UNIVERSITY OF SZEGED

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New peptidic Cu(I) chelators as potential candidates for the treatment of Wilson's disease

Theses of Doctoral (Ph.D.) Dissertation

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I. Introduction

The essential micronutrient copper participates in several biological processes, like respiration, iron homeostasis, antioxidant defense or pigment formation. However, excess of copper can promote ROS formation and thus induce oxidative damages. Therefore, intracellular copper concentration is under strict control. Menkes and Wilson's disease are genetic disorders causing impairment in copper homeostasis leading to copper deficiency or overload, respectively. Wilson's disease is treated by chelation therapy, but the presently used drugs have several adverse side effects. New peptide or tripodal pseudopeptide ligands that display high affinity for the intracellular Cu(I) and target hepatic cells have been developed by Delangle et al. Some of them are currently proposed as highly specific drugs for localized copper chelation therapy in Wilson's disease.

II. Aims and objectives

My Ph.D. work consisted of the design of three groups of cysteine containing peptides and the characterization of their Cu(I) complexes to determine whether they are appropriate candidates for the treatment of Wilson's disease. The interaction of some peptides with Hg(II), which is a metal ion possessing similar coordination properties to Cu(I) and therefore an often used probe of the oxygen and water sensitive Cu(I), and with the ubiquitous Zn(II), which is a potential intracellular competitor, was also studied.

The peptides were designed by three different approaches. In a first strategy, we attempted to take advantage of the outstanding selectivity and sensitivity of the bacterial copper efflux regulator protein CueR by studying oligopeptides based on the metal binding motif of CueR involving two cysteine residues (Figure 1).

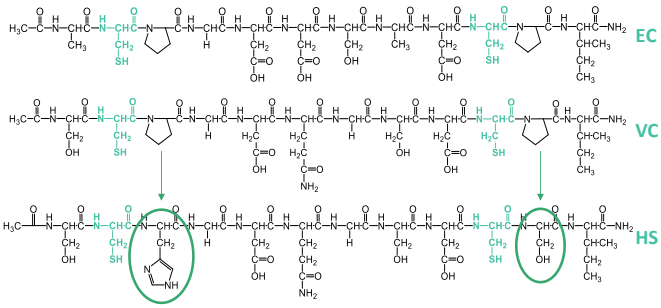

Figure 1. Schematic structures of the CueR model peptides

Second, three-cysteine containing linear and cyclic peptides (Figure 2) were designed with the aim of merging the advantages of peptides and tripods previously studied in the Delangle lab, namely the large Cu(I) binding affinity of tripodal pseudopeptides and the better internalization by the hepatocytes of peptide based molecules.

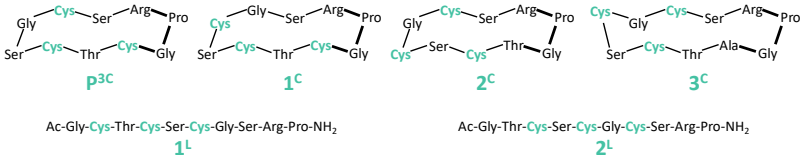

Figure 2. Schematic representation of the three-cysteine-containing peptides.

Finally, the advantages of a highly preorganized peptide structure were exploited in a short, rigid tetrapeptide where two cysteines were linked by a turn motif (CDPPC). For comparative purposes studies were also performed with another, less rigid tetrapeptide ligand containing the PG unit as a turn inducing motif (Figure 3).

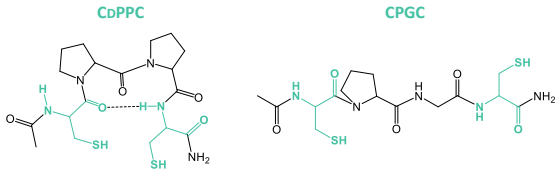

Figure 3. Schematic structures of the tetrapeptides with β turn inducing motifs. The dashed line symbolizes the H-bond in the turn present in CDPPC

The interaction of the peptides with the metal ions were studied by UV-vis, CD and NMR spectroscopies and ESI-MS. The stabilities of the peptide-metal ion complexes were determined by competition reactions with well-known ligands forming colored species with the relevant metal ions.

III. Methods

Terminally protected linear peptides were synthesized by solid phase peptide synthesis following the Fmoc protocol. For the cyclic peptides, linear precursors with unprotected termini were assembled by the same method. After cleaving the precursors off the resin, cyclization reaction was performed in a highly diluted solution by general coupling agents. The crude products were purified by reversed phase HPLC. The purity was checked by analytical HPLC and ESI-MS.

Since the cysteine moieties and Cu(I) are sensitive for oxidation, all sample manipulations were performed in a glovebox under argon atmosphere. The majority of the experiments were carried out at pH = 7.4 in buffered media. In a few cases, pH titrations were also executed at constant metal-to-ligand concentration ratios.

The typical ligand to metal charge transfer (LMCT) bands were followed by UV and CD spectroscopies. The concentration of the peptide samples was generally $c \sim 30$ μM and these samples were titrated by the addition of aliquots of the solution of the appropriate metal ion.

The composition of the metal ion complexes was analyzed by ESI-MS in both positive and negative ion modes. Spectra were recorded on samples containing the peptide in $c \sim 100$ μM concentration and different equivalents of the appropriate metal ion.

^{1}H NMR spectroscopy was applied for the identification of the donor groups participating in metal ion coordination in case of the CueR model peptides and for a more extensive analysis of the systems of CDPPC and CPGC. Complete assignment of the proton resonances in the free and Cu(I)-bound tetrapeptides were achieved by the combination of COSY, TOCSY, NOESY and ROESY measurements. The composition of the $Cu_4(CDPPC)_3$ cluster was proved by DOSY NMR experiments. Samples were prepared in deuterated solvents in a typical concentration of $c \sim 1.0$ mM.

Hydrogen bonds between the $C{=}O_i$ and $NH_{(i+3)}$ groups, characteristic for β turns, were detected by measuring the temperature coefficients of the amide proton resonances. NMR spectra were recorded over the temperature range of 278-318 K.

Cu(I) complex stabilities were determined by competition with BCA (bicinchoninate anion) or BCS (bathocuproin disulfonate), I^- ions competed with the peptides for Hg(II) and zincon was used for the determination of the Zn(II) complex stabilities. The withdrawal of the metal ion was followed by UV-vis spectroscopy.

IV. New scientific results

1. Mononuclear Cu(I) complexes of the CueR model peptides, **EC**, **VC** and **HS** are formed at pH = 7.4 in excess of the ligands and these transform into polynuclear species in the presence of Cu(I) excess. Based on the observed thiolate to Cu(I) charge transfer transitions in the UV and CD spectra, Cu(I) is bound by the two cysteine residues of the ligands. ^{1}H NMR spectra indicates the involvement of the histidine residue of **HS** in metal ion coordination.

2. The complex formation is complete above pH = 7.0 and the decrease of pH leads to the decoordination of the cysteines in two steps. Apparent pK_{a1} = 1.3-3.0 and pK_{a2} = 3.6-5.5 constants were determined for the two successive protonation accompanying the reverse processes.

3. The $\log\beta_{11}^{pH7.4} \sim 16$ stabilities of the mononuclear complexes at pH = 7.4 are rather large and similar to those obtained for peptides incorporating the CxxC or the CxxxxC motif. This result shows that the length of the spacer between the Cys residues affects little the affinity towards Cu(I). Therefore,

the driving force for the coordination of the soft Cu(I) cation is expected to be mainly the formation of the two strong bonds with the soft thiolate donors.

4. Striking differences were observed in the coordination of Cu(I) and Hg(II) by the series of three-cysteine containing peptides incorporating the CxCxxC and CxCxC motifs, typically appearing in metallothioneins. The peptides proved to be too flexible to control the speciation of Cu(I) complexes and hereby leading to the formation of several species, but they are adapted for an efficient trithiolate coordination of Hg(II). Mononuclear HgP complexes are formed in the presence of ligand excess and Hg(II) is coordinated by all the three Cys residues of the peptides. In the presence of Hg(II) excess, the mononuclear complexes transform into polymetallic species adopting the more favorable HgS_2 coordination mode. The observed difference demonstrates that the use of Hg(II) as a probe for Cu(I) coordination with sulfur-rich peptides or proteins under physiological conditions may not always be fully appropriate.

5. Structural differences in the three-cysteine containing peptides have minor effect on the affinity of the ligands towards Cu(I) and Hg(II) ions. Furthermore, these differences influence little the pH dependent transformation between the HgS_2 and HgS_3 species.

5.1. All the six peptides have similar overall affinities for Cu(I) that reflect remarkable Cu(I)-capturing abilities of the peptides. The $\log\beta_{11}^{pH7.4} \sim 18$ apparent stability constants of the theoretical mononuclear CuP complexes are in the range of Cu(I) chaperon proteins.

5.2. The apparent stabilities of the HgP complexes determined at pH = 2.0 fall in the narrow range of $\log\beta_{11}^{pH2.0}$ = 27.0-27.5. The apparent stability constants estimated for pH = 7.4 reflect a more than 20 orders of magnitude larger affinity of the peptides for Hg(II) than for Cu(I) due to the significantly larger thiophilicity of Hg(II). Stabilities of the HgP complexes are of the same magnitude as that of the Hg(II) complex of the well-known heavy metal chelator BAL.

5.3. UV-pH titrations showed an $HgS_2 \rightleftharpoons HgS_3$ transformation with pK_a = 4.3-5.1 values characterizing the deprotonation of the third cysteine

thiol. The observed tendency of pK_a values reveal that the CxCxxC motif in $\mathbf{P^{3C}}$ is more favorable for the formation of the HgS_3 structure, just like the longer distance from the turn motif in $\mathbf{2^C}$ and $\mathbf{3^C}$ compared to $\mathbf{1^C}$, and the cyclic structure against the linear.

6. Preorganization of the peptide structure is a key element in the control of Cu(I) complex speciation and in the affinity of the ligands for Cu(I).

6.1. UV, CD and NMR titrations reveal that CDPPC forms a single Cu(I) complex up to 1.33 equivalents of Cu(I). The results of DOSY NMR and ESI-MS experiments prove that this species is a Cu_4P_3 cluster with a Cu_4S_6 core where the Cu(I) ions are in a highly favorable trithiolate coordination environment. It is worth to note that this simple peptide is able to mimic the Cu(I)-thiolate cluster formation that are typical in proteins like Ctr1 or Cox17.

6.2. The CPGC-Cu(I) system is characterized by a more complicated complex formation. Since the PG motif provides higher flexibility, the peptide cannot control the speciation of the Cu(I) complexes.

6.3. The apparent stability constant calculated for a theoretical CuP mononuclear complex is one order of magnitude larger for CDPPC ($\log\beta_{11}^{pH7.4} = 17.5$) than for CPGC ($\log\beta_{11}^{pH7.4} = 16.4$).

7. The Zn(II) complexes of CDPPC and CPGC were demonstrated to be mononuclear ZnP or ZnP_2 species. The apparent stability constants at physiological pH calculated for the ZnP complexes are in a magnitude of 10^6, and the selectivity in favor of Cu(I) with respect to Zn(II) is therefore extremely large.

8. Since CDPPC forms a single Cu_4P_3 cluster with high stability and displays large selectivity for Cu(I) with respect to the ubiquitous Zn(II), it is an interesting simple peptide candidate to be targeted to the liver cells for the localized treatment of Cu overload in Wilson's disease.

V. List of publications

Identification number in the Hungarian Collection of Scientific Publications (MTMT): 10054976

Publications related to the dissertation

1. **E. Mesterházy**, B. Boff, C. Lebrun, P. Delangle and A. Jancsó, Oligopeptide models of the metal binding loop of the bacterial copper efflux regulator protein CueR as potential Cu(I) chelators, *Inorg. Chim. Acta*, 2018, **472**, 192-198.
   IF = 2.264

2. **E. Mesterházy**, C. Lebrun, A. Jancsó and P. Delangle, A Constrained Tetrapeptide as a Model of Cu(I) Binding Sites Involving Cu_4S_6 Clusters in Proteins, *Inorg. Chem.*, 2018, **57**, 5723-5731.
   IF = 4.700

3. **E. Mesterházy**, C. Lebrun, S. Crouzy, A. Jancsó and P. Delangle, Short oligopeptides with three cysteine residues as models of sulphur-rich Cu(I)- and Hg(II)-binding sites in proteins, *Metallomics*, 2018, DOI: 10.1039/C8MT00113H.
   published online
   IF = 4.069

   ΣIF = 11.033

Further publication

1. A. Jancsó, B. Gyurcsik, **E. Mesterházy** and R. Berkecz, Competition of zinc(II) with cadmium(II) or mercury(II) in binding to a 12-mer peptide, *J. Inorg. Biochem.*, 2013, **126**, 96-103.
   IF = 3.274

Oral presentations and posters

1. **Mesterházy, E.**, Jancsó, A., Lebrun, C., Tömösi, F., Delangle, P., A Wilson betegség kezelésére potenciálisan alkalmas Cu(I) kelátorok
   50. Komplexkémiai Kollokvium, Balatonvilágos, 2016.

2. **Mesterházy, E.**, Jancsó, A., Lebrun, C., Tömösi, F., Delangle, P., New peptidic Cu(I) chelators as potential candidates for the treatment of Wilson's disease
   13th European Biological Inorganic Chemistry Conference, Budapest, 2016.

3. **Mesterházy**, **E.,** Jancsó, A., Lebrun, C., Delangle, P., Interaction of copper(I) with 12-mer peptides mimicking the metal binding domain of CueR, a copper-efflux regulator
   Journées de Chimie de Coordination de la SCF, Grenoble, 2017

4. **Mesterházy, E.**, Lebrun, C., Jancsó, A., Delangle, P., An Efficient Peptidic Copper(I) Chelator with Two Cysteines Linked by a Strong Turn
   14th International Symposium on Applied Bioinorganic Chemistry, Toulouse, 2017

5. **Mesterházy, E.**, Lebrun, C., Jancsó, A., Delangle, P., Cu(I)ionok hatékony megkötésére alkalmas β-turn motívumot és két ciszteint tartalmazó tetrapeptid
   52. Komplexkémiai Kollokvium, Balatonvilágos, 2018.

6. **Mesterházy, E.**, Lebrun, C., Crouzy, S., Jancsó, A., Delangle, P., Metalloproteinek cisztein-gazdag Cu(I)- és Hg(II)-kötőhelyeit utánzó modellpeptidek
   52. Komplexkémiai Kollokvium, Balatonvilágos, 2018.

7. **Mesterházy, E.**, Lebrun, C., Crouzy, S., Jancsó, A., Delangle, P., Oligopeptide models of cysteine-rich Cu(I)- and Hg(II)-binding metal sites of metalloproteins
   14th European Biological Inorganic Chemistry Conference, Birmingham, 2018.

8. **Mesterházy, E.**, Jancsó, A., Gyurcsik, B., Berkecz, R., Zinc(II) interaction of a 12-mer peptide and competition of group 12 ions in binding to the ligand
   REGIONAL CONFERENCE "Heavy metal as contaminants of the environments". Timisoara, Romania, 2013

9. Balogh, R.K., **Mesterházy. E.**, Gyurcsik, B., Jancsó, A., Christensen, H. E. M., Asaka, M.N., Kato, K., Nagata, K., Réz(I)ionok szelektív kimutatása a CueR fémszabályzó fehérje segítségével
   XXXVIII. KÉMIAI ELŐADÓI NAPOK, Szeged, 2015.

10. Balogh, R.K., **Mesterházy. E.**, Kato, K., Nagata, K Jancsó, A., Gyurcsik, B. Detection of toxic metal ions by the CueR metalloregulator
    14th European Biological Inorganic Chemistry Conference, Birmingham, 2018.