figure 4a).



- In the case of Cu(II) complexes with an $[N_{Im}, N^-, N^-, N_{Im}]$ coordination environment, a more significant difference was observed (Figure 4b), as the redox potential value of the $[CuH_2L]$ complex of peptides containing aspartic acid increased compared to that of previously investigated peptides. This change can be explained by the coordination of the carboxylate group of aspartic acid in amide nitrogen-coordinated

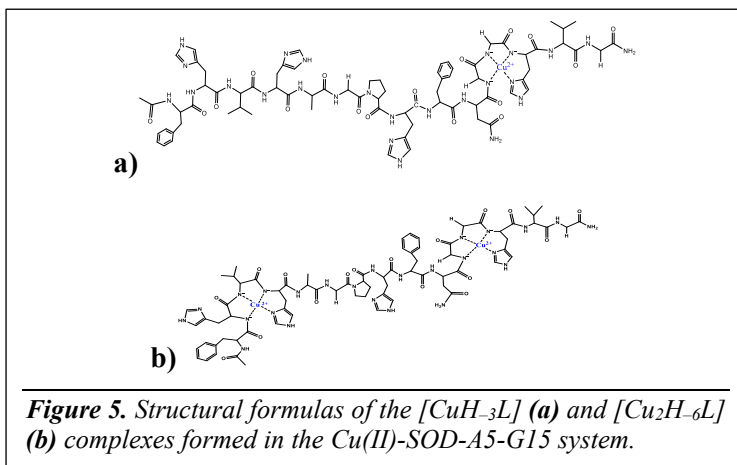
Cu(II) complexes, which results in increased redox potential values for the [CuH₁L] and [CuH₂L] complexes, as well as greater reducibility due to a more distorted geometry.

- Based on these findings, it can be concluded that the rigid structure of Cu(II) complexes does not fulfil the coordination requirements of Cu(I) complexes. Thus, Cu(II) complexes in which the coordinated donor groups are arranged in a single plane have low (negative) redox potential values.
- When the geometry of Cu(II) complexes is distorted compared to this arrangement, a tetrahedral geometry can easily form during the reduction process of the species containing the Cu(II) ion, leading to increased stability of the Cu(I) complexes. Consequently, the redox potential values of the [CuH₂L] complexes of multihistidine peptides fall into the positive range due to their distorted geometry, and the geometry of the complex is further distorted by the interaction between the metal center and the carboxylate group of aspartic acid.
- These results confirm that the redox properties of Cu(II) complexes are significantly influenced not only by the nature and number of coordinated donor groups but also by their spatial arrangement and the structural features of the ligand.

3.3. Based on the results obtained for small-ring oligopeptides, I designed and synthesised terminally protected multihistidine peptides containing amino acid sequences corresponding to the Cu(II) and Zn(II) binding sites of the CuZnSOD enzyme. These sequence segments are, namely, the Zn(II) binding HVG D sequence (residues 80-83), the Cu(II) binding HVH sequence (residues 46-48), and histidine (His63), which serves as an imidazolate bridge connecting the two metal ions in the active site of the enzyme.

3.4. Based on potentiometric and spectroscopic studies, I demonstrated that in equimolar solutions of the Cu(II)-L (L = SOD-A5-G15 and SOD-E5-D16) systems, the main coordination site is the –GGHVG(D)– sequence. In the presence of an excess of metal ions, the multinuclear complexes bind the metal ions to both the –FHVH– and –GGHVG(D)– motifs.

- In the studied peptides, all histidine imidazole rings can act as anchoring groups for the Cu(II) ion.
- In the physiological pH range, imidazole-coordinated complexes predominate, whereas in alkaline media, following the deprotonation of amide nitrogens, the coordination sphere of the Cu(II) ion becomes saturated with $[N^-, N^-, N^-, N_{im}]$ donor atoms.
- UV–visible and CD spectroscopic studies confirmed that, in equimolar solutions, the –GGHVG(D)– sequence of the peptides is the preferred binding site (Figure 5a), modeling the Zn(II)-binding site of the native enzyme.
- In solutions with an excess of Cu(II) ions, dinuclear complexes are formed in which the Cu(II) ion binds in nearly equal proportions to the –FHVH– and –GGHVG(D)– sequences (Figure 5b).



3.5. Based on potentiometric and spectroscopic results for mixed systems containing Cu(II), Zn(II), and Ni(II) ions, I concluded that the distribution of the metal ions among the binding sites changes compared to complexes containing only one metal ion, and that in the model peptides, Cu(II) and Zn(II) ions coordinate in a manner consistent with the active sites of the native enzyme.

- In the Ni(II)-SOD-A5-G15 system, in the alkaline pH range, spectral parameters indicated the formation of a $[\text{NiH}_3\text{L}]$ complex, in which the preferred binding site of the Ni(II) ion is the C-terminal –GGHVG(D)– sequence.
- CD spectroscopic studies of the Cu(II)-Ni(II)-SOD-A5-G15 mixed system showed that in the physiological pH range, Cu(II) complexes are the predominant species, whereas in alkaline media, the Cu(II) ion binds to the sequence modeling the Cu(II) binding site, while the Ni(II) ion binds to the sequence modeling the Zn(II)-binding site.
- In the Cu(II)-Zn(II)-L (L = SOD-A5-G15 or SOD-E5-D16) mixed systems, the presence of Cu(II)–Zn(II) heteronuclear complexes could only be detected in the alkaline range. Spectroscopic results indicated that increasing the Zn(II) ion concentration induces a redistribution of the Cu(II) ion among potential binding sites, with the Zn(II) ion binding to the C-terminal region of the peptides, corresponding to the Zn(II)-binding site of the native CuZnSOD enzyme.

3.6. Based on superoxide dismutase activity assays of the Cu(II) complexes of SOD-A5-G15, SOD-E5-D16, and Ac-HDHAHDH-NH₂ peptides, I demonstrated that in the physiological pH range (pH = 6.8) all three peptides form Cu(II) complexes with outstanding SOD activity. Although the measured IC₅₀ values indicate lower activity compared to the native enzyme, they significantly exceed the values reported for other known SOD model complexes.

VI. POSSIBLE APPLICATION OF THE RESULTS

The development of structural and functional models of naturally occurring metalloenzymes, including superoxide dismutase (SOD) enzymes, is a prominent research area of bioinorganic chemistry. The main objective of my doctoral thesis was the synthesis of an oligopeptide capable of mimicking both the structure and the function of the active site of the CuZnSOD enzyme.

During the research, a detailed comparative analysis was carried out on the effects of the number and spatial arrangement of histidine side chains on the complex formation of Cu(II)-, Zn(II)-, and Ni(II) ions. Based on the results, the synthesised multihistidine peptides, due to their structural characteristics, form stable, imidazole-coordinated metal complexes, several of which exhibited outstanding SOD activity and the activity values exceeded those reported for similar models in the literature.

These findings may represent a significant step forward in the development of artificial antioxidant systems. The investigations contribute to understanding of the modelling of peptide-based metalloenzymes, open new opportunities in the research of bioactive complexes, and provide a potential basis for the development of antioxidant compounds that could be applied for future therapeutic purposes.



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List of publications related to the dissertation

Foreign language scientific articles in international journals (2)

1. **Székely, E.**, Molnár, M., Lihi, N., Várnagy, K.: Characterization of Copper(II) and Zinc(II) Complexes of Peptides Mimicking the CuZnSOD Enzyme.
Molecules. 29 (4), 1-27, 2024. ISSN: 1420-3049.
DOI: <http://dx.doi.org/10.3390/molecules29040795>
IF: 4.6
2. **Székely, E.**, Csire, G., Balogh, B. D., Erdei, J. Z., Király, J. M., Kocsi, J., Pinkóczy, J., Várnagy, K.: The Role of the Side Chains in the Fine-Tuning of the Metal-Binding Ability of Multihistidine Peptides.
Molecules. 27, 1-29, 2022. ISSN: 1420-3049.
DOI: <http://dx.doi.org/10.3390/molecules27113435>
IF: 4.6

Hungarian conference proceedings (1)

3. **Székely, E.**, Várnagy, K.: Az oldalláncok szerepe a peptidek fémionkötő képességének finomszabályozásában.
In: Tavaszi Szél = Spring Wind 2020: Konferenciakötet. Szerk.: Szabó Csaba,
Doktoranduszok Országos Szövetsége, Budapest, 626-642, 2020.

Total IF of journals (all publications): 9,2

Total IF of journals (publications related to the dissertation): 9,2

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.



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