Theses of Ph.D Dissertation

Study of the metal binding properties of Cu-efflux regulator CueR protein via model peptides

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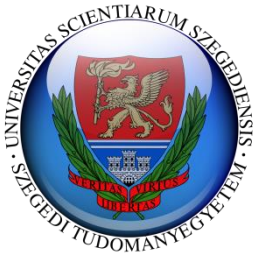

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1. INTRODUCTION

The ions of transition metal, such as manganese, iron, cobalt, nickel, copper, zinc, molybdenum are essential components of biological systems. These metal ions can be found in the active site of metalloenzymes, as a catalytic cofactor involved in the hydrolytic or reversible oxidation and reduction reactions and electron transfer processes. Their concentration should be within a narrow range; its extreme increase or decrease can lead to cell damage. The maintenance of optimal can be achieved with the equilibrium of uptake, storage, and efflux of the metal ion.

Using the tools of bioinorganic chemistry, we can design and prepare model compounds to understand the function of proteins and enzymes, having essential roles in metal ion homeostasis. The structural investigation of these compounds in solution may help us to understand the interactions between the metal ions and biomolecules, the effect of the metal ion on the protein structure, and the metal ion selectivity of the proteins. My dissertation presents the investigation of the metal binding domain of two bacterial CueR proteins through their model peptides.

2. AIMS AND OBJECTIVES

The metal ion homeostasis of the bacterial cells is maintained by proteins (*e.g.* the members of MerR family, which have been chosen), controlling the amount of both the essential and the toxic metal ions. Their operation is based on the selective binding of metal ions. These regulatory proteins are able to influence at the level of transcription the expression of redox enzymes catalyzing the reduction/oxidation of the metal ions or proteins carrying out metal ion transportation storage, transport and efflux.

The aim of my work was to explore the metal binding properties of bacterial CueR transcriptional metalloregulatory protein (selective for monovalent d^{10} metal ions such as Cu^{+}, Ag^{+}, Au^{+}) from *V. cholerae and E.coli* bacteria (*Fig. 1.*).

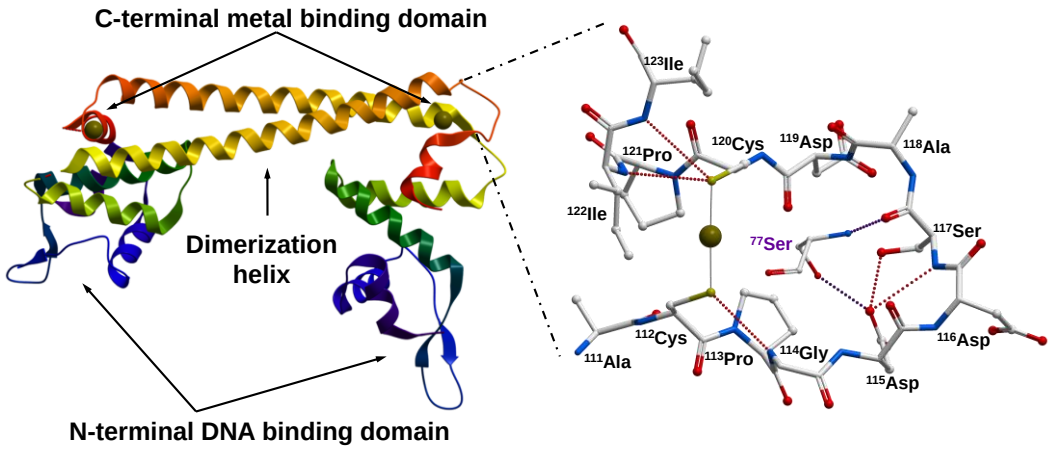


Fig. 1. The structure of CueR regulatory protein and its metal binding region

Our plan was to investigate, whether we can achieve metal ion selectivity on the level of peptide models representing the metal binding domains of the above mentioned CueR proteins. Therefore, our aim was to synthesize two peptides, with the amino acid sequence identical to their metal binding loops.

O'Halloran and co-workers have demonstrated that the CueR protein senses only monovalent transition metal-ions, while divalent metal-ions do not induce biological response. To better understand this, measurements with divalent metal ions were planned by means of which we could study the metal ion affinity of the peptides, and the effect of the metal ions on the peptide structure. There are proteins among the members of MerR family, which are selective for divalent metal ions. Examples include MerR and ZntR, which act as specific sensors of Hg^{2+} and Zn^{2+}, respectively, and CadR, which senses Cd^{2+}. In these proteins, His and Cys residues are responsible for the selectivity towards divalent metal ions.

This inspired us to explore, how the metal binding ability and the solution structure of the complexes are affected by a further potential donor group in the peptide sequence. Two peptides containing less proline residues and/or having an extra histidine were designed from the native sequence of metal binding domain of CueR protein from *V. cholera* bacteria. The position of substitution was chosen to gain information about ligand-flexibility as well. The relevance of the investigation of such modified peptides has been shown in a recent study, demonstrating that the mutation of ^{77}Ser to ^{77}Ala or ^{77}Cys did not significantly affect the response of CueR to monovalent ions, but the introduction of a potential donor group *e.g.* Cys residue at position 77 gave rise to sensitivity to divalent metal ions as well.

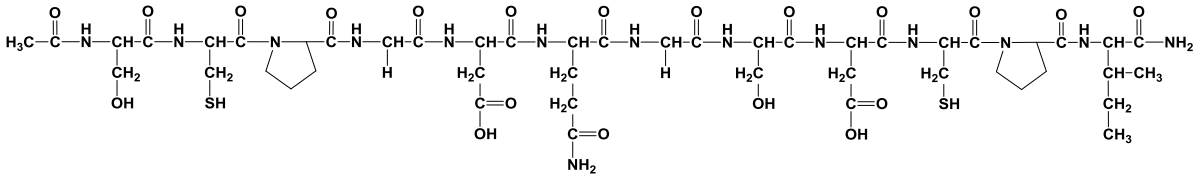
To better understand the metal ion selectivity of the CueR protein, measurements were designed using Ag^{+}, a monovalent transition d^{10} metal ion. The detailed investigations of metal ion-peptide system may help us to decide if the selectivity depends only on the bond strength between the metal ion and the protein, or other factors also contribute to the selective function.

3. EXPERIMENTAL METHODS

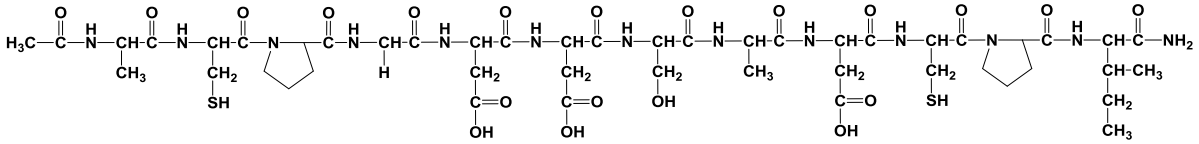
The synthesis of the ligands

The N- and C- terminally protected peptides were prepared by solid phase peptide synthesis. The crude products were purified by RP-HPLC (Reversed-Phase High Performance Liquid Chromatography), and the peptides were identified by ESI-MS (Electrospray Ionization Mass Spectrometry)

Ac-SCPGDQGSDCPI-NH_2 (Ac-Ser-Cys-Pro-Gly-Asp-Gln-Gly-Ser-Asp-Cys-Pro-Ile-NH_2) (**PP**, The metal binding domain of CueR protein from *V. cholerae* bacteria):



Ac-ACPGDDSADCPI-NH_2 (Ac-Ala-Cys-Pro-Gly-Asp-Asp-Ser-Ala-Asp-Cys-Pro-Ile-NH_2) (**EC**, The metal binding domain of CueR protein from *E. coli* bacteria):



The two modified peptides were: **PS**: Ac−SCPGDQGSDCSI−NH_2, in which the C-terminal proline of the **PP** peptide was changed to serine, and the **HS** (Ac−SCHGDQGSDCSI−NH_2), where one more potential donor group was built in the molecule beside the Asp carboxylic and Cys thiolate groups.

Experimental methods

The assignment of the hydrogen resonances to the amino acids was carried out by ^{1}H-^{1}H COSY (Correlation Spectroscopy), ^{1}H-^{1}H TOCSY (Total Correlation Spectroscopy), and ^{1}H-^{1}H ROESY (Rotating-frame Overhauser Spectroscopy) respectively.

The protonation and the coordination equilibria at various metal to ligand ratios were investigated by potentiometric titrations in aqueous solution. In the case of Zn^{2+}-, and Cd^{2+}-ions the pH dependent speciation of the metal ion, and the formation constants of the species were determined by PSEQUAD program.

The coordination of the thiolate groups was primarily was primarily followed by monitoring the pH dependent UV spectral series for the S^{-}−M^{n+} ligand to Metal Charge Transfer band (LMCT).

In order to gain information on the metal ion induced pH dependent conformational change of the peptides, SRCD (Synchrotron Radiation Circular Dichroism) spectra of the solution of the metal complexes were recorded.

In addition to ^{1}H NMR and UV spectroscopic measurements, the local environment and coordination geometry of Hg^{2+} was also monitored by ^{199m}Hg PAC (perturbed angular correlation of γ-rays) spectroscopy.

4. NEW SCIENTIFIC RESULTS

1. **We demonstrated by solution and structural studies, that the peptides (not containing histidine residue) form stable complexes in the presence of one equivalent amount of Zn^{2+}-, and Cd^{2+}-ions. The two cysteine residues simultaneously bind to the metal ions in spite of their considerable distance in the amino acid sequence, showing the dominant role of the Cys thiolates among the potential donor groups.**

   1.1. ML parent complexes are formed with **PP**, **PS** and **EC**, with a very similar structure in the presence of one equivalent of Zn^{2+}-, or Cd^{2+}-ions. Based on pH-potentiometric, UV-, and NMR spectroscopy studies, the metal ion induced thiol group deprotonation occurs at more acidic conditions (several pH units lower) in each case than in the free ligands.

   1.2. The changes of the Asp signals in the ^{1}H NMR spectra demonstrated that the carboxylic groups of these amino acids take a part in the coordination of Zn^{2+}- and Cd^{2+}-ions above physiological pH. The coordination sphere of the metal ions are completed by coordinated H_2O/COO^-. The pH potentiometric and UV spectroscopic results revealed that the deprotonation of ZnL and CdL complexes led to the $ZnH_{-1}L$ and $CdH_{-1}L$ species, respectively under alkaline conditions. The $MH_{-1}L$ composition is better described as M(OH)L, since it most probably contains a deprotonated water ligand. The pK_a value of the deprotonation process is ca. 1.5 lg units lower for the Zn^{2+}-complex, than the pK_a determined for the same type of proton release in the Cd^{2+}-complex. The difference may be due to the stronger Lewis acidic character of the Zn^{2+}-ion compared to Cd^{2+}-ion, and thus more significant affinity for hydroxide ion.

   1.3. Similar conformational distribution was observed in the ZnL and CdL species by (SR)CD measurements, resembling to the random structure. Based on the line broadening of the ^{1}H resonances of the ligands, the exchange is falling into the intermediate fast/slow exchange compared to the NMR time regime (ms–s). Accordingly, the flexibility of the ligand is kept in the complex.

2. **Only one dominant species was observed from acidic to alkaline pH (pH 3-11) in the equimolar solutions of Hg^{2+}-ion and peptides (not containing His residues). The peptides are demonstrated to form a loop-like structure in a {2×S^-} coordination fashion, *i.e.* via binding of their two cysteines to Hg^{2+}. [1]**

2.1. The apparent stability constant for the Hg(**PS)** complex was defined at pH = 2.0 by a ligand competition method. The lg*K*' = 25.7 shows outstanding affinity of the Hg^{2+}-ion to our Cys containing peptides. This value is several orders of magnitude higher compared to the affinity of Zn^{2+} and Cd^{2+} complexes of the same peptides. The ligand in the HgL complex may have several conformational states, the transition of which into each other is intermediate fast/slow compared to the NMR time regime (ms–s). This is the reason, that several resonances of the ligand show line broadening in the NMR spectra in the presence of metal ion, while their chemical shifts are unchanged.

2.2. Based on UV-, and ^{1}H NMR spectroscopy measurements we demonstrated, that the coordination mode is exclusively {2×S^-} in the whole studied pH range (pH = 3-11). The chemical shifts of $C_\beta H_2$ from Asp residues are very similar to those of the free ligand. Based on this, there is no sign for the participation of Asp side chain donors in Hg^{2+}-coordination, unlike in the Zn^{2+} and Cd^{2+}-complexes. The coordination of two donor groups around the metal ion was supported by PAC (Perturbed Angular Correlation) spectroscopy, too.

3. Bis-ligand ML_2 complexes are also formed in significant amount, in addition to monomeric species, when the ligands (PP, PS, EC) are in twofold excess over the Zn^{2+}-, and Cd^{2+}-ions. The coordination type is {4×S^-} in these complexes. In contrast to Cd^{2+} and Zn^{2+}, formation of HgL_2 complex was not observed.

3.1. MHL and ML complexes dominate in the acidic/neutral pH range, when the ligands are present in twofold excess over Zn^{2+}-, and Cd^{2+}-ions, but formation of bis-ligand complexes was observed above pH = 6.0. The overall stability constants calculated for the ML_2 complexes (ZnL_2: lgβ = 14.5-15.0, and CdL_2: lgβ = 17.6-18.2 ranges) and the derived lgK_2 for the stepwise binding of the second ligand in ML_2 (Zn^{2+}: lgK_2 = 3.70-4.70 and Cd^{2+}: lgK_2 = 5.30-6.21 ranges) show notably weaker binding of the second ligand as compared to the stability of the ML complexes (ZnL: lgβ = 9.80-10.60, and CdL_2: lgβ = 11.60-12.24 ranges). In one hand, the binding of the second ligand is not favorable because of the size of the peptide. In the other hand, the ligands have 4-5 negative charges under alkaline pH, so the electrostatic repulsion may discourage the coordination of the second ligand and the formation of the stabile bis-ligand complexes, as well.

3.2. Based on pH-potentiometric titrations and ^{1}H NMR spectroscopic measurements, we demonstrated that the CdL_2 species is dominant under alkaline condition, while the ZnL_2 complex dissociates above pH = 9 to form the $ZnH_{-1}L$ species and free ligand. This can be attributed to the fact that the Zn^{2+}-ion is stronger Lewis-acid than Cd^{2+}, and instead of the $\{4\times S^{-}\}$ coordination fashion, it prefers hydroxide-ions as ligands, which can replace the thiolates of the second peptide molecule in a stepwise manner above pH = 8.

3.3. In contrast to Zn^{2+}-, and Cd^{2+}-ions, formation of bis-ligand (HgL_2) complexes was not observed even pH > 9 and in the presence of twofold ligand excess over the Hg^{2+}. The results from UV-spectroscopy show that in the whole studied pH range (pH = 3-11) 50% of the ligand molecules bind to the metal ion forming the $\{2\times S^{-}\}$ coordination type, and the excess of the ligand deprotonates without coordination. Beyond the allosteric hindrance and charge repulsion a further reason for this is the outstanding preference of Hg^{2+} for coordination number of two with linear geometry, in contrast to Zn^{2+}-, and Cd^{2+}-ions. [1]

3.4. It has been confirmed by ^{199m}Hg PAC measurements that the coordination fashion is similar in the systems containing ligand excess and 1:1 metal to ligand ratio. There is also no indication for the participation of other side chain donor groups in Hg^{2+}-coordination except the thiolates of Cys residues. Based on ^{1}H NMR measurements, the ligand exchange rate between the free and bound forms gradually increases from the slow/intermediate in acidic solutions (pH = 2-6) to the intermediate/fast time regime (pH 7-11) in parallel with the deprotonation of the unbound thiol groups of the free ligand being present in the Hg^{2+}-**HS** 0.5:1 system. [1]

4. We demonstrated by solution studies that the imidazole moiety of His in HS peptide participates in the coordination of the Zn^{2+}-, and Cd^{2+}-ions, but stability increase is only observed in the Zn^{2+}-HS complexes. In contrary, no His imidazole-coordination was observed to the Hg^{2+}-ion. [2,3]

4.1. The ^{1}H NMR and pH-potentiometric results demonstrate, that the His imidazole moiety of the **HS** peptide coordinates to Zn^{2+}-, and Cd^{2+}. The interpretation of the ^{1}H NMR, UV absorption, and potentiometric data at pH ~ 5.5, leads us to propose co-existence of binding isomers of the ZnHL species, with the participation of two Cys-thiolates or one of the Cys-thiolates and the His side chain in metal ion binding. In contrary, His imidazole group does not bind to the Cd^{2+}-ion in the CdHL species.

However, the coordination fashion in the ML parent complexes of both metal ions is $\{2\times S^{-},N_{im},X\}$, where X may be oxygen from either water or Asp residue.

4.2. Interestingly, the coordination of His imidazole moiety affects only the stability of the Zn^{2+}-**HS** complex, which can be explained with the preference of the two metal ions towards different donor atoms. The deprotonation process of the ML parent complexes started earlier, than it was observed for the same type of proton release of the Zn^{2+}-, and Cd^{2+}- complexes of peptides lacking His residue. This can be explained by the increase of the Lewis acidity of the metal ion upon the coordination of the His imidazole moiety of **HS**.

4.3. The chemical shift values of His and Asp residues are practically independent on the metal ion to ligand ratio at all selected pH values. These findings indicate that the deprotonation processes of both the Asp carboxylic groups and the His imidazole are practically unaffected by the presence of Hg^{2+} and thus, these groups do not participate in Hg^{2+}-binding. The coordination sphere around the metal ion is $\{2\times S^{-}\}$, similarly to the complexes of peptides lacking His residue.

5. The HS ligand can form bis-ligand complexes with the participation of the His imidazole moiety in Zn^{2+}-, and Cd^{2+}-ion binding. On the other hand, formation of the HgL_2 species was not observed with HS, similarly to the investigated peptides, which do not contain His. [2,3]

5.1. ^{1}H NMR results demonstrated that bis-ligand complexes are formed in the pH = 7-10 range as well, in the presence of Zn^{2+}-ion. At pH ~8 and 0.5:1 metal to ligand ratio at least three broad peaks are observed for both the His $C_{\varepsilon 1}H$ and $C_{\delta 2}H$ resonances. These bands can be assigned to three coexisting species in different chemical environment and in slow to intermediate exchange rate relative to the NMR timescale. The result suggests that the His-imidazole moiety of at least one of the two ligands plays a role in Zn^{2+}-coordination in some or all of the bis-complexes.

5.2. UV absorption and ^{1}H NMR spectroscopic measurements show that ZnL_2 complex dissociates to $ZnH_{-1}L$ species and free ligand above pH = 9.5, similarly to that observed in the bis-complexes of **PP**, **PS** or **EC** peptides.

5.3. Based on UV measurements, the deprotonation of the thiol moieties take place in two steps when the peptide is in twofold excess over Cd^{2+}. LMCT bands appear in the pH range 4.5-6.5, showing the formation of the CdH_2L_2 complex at pH = 7. Further increase of the absorbance occurs between pH 7.5-9.5 to its final level, comparable to that observed for the 1:1 stoichiometry. This indicates that only a fraction of cysteines coordinates to the metal ion at pH 6-7.5, and the $\{4\times S^-\}$ coordination mode evolves above pH 8.

5.4. When the CdH_2L_2 species is formed, the chemical shift of the His $C_{\varepsilon 1}H$ and $C_{\delta 2}H$ signals are very similar to the signals observed in the spectra of CdL complex. A metal ion bridged species with $\{2\times S^-,2\times N_{im}\}$ donor group set would account for these ^{1}H-NMR data. At high pH (pH = 7.5-9.5) the His signals in the NMR spectra, however, become very similar to those characteristic for the free peptide. This indicates at least partial loss of His coordination, *i.e.* the formation of the CdL_2 species with $\{4\times S^-\}$ coordination type.

6. The formation of polynuclear complexes was also observed in the presence of twofold excess of Hg^{2+}-ions over the peptide.

6.1. In the presence of two equivalents of Hg^{2+}, the ^{1}H NMR spectra look different, compared to the 1:1 metal to ligand ratio. This indicates the formation of a complex different from the HgL species. This finding has been supported by (SR)CD measurements, as the spectra are different in the presence and absence of metal ion excess. It suggests that the conformation of the ligand is influenced by the number of the interacting metal ions.

6.2. Based on PAC spectra recorded in acidic pH range (pH < 7), we suggest the formation of a complex in which the two metal ions are connected by a thiolate bridge. In the alkaline pH (pH > 9), one thiolate and one hydroxide ion may coordinate to each metal ion. Above pH = 11, the structure of the polynuclear Hg^{2+}-complexes changes in time to HgL species with $\{2\times S^-\}$ coordination fashion, and hydrolyzed metal ion.

7. We investigated the Ag^+ complexes of PP and EC peptides. Based on the structural studies, it was found that a part of the thiol groups is coordinated to the metal ion, already at low pH (pH < 2) in deprotonated form. A further ligand related deprotonation occurs close to the physiological pH. We suggest the coordination of a thiol group in its protonated form below this pH. [1].

7.1. Based on UV spectroscopic measurements, the peptides – similarly to that observed in the Hg^{2+}-peptide system – are coordinated to the metal ion already at acidic pH (pH < 2). The increase of the absorbance in the physiological pH range (pH = 6-8) is observed in equimolar systems. In parallel, the ellipticity also increases in CD spectra, implying significant change in the peptide conformation. An isodichroic point at approximately 218 nm refers to the presence of two species.

7.2. The potentiometric titration curve of Ag^{+}-**PP** 1:1 system reveals three proton release up to pH 5.5, meaning that the carboxylic groups of the two Asp residues and one of the thiol groups are already deprotonated at pH = 5.5. This suggests that the ligand binds to the metal ion through at least one thiolate moiety below pH = 6. One more deprotonation step was observed on the titration curve between pH 5.5-7. In all probability, this belongs to the deprotonation and coordination of the second thiol group of the peptide. The pK_a for the deprotonation process is ~6.5, in agreement with the UV-, and CD spectroscopic results, as well. This unique process occurs only in the presence of this monovalent metal ion, that is, it was not observed for the studied divalent metal ions.

7.3. In the equimolar solutions of Ag^{+}-ion and **PP** two separated Cys $C_\beta H_2$ signals were observed at pH = 6 in the ^{1}H NMR spectra. One of these resonances resembles closely that observed for the free peptide, while the other, broad resonance appears at higher chemical shifts. This result supports that only a part of the thiol groups coordinates to the metal ion in the acidic pH range (pH = 2-6), while the rest is still protonated. The coalescence of the two separated Cys $C_\beta H_2$ signals can be observed parallel with increasing pH (pH = 6-11).

7.4. We demonstrated by structural studies, that the two thiol groups of the peptides have significantly different affinity towards the Ag^{+}-ion, unlike towards the divalent metal ions. Only one thiol group binds to the metal ion below pH = 7 in its deprotonated form, while the other can only coordinate in its protonated from. The bis(thiolate) coordination type characteristic for Hg^{2+}, was only observed only above physiological pH. The above data indicate that the presence of a protonated Cys residue at the metal site of CueR protein cannot be excluded, as well. Quantum-chemical calculations supported this assumption and revealed important structural consequences of protonating ^{112}Cys.

The coordination of this residue ”pulls” the ^{77}Ser backbone oxygen atom via a hydrogen bond closer to the metal ion binding loop. In this way, the metal binding gives rise to a considerable movement of ^{77}Ser, inducing structural changes in the protein, which may account for an efficient allosteric mechanism and selectivity towards the monovalent metal ions.

5. PUBLICATION LIST

Identification number in the Hungarian Collection of Scientific Publications (MTMT): 10034748

Publications related to the dissertation

[1] **D. Szunyogh**, H. Szokolai, P. W. Thulstrup, F.H. Larsen, B. Gyurcsik, N. J. Christensen, M. Stachura, L. Hemmingsen, A. Jancsó: Specificity of the Metalloregulator CueR for Monovalent Metal Ions: Possible Functional Role of a Coordinated Thiol?
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IF = 11.261

[2] **D. Szunyogh**, B. Gyurcsik, F.H. Larsen, M. Stachura, P.W. Thulstrup, L. Hemmingsen, A. Jancsó: Zn(II) and Hg(II) binding to a designed peptide that accommodates different coordination geometries.
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1. A. Jancsó, B. Gyurcsik, **D. Szunyogh**, A. Angyal, L. Hemmingsen: Mercury(II)- interaction of dodecapeptides designed for the sequestering of toxic metal ions.
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2. L. Hemmingsen, A. Jancsó, **D. Szunyogh**, F.H. Larsen, P.W. Thulstrup, N.J. Christensen, B. Gyurcsik: Metal Ion Controlled Polymorphism of a Peptide.
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