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Peptide Models of Cu(I) and Zn(II) Metallochaperones: The Effect of pH on Coordination and Mechanistic Implications

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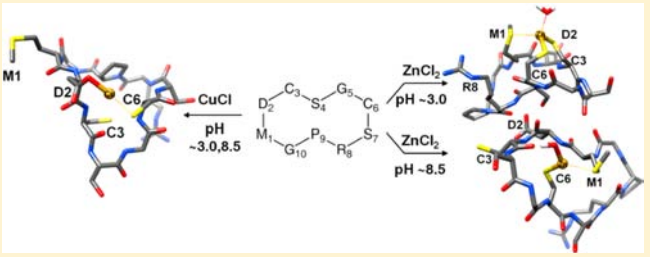
Supporting Information

ABSTRACT: The first NMR structures of Cu(I) and Zn(II) peptide complexes as models of metallochaperones were derived with no predetermined binding mode. The cyclic peptide MDCSGCSRPG was reacted with Cu(I) and Zn(II) at low and moderate pH. This peptide features the conserved sequence of copper chaperones but with Asp at position 2 as appears in the zinc binding domain of ZntA. The structures were compared with those of the Cu(I) complexes of the wild-type sequence peptide MTCSGCSRPG. All analyses were conducted first with no metal-binding constraints to ensure accurate binding ligand assignment. Several structures included metal-Met binding, raising a possible role of Met in the metal transport mechanism. Both Cu(I) and Zn(II) gave different complexes when reacted with the peptide of the native-like sequence under different pH conditions, raising the possibility of pH-dependent transport mechanisms. Cu(I) bound the MTCSGCSRPG peptide through one Cys and the Met under acidic conditions and differently under basic conditions; Zn(II) bound the MDCSGCSRPG peptide through two Cys and the Met residues under acidic conditions and through one Cys and the Met under basic conditions, while Cu(I) bound the non-native Asp mutant peptide through the Asp and one Cys under both conditions, suggesting that Asp may inhibit pH-dependent binding for Cu(I). NOESY and ESI-HRMS supported the presence of an aqua ligand for Zn(II), which likely deprotonated under basic conditions to give a hydroxo group. Coordination similarities were detected among the model system and native proteins, which overall suggest that coordination flexibility is required for the function of metallochaperones.



INTRODUCTION

A broad variety of metal ions participate in many biological processes in all types of organisms. Many of the metals are found in the active sites of proteins and play important roles in their functions. In particular, copper serves as an important cofactor in the chemistry of proteins that carry out fundamental cellular functions. The ability of copper to readily shuttle between +1 and +2 oxidation states enables the metal functionality, but at the same time, may trigger formation of toxic reactive oxygen species (ROS) upon metal accumulation.[1−8] Zinc is also an essential metal that participates in many biological functions and must be regulated tightly.[9] Breakdown in either copper or zinc regulation may lead to numerous neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and more.

Copper metallochaperones are intracellular proteins that specifically and tightly bind Cu(I) ions, protect them from harmful oxidation reactions, and deliver them to the target proteins via protein−protein interactions.[3,10−17] The exact mechanism of metal delivery and the parameters influencing its release at the target are yet unknown. Similarly, a metal binding domain in the protein ZntA [46−118] binds Zn(II) selectively and controls the free Zn(II) concentration.[18,19] These metallochaperones contain a conserved sequence at their binding site: $MX_1CX_2X_3C$ (single letter abbreviation of amino acids; X is any amino acid), "X_1" in Cu(I) metallochaperones is H or T and in ZntA is D, and "X_2", and "X_3" are varying amino acids. In the several copper chaperones characterized thus far by NMR or crystallography, the Cu(I) ion binds tightly to the two soft thiolato ligands of the cysteine residues with a low coordination number of two, although possible participation of a third external ligand has also been suggested.[20−26] The net charge of the binding site is therefore (−1), which is stabilized by the positive nitrogen atom of Lys~60[17,20−23,27,28] that functions as a counterion proximate to the binding site. In addition, it is generally accepted that the methionine residue does not participate in copper binding, but rather is located in a hydrophobic core.[10,11,20,21,29−31] The Zn(II) binding domain in ZntA has been structurally characterized by NMR and it was deduced that the metal binds the two thiolato groups of the cysteine residues and the carboxylato group of the aspartate.[19] Another external aqua ligand was proposed to complete the coordination sphere to give the preferred coordination number of four.

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Table 1. Average Distances between Oxygen and Sulfur Atoms of Asp, Met, and Cys Residues of the Peptide Complexes Calculated with No Metal Binding Constraints[a]

	O–S and S–S atom distances (Å) (STD)					
peptide complex	M1–C3[b]	M1–C6[b]	C3–C6[b]	M1–D2[b]	D2–C3[b]	D2–C6[b]
Cu(I); acidic A[c]	8.1 (1.7)	12.1(1.0)	9.4 (1.2)	6.9 (1.5)	7.2 (0.8)	*5.5 (1.7)*
B[c]	9.8 (1.0)	10.6 (1.5)	9.3 (1.7)	7.2 (1.1)	7.8 (0.9)	*4.8 (0.8)*
Zn(II); acidic	*4.8 (0.9)*	*4.8 (0.9)*	*3.9 (0.5)*	8.5 (1.0)	7.3 (1.1)	12.2 (1.0)
Zn(II); basic	8.7 (1.1)	*3.8 (0.7)*	9.6 (1.5)	8.0 (1.2)	7.3 (0.8)	7.3 (0.8)

[a]Distances indicative of metal binding are italicized. [b]Single letter abbreviation of amino acids. [c]A and B represent the two low-energy ensembles found among the nonviolated conformers.

Table 2. Average Distances between the Metal Center and the Oxygen and Sulfur Atoms of Asp, Met, and Cys Residues of the Peptide Complexes Analyzed, As Calculated with Metal Binding Constraints[a]

		M–S and M–O distances (Å) (STD)			
peptide complex	constraint[b]	M–S(M1[b])	M–O(D2[b])[c]	M–S(C3[b])	M–S(C6[b])
Cu(I); acidic	D2, C6	9.4 (0.8)	≡*1.98*	7.4 (0.7)	≡*2.70*
	C3, C6	10.5 (0.9)	5.6 (0.9)	≡*2.26*	≡*2.25*
Zn(II); acidic	M1, C3, C6	≡*2.70*	8.0 (1.4)	≡*2.25*	≡*2.25*
	C3, C6	5.6 (1.0)	9.8 (0.8)	≡*2.27*	≡*2.25*
	M1, C3, C6, H_2O	≡*2.70*	8.7 (0.9)	≡*2.26*	≡*2.25*
Zn(II); basic	M1,C6	≡*2.70*	8.3 (0.9)	5.3 (1.5)	≡*2.25*
	C3, C6	4.5 (0.9)	6.3 (1.1)	≡*2.25*	≡*2.25*
	M1, C6, OH^-	≡*2.70*	6.4 (1.2)	6.8 (1.0)	≡*2.25*

[a]Set distances are italicized and designated with the symbol ≡. [b]Single letter abbreviations of amino acids. [c]shortest M–O_{Asp} distance

One approach to studying metal binding, transport, and the release mechanism of these metallochaperones is based on synthetic models.[32] Although such models do not include the entire protein structure and do not presume to accurately mimic the biological environment, focusing on the metal binding site often leads to insights on the factors governing metal coordination under various conditions. Peptide-based models containing the conserved sequence of the metallochaperones have previously been developed, and their structures were examined by NMR.[33−35] Structural characterization of peptide complexes with other metals such as Hg(II) supported the binding mode observed in the natural proteins, where the metal bound the two thiolato groups of the cysteine residues.[34] Our recent communication reported two peptide-Cu(I) complexes that were produced from different pH conditions and were analyzed by NMR.[33] The NMR-derived structure of the complex produced under acidic conditions showed that the Cu(I) bound one Cys and the Met residues to give a neutral metal center, while a different coordination was observed under basic conditions, which likely involved the two Cys residues. This raised the possibility of a pH-dependent binding and release mechanism that may involve the Met residue.[33]

This extended study includes the full NMR structural characterization of Cu(I)- and Zn(II)-peptide complexes containing the Asp2 mutant peptide, produced under different pH conditions. Unexpected binding modes shed new light on possible roles of Met and Asp in the metal binding sequence, the possibility of external ligands in the metallochaperones ligation, and the possible role of pH in the metal coordination and transport.

RESULTS

The head-to-tail cyclic peptide with the sequence MDCSGCSRPG was reacted separately with CuCl and $ZnCl_2$ under two different pH conditions each, at room temperature and in an inert environment. The different pH conditions were achieved by lyophilizing the apo-peptide samples from aqueous solutions at one of two possible predetermined pH values, ∼3.0 and ∼8.5. The Cu(I) complexes were analyzed in DMSO-d_6 since the amide signals were lost in water and to avoid disproportionation reactions of the copper, while the Zn(II) complexes were analyzed in 10% TDW in DMSO-d_6 because of solubility considerations. All one-dimensional (1D) and two-dimensional (2D) ^{1}H NMR spectra were measured under identical conditions, processed (TopSpin, Bruker), analyzed (Sparky[36]) and structures were determined (Xplor[37]) by standard methodology.[38] Supporting Information, Table S4 provides NOE interactions statistics, and Supporting Information, Table S5 summarizes backbone and heavy atom RMSD values for the low-energy ensembles chosen for evaluation in all calculations. Importantly, the structures were initially analyzed and determined with no preset bonds to the metal atoms, to determine which atoms were positioned at distances appropriate for metal coordination in an unbiased manner, without forcing a particular coordination mode. Table 1 provides selected distances between particular residues obtained for all peptides analyzed without any metal binding constraints. Calculations were then repeated including the determined metal bonds (set binding values as provided in Supporting Information, Table S6); selected distances measured between the metal center and relevant residues are summarized in Table 2. ESI-HRMS results supported only monomeric complexion for all peptide-based complexes described herein (Supporting Information, Figures S14−S16).

Cu(I) Complex from Acidic Conditions. Comparison between the 1D and the TOCSY ^{1}H NMR spectra of the apo and the copper-containing samples obtained under acidic conditions indicated that a complex had formed (Supporting Information, Figure S1): The amide protons of the apo peptide were spread over a 1.2 ppm range of 7.4−8.6 ppm; after adding

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CuCl, the range expanded to 2.2 ppm, 6.9−9.1 ppm, in agreement with a significant conformational change.

The chemical shifts of the amide protons changed dramatically upon complexation. In particular, Met1 shifted most strongly downfield ($\Delta\delta$ 0.64 ppm). Additionally, Cys6 shifted strongly downfield ($\Delta\delta$ 0.33 ppm) and Asp2 shifted upfield ($\Delta\delta$ 0.24 ppm). Other amide chemical shifts also occurred, as expected of the major structural change to a bicyclic structure upon complexation.

Altogether 94 NOE interactions were identified in the NOESY spectrum of the reacted peptide (Supporting Information, Table S4). Analysis and structure determination with no preassigned bonds to Cu(I) revealed that the most rigid area in the peptide is that of amino acids R8-D2 with local backbone and heavy atoms RMSD values of 0.21 Å and 1.02 Å, respectively. The 27 nonviolated conformers gave a low energy ensemble with backbone and heavy atom RMSD values of 0.74 and 1.36 Å, respectively. Further analysis identified two low-energy ensembles containing 11 (lower energy; ensemble A; Supporting Information, Figure S2a, S2b) and 10 (higher energy; ensemble B; Figure 1a, Supporting Information, Figure S2c) conformers (Supporting Information, Table S5). These differed in that ensemble A included numerous hydrogen bonding distances between the backbone oxygens of Cys3 and Gly5/Cys6, and between the Arg8 guanidinium group and Ser4/Gly5.

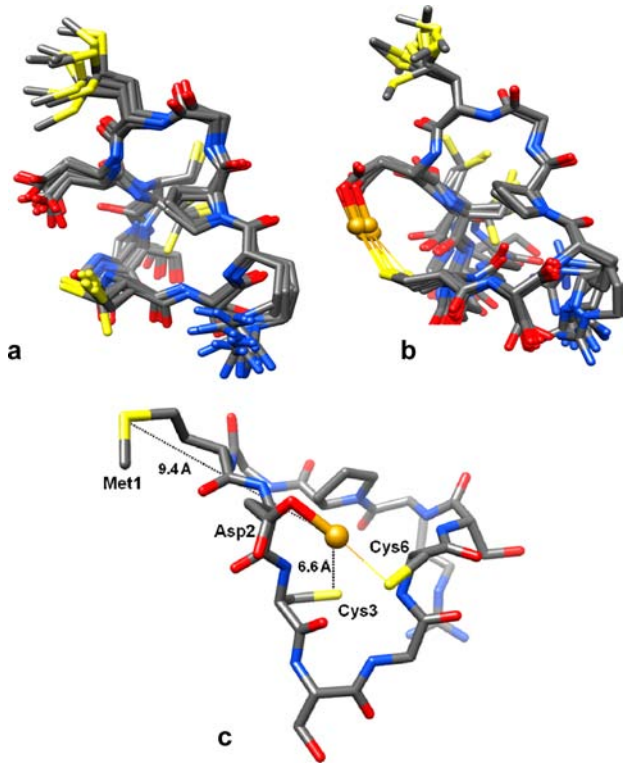

Figure 1. NMR-derived structures of copper peptide from acidic conditions: (a) superposition of 10 low-energy structures, ensemble B, obtained without Cu(I) constraints; (b) superposition of 13 low-energy structures obtained with preset dicoordinated metal binding; (c) calculated lowest energy structure obtained with preset dicoordinated metal binding.

The average distances between the residues suspected of metal binding in both ensembles suggested that the only atoms at a distance able to bind the Cu(I) ion were one of the side chain oxygen atoms of Asp2 in monodentate binding and the sulfur atom of Cys6 (Table 1). The average O−S distances were 5.5 Å ± 1.7 Å and 4.8 Å ± 0.8 Å for ensemble A and ensemble B, respectively. Further support for this structure came from the NOESY spectrum that showed a clear interaction between Hα of Asp2 and Hα of Cys6 (Supporting Information, Figure S3). The average distances between other relevant residues including Met and Cys3 were all above 8 Å, suggesting that these residues could not participate in chelating metal binding.

After deducing that Asp2 and Cys6 are the coordinating residues, the calculation was repeated including Cu(I) binding (Supporting Information, Table S6). The Cu(I)−Cys bond was set to 2.70 ± 0.05 Å, assuming that the Cys is protonated under acidic conditions and as no other charge neutralization interactions were apparent, consistent with a neutral metal center. The Asp−Cu(I)−Cys angle was set to 170° ± 5° to accommodate a pseudo linear geometry. A better-defined low energy ensemble resulted (Supporting Information, Table S5, Figure 1b, 1c). In half of the ensemble members, the Cys3 carbonyl pointed into the plane of the ring and showed hydrogen bond distances to Gly5 or Cys6. Notably, similar ensemble parameters resulted when setting a shorter Cu(I)−Cys bond to reflect a deprotonated Cys (Supporting Information, Table S6), with 22 nonviolated conformers.

Since peptides are small and flexible, control calculations were performed to test the degree to which the proposed metal-binding mode was the only or best solution for the empirical NMR data. One such calculation included set bonds to the two Cys residues, as would be anticipated based on the known binding mode of Cu(I) chaperone proteins (Supporting Information, Table S6).[20−26] Notably higher RMSD values identified the low energy ensemble (Supporting Information, Table S5), where the proximate Asp (shortest Cu(I)−O_{Asp} distance of 5.6 ± 0.9 Å; Table 2) supported its assigned metal binding.

Cu(I) Complex from Basic Conditions. The 1D NMR spectrum of the peptide with Cu(I) obtained under basic pH (~8.5) was identical to the one obtained under acidic conditions, suggesting that similar structural complex had formed (Supporting Information, Figure S4). ESI-HRMS of the peptide obtained under basic conditions differed by a single proton mass relative to that of the peptide obtained under acidic conditions, supporting Cys6 deprotonation (Supporting Information, Figure S14).

Zn(II) Complex from Acidic Conditions. The 1D and the TOCSY ^{1}H NMR spectra of the zinc containing sample from acidic conditions indicated that a complex had formed: After adding $ZnCl_2$, the range of the amide protons expanded from 1.1 to 1.8 ppm (7.6−8.7 to 7.2−9.0 ppm) (Supporting Information, Figure S5). Altogether 155 NOE interactions appeared in the NOESY spectrum of the reacted peptide (Supporting Information, Table S4).

In the calculations not including preset metal bonds, the low-energy ensemble (Supporting Information, Table S5; Figure S6) featured average distances of 4.8 ± 0.9, 4.8 ± 0.5, and 3.9 ± 0.5 Å between the sulfur atoms of Met1 and Cys3, Met1 and Cys6, and Cys3 and Cys6, respectively. The average distances between the three sulfur atoms of Met1, Cys3, and Cys6, and either of the two oxygen atoms of the carboxylate group of Asp2 were all above 7 Å (Table 1). This suggested that Met1, Cys3, and Cys6 may bind the zinc ion under acidic conditions with a coordination number of 3. This result was unexpected,

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since ZntA was reported to bind Zn(II) through the Asp residue,[19] and the binding observed for this peptide to Cu(I) attested to the steric feasibility of Asp to bind metal.

The calculation was repeated to include metal binding to the proposed ligands (Supporting Information, Table S6). The low-energy ensemble (Supporting Information, Table S5, Figure S7) exhibited average distances between the backbone and ε nitrogen atoms of Arg8 and Zn(II) of 6.5 ± 0.3 and 5.9 ± 0.1 Å, respectively. The average distance between the nearest oxygen atom of Asp2 and the Zn(II) was 8.0 ± 1.4 Å, again, too far for metal binding. No other potential coordinative atoms were found proximate to the metal ion.

To ascertain that Met1-metal binding was the best solution for the calculated structure, control calculation was performed with bonds only between Cys3 and Zn(II), and Cys6 and Zn(II) in a pseudolinear geometry (Supporting Information, Table S6). The calculation gave only 11 nonviolated conformers (Supporting Information, Table S5). The nonviolated ensemble supported the trigonal structure where Met1 was proximate to the metal site (Zn(II)−S_{Met} = 5.6 ± 1.0 Å) (Table 2). Another control calculation included binding constraints to Cys3, Cys6, and Asp2. All conformers of the 50-member calculation (RMSD 1.31 (backbone) and 2.43 Å (heavy atom)) were violated (Supporting Information, Table S5). These results supported the determined binding of all three residues, Met1, Cys3, and Cys6 to the metal center, and called for further exploration of a possible fourth external ligand, as proposed for the natural protein ZntA.[19]

In preliminary ESI-HRMS measurement, the only species identified except for the unreacted peptide was the monomeric complex (m/z 1056.2684). Applying milder fragmentation conditions enabled identification of a new signal at m/z 1092.2304 that may be attributed to a monomeric Zn(II) complex with sodium as a counterion and an additional oxygen atom (Supporting Information, Figure S15). Because of these observations, the preferred Zn(II) coordination number being four, and the proposed fourth aqua ligand in ZntA,[19] an additional aqua ligand was suspected to coordinate to the solvent accessible metal center. Such a ligand may also participate in hydrogen bonding with the nitrogen atom of Arg8 near the binding core, stabilizing the metal center.

This hypothesis was tested by measuring the NOESY spectrum of the Zn(II) peptide complex to which 30 μL of D_2O were added to exchange all solvent-labile peaks. After 58 h, an unassigned peak (3.76 ppm) in the NOESY spectrum that interacted with the amide proton of Arg8, disappeared. This peak is suspected to be an NOE interaction of the amide proton with the aqua ligand that underwent a minor upfield shift ($\Delta\delta$ 0.14 ppm relative to water in DMSO-d_6 at 3.9 ppm) because of possible hydrogen bonds and metal binding.

A new calculation was performed, containing a fourth aqua ligand in addition to the Zn(II)−Met and Zn(II)−Cys constraints as described above (Supporting Information, Table S6). The Zn(II)-O(aqua) bond was set to 2.1 ± 0.05 Å according to the solved X-ray structure of carbonic anhydrase II.[39] In the low-energy ensemble (Supporting Information, Table S5, Figure S8, Figure 2a), the aqua ligand appeared in the solvent accessible site above the peptide surface, as expected. No other heteroatoms appeared at a binding distance from the metal ion or from the aqua ligand.

Zn(II) Complex from Basic Conditions. Comparing the 1D and the TOCSY ^{1}H NMR spectra of the apo and the zinc containing samples obtained under basic conditions indicated

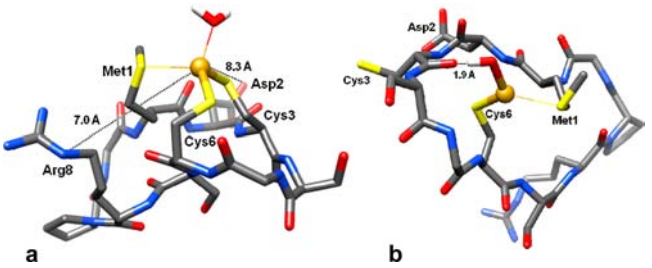

Figure 2. Calculated lowest energy structure obtained for: (a) Zn(II) complex from acidic conditions with preset tetra-coordinated metal binding, obtained out of 10 conformers in the lowest energy ensemble (RMSD values 0.31 Å for backbone and 0.89 Å for heavy atoms); (b) Zn(II) complex from basic conditions with preset tricoordinated metal binding, obtained out of 10 conformers in the lowest energy ensemble (RMSD values 0.43 Å for backbone and 0.89 Å for heavy atoms) (Supporting Information, Table S5, Figures S8, S13).

the formation of two species, neither of which were identical to the apo form (Supporting Information, Figure S9). The TOCSY and the 1D NMR gave two complete sets of peptide peaks. In the 1D NMR, one set was characterized by narrow peaks corresponding to the same complex as that which had formed under acidic conditions, and the second included broad signals representing a new complex (Supporting Information, Figure S10). For the latter, 85 NOE interactions were identified in the NOESY spectrum and assigned (Supporting Information, Table S4).

Throughout analysis with no metal binding constraints, 49 of 50 structures had no violations (Supporting Information, Table S5). The low-energy ensemble (Supporting Information, Figure S11a, S11b) featured an average distance of 3.8 ± 0.7 Å between the sulfur atoms of Met1 and Cys6, whereas the average distances between the sulfur atoms of Met1 and Cys3, Cys3 and Cys6 and the three sulfur atoms of Met1, Cys3, and Cys6 and between the two oxygen atoms of the carboxylate group of Asp2 were all above 7 Å (Supporting Information, Table S4). It was particularly noticeable that Cys3 pointed away from the metal binding site, despite four constraints (two sequential) to the Hβ atoms. This suggested that Met1 and Cys6 bind the zinc ion under basic conditions in a low coordination number, with no participation of Cys3 in binding, unlike under acidic conditions.

The calculation was repeated including bonds between Zn(II) and the suspected coordinating residues, Met1 and Cys6, assuming a deprotonated Cys (Supporting Information, Table S6). In the low-energy ensemble (Supporting Information, Table S5, Figure S12), the average distance between the sulfur atom of Cys3 and the Zn(II) was 5.3 ± 1.5 Å (Table 2). The average distances between the backbone and ε nitrogen atoms of Arg8 and the Zn(II) were 6.0 ± 0.6 Å and 4.3 ± 1.0 Å, respectively; this suggested a possible stabilizing interaction similar to that observed under acidic conditions when inserting metal bonds to peptide ligands only. The average distance between the nearest oxygen atom of Asp2 and the Zn(II) was 8.3 ± 0.9 Å, precluding Asp2 from participating in metal binding. No other potential donor atoms appeared at an appropriate distance from the metal center, which should yield a nonstabilized positively charged metal site.

In a control calculation, binding constraints were added between the metal and the two Cys residues giving 41 nonviolated of 50 total conformers (Supporting Information, Table S5). The distance between the Zn(II) and the Met1

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residue remained short, with a value of 4.5 ± 0.9 Å. In an additional control calculation, metal binding was set to Met1, Cys3, and Cys6; 40 nonviolated of 50 total conformers resulted with similar ensemble parameters.

The ESI-HRMS spectrum that was performed under regular fragmentation conditions featured three clear signals: One corresponded to the free peptide, the second agreed with a monomeric Zn(II)-bound species, and the third at m/z 1091.2299 agreed with a monomeric Zn(II) complex with sodium as a counterion and an additional oxygen atom (Supporting Information, Figure S16). As no additional possible counterions were identified near the metal center, and because of the basic conditions employed, an external hydroxo ligand was suspected to accommodate the solvent accessible Zn(II), favoring higher coordination numbers than 2. Such a ligand may again participate in hydrogen bonding with the Arg8 residue.

A new calculation was performed to include a third hydroxo ligand. The Zn(II)−Cys and Zn(II)−Met bonds were set as described above (Supporting Information, Table S6), and the Zn(II)−O(hydroxo) bond was set to 2.0 ± 0.05 Å, according to the X-ray structure of carbonic anhydrase II with a hydroxo ligand.[40] Out of the 46 nonviolated of 50 total conformers, the calculated low-energy ensemble (Supporting Information, Figure S13, Figure 2b) featured markedly improved RMSD values (Supporting Information, Table S5), with an average distance of 2.3 ± 0.7 Å between the hydroxo proton and the backbone oxygen of Cys3, suggesting a stabilizing hydrogen bonding interaction, and overall supporting the assigned ligation.

DISCUSSION

This study presents two peptide-models for the binding site of metallochaperone proteins that differ in their second residue. All structures were first determined with no metal bonds to identify the atoms that participate in metal binding without any prior assumptions, to ensure reliable structure determination. In fact, the results evince that no particular metal−ligand bonds should be taken for granted and assumed constraints can actually be misleading: All control calculations supported assignments based on metal-free calculations, and worse ensemble parameters resulted from incorrect assignments. However, using predetermined *incorrect* metal bonds did not necessarily prevent achieving a calculated structure, emphasizing the importance of nonbiased evaluation. This work presents the first complete NMR-derived structure determination of Cu(I) and Zn(II)-peptide models of metallochaperone proteins.[33]

Our previous communication reported that the peptide with the sequence MTCSGCSRPG,[34] corresponding to natural copper proteins binding sites, yielded two different structures with Cu(I) under different pH conditions.[33] Under acidic conditions, Met participated in copper binding along with only one Cys. This coordination sphere yielded a neutral metal center that did not require further stabilization from a counterion, unlike in the natural systems where lysine at position ∼60 electrostatically stabilizes the anionic metal center.[20−23,27,28] Nevertheless, this raised the possibility that the Met residue may play a role in metal transport and release upon possible protein conformational change, which is reasonable considering the high affinity of Cu(I) to sulfur ligands[41] and the conserved nature of this residue in the many copper chaperone proteins analyzed. Furthermore, since a different binding mode occurred under basic conditions that seemed not to involve Met binding, it is likely that the metal transport and release is pH dependent,[42] as also described for the Menkes protein.[43] Metal release mechanisms based on pH variations and ligand protonation are well-known.

The mutant peptide MDCSGCSRPG formed complexes with both Zn(II) and Cu(I) under two different pH conditions. This peptide has Asp at position 2 as present in the binding site of the natural Zn(II)-binding protein ZntA.[19] It was previously suggested that the Asp residue in the natural system enables Zn(II) selectivity,[16,32] as it increases the coordination number and stabilizes the higher oxidation state of Zn(II) when compared with the Cu(I). In the current model, the Asp mutant peptide actually did bind Cu(I) through the Asp and only one Cys residue. This binding mode did not change even upon marked increase of pH. It is thus clear that the Cu(I), despite its low coordination number, prefers to bind the Asp residue over any S ligand at least in this particular loop, where the dependence of binding on pH is lost. If indeed the pH dependence is an important mechanistic step, this observation may offer another explanation for the absence of Asp from the binding site of copper chaperone proteins. It is therefore tempting to propose that binding Asp may inhibit proper release of the metal ion at its target location.

Interestingly, when analyzing binding of the Asp mutant peptide to its native cofactor Zn(II),[19] again different structures were obtained under different pH conditions. In fact, Asp did not participate in metal coordination under any of the conditions employed: Only sulfur-donors, namely, the Met and the two Cys, served as ligands. This observation further shows that Met is able to participate in metal binding in this model, despite the opposite observations reported for natural chaperones.[10,11,20,21,29−31] Even in the control calculation that included only Cys−Zn(II) bond constraints, the calculation gave the proximate Met1. Overall this supports the metal inability to bind Met in natural systems arising from steric effects occurring in the entire protein structures. Most notably, the absence of Asp from the coordination sphere was unexpected for several reasons: (a) Asp binding was reported to occur in the natural protein ZntA;[19] (b) the harder CO_2^- relative to the softer S^- donor is expected to bind more strongly to the Zn(II) relative to Cu(I); and (c) the Asp binding to Cu(I) in a non-pH dependent manner attests to its steric feasibility to bind metal. One possible explanation may relate to the strong preference of Zn(II) to form coordination numbers higher than two, because of its higher oxidation state when compared with Cu(I). Perhaps such binding, that forms multiple cyclizations and thus severe steric strain, is only possible near the S donors of the peptide.

When considering only ligands available from the peptide, the coordination change in the second Zn(II) complex that formed upon increasing pH is counterintuitive: One would expect that under conditions favoring ligand deprotonation, more Zn(II)−Cys bonds should form, especially where the metal center is not coordinatively saturated. Instead, increasing the pH lowered the coordination number to peptide ligands in the new complex by loss of one Cys ligand. Additionally, the positively charged Arg positioned proximate to the metal center cannot be attributed to charge stabilization in both structures, since the metal site should be either neutral (acidic conditions) or positive (basic conditions) based on the assigned ligands. Thus, the proximity of Arg to the binding center may reflect hydrogen bonding to a moiety that could not be clearly

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identified by NMR. This prompted us to hypothesize that an additional external aqua ligand might be coordinated, since the analysis of Zn(II) complexes was conducted in 10% water, and as such ligation was proposed for the natural ZntA protein based on solvent accessibility.[19] Carbonic anhydrase and related proteins with similar coordination spheres show a coordinating water molecule with a pK_a of ∼7 because of metal binding and hydrogen bonding;[39,40] thus, under the basic conditions employed herein, deprotonation of the coordinated water molecule may have occurred. This can explain the lower coordination number formed for the new complex, as a stronger bond to the hydroxide ion was achieved, while charge neutralization was maintained. Calculations conducted with coordinated water/hydroxide ligands produced stable structures: similar numbers of nonviolated structures and RMSD values resulted; and for the structure from basic conditions the RMSD values were even markedly improved and an additional stabilizing hydrogen bonding interaction occurred between the hydroxide ligand and the backbone oxygen of Cys3. This conclusion is also supported by ESI-HRMS experiments that suggested an additional O atom with the Zn(II)-peptide species, evident more clearly under basic conditions where the bond should be stronger. Additional support came from a NOESY experiment, where adding D_2O exchanged-out a signal attributed to an interaction of the water molecule with Arg8 amide proton. It is noteworthy that in calculations including H_2O/OH bonds, the Arg residue did not occur at a binding distance from the metal center, and therefore was unable to provide additional stability. These findings support the assignment of an aqua ligand in the natural system,[19] and raise the possibility that a pH-dependent mechanism of metal transport may also exist for Zn(II).

Overviewing Zn(II) coordination spheres in different proteins, where the role of the metal is purely structural, Zn(II) usually binds four ligands of which at least two are cysteines, giving coordinative saturation and therefore reduced flexibility in coordination number. In contrast, catalytic enzymes, for which substrate binding and release is required, largely include an Asp or Glu ligand and a water ligand that may or may not participate in the catalytic reaction.[44] Other catalytic enzymes such as some alcohol dehydrogenases,[44,45] have coordination spheres similar to that of the peptide-Zn(II) complex model obtained under acidic conditions: Such proteins contain two Cys ligands, one coordinative bond to His, and an external aqua ligand that does not participate in the catalytic reaction but rather serves to afford flexibility in coordination number. It may be meaningful that the ZntA protein includes a coordination site more similar to a Zn(II) catalytic center than a structure-determining one.[16,19] This may reflect the required flexibility in metal binding in the metallochaperone proteins, where different ligation may be essential for metal transport among different proteins.

CONCLUSION

The first structures of Cu(I) and Zn(II)-peptide complexes were solved to serve as models for metallochaperone proteins; the coordination parameters that govern metal binding were determined with no prior bias and may shed light on the mechanism of metal transport and release in natural systems.[33] In particular, the results suggest that the Met residue in the conserved sequence should not be ignored and may participate in metal binding under certain conditions through conformational changes. Additionally, pH conditions may drastically affect the coordination sphere of the metal and thus might serve as a mechanistic force for metal release. Particularly, it appears that a water ligand in the Zn(II) model may undergo deprotonation under basic conditions similar to those influencing known Zn(II) proteins,[39,40,46] in conjugation with losing other covalently bound ligands. This suggests the possibility of a pH-dependent metal release mechanism in zinc proteins as well, although in a negatively charged metal center such as that reported for ZntA, deprotonation would be inhibited.[16] Nevertheless, the coordinated water ligand in ZntA is similar to those in other zinc catalytic enzymes that require coordination flexibility, which may possibly manifest the flexibility required for delivery proteins that should enable appropriate metal release. Further studies on both natural and model systems are essential to elucidate additional influencing parameters and draw conclusions regarding the connection between the behavior of models and natural systems.

EXPERIMENTAL SECTION

The head-to-tail cyclic peptide with the sequence MDCSGCSRPG was purchased from Biochimica Ltd. (Montreal, Quebec, Canada) with a purity of 95.31%. Samples were prepared in NMR tubes as detailed below. The pH was determined using pH indicator strips (nonbleeding) from Merck Millipore Ltd. Accurate-Mass Q-TOF LC/MS measurements were carried on an Agilent Technologies 6520 (mass accuracy <2.0 ppm) through direct injections, and the samples were prepared under conditions identical to those used for NMR measurements. Regular conditions included temperature of 350 K and fragmentor voltage of 150 V. Repeated measurement for zinc peptide obtained from acidic conditions was performed under temperature of 300 K and fragmentor voltage of 100 V.

Sample Preparation. The analyses were carried out in pure or a large excess of DMSO-d_6, which has been shown to be a close mimic of the aqueous physiological environment,[48,49] since the amide region of the spectrum underwent exchange with water under the experimental conditions and the amide signals were broadened out. All samples were prepared in NMR tubes under inert conditions in an oxygen-free M. Braun glovebox to avoid Cu(I) oxidation. The NMR tubes remained sealed for the entire measurements. Additional information is provided in the Supporting Information. In a reference measurement for copper-bound peptide analysis, the metal was allowed to oxidize to the +2 state under air. A clear color change to dark green resulted, giving a new spectrum that was impossible to interpret as characteristic for paramagnetic compounds. This assured that the measured samples included copper in the (+1) state.

NMR Structure Determination. NMR experiments were performed on a Bruker Avance 600 MHz DMX spectrometer operating at the proton frequency of 600.13 MHz, using a 5-mm selective probe equipped with a self-shielded xyz-gradient coil at 25.0 ± 0.1 °C. The transmitter frequency was set to 5.30 ppm for the nonaqueous samples or on the hydrogen−deuterium exchange signal in water. The samples were calibrated to 2.50 ppm on the residual DMSO signal.

COSY, TOCSY, and NOESY spectra were acquired under identical conditions for all samples. These were acquired in phase-sensitive mode[50−52] in nonaqueous solutions and with Watergate suppression of water[53] in the aqueous samples. The MLEV-17 TOCSY spinlock[54,55] was 150 ms, and the NOESY mixing time was 150 ms.

Spectra were processed and analyzed with the TopSpin (Bruker Analytische Messtechnik GmbH) and SPARKY3 software.[36] Resonance assignment followed the sequential assignment methodology developed by Wüthrich.[38] Peak intensities were manually assigned as strong, medium, weak, and very weak, from the van der Waals radius until the top of the square well, as specified in the Supporting Information.

The three-dimensional structures of the peptides were calculated using Xplor-NIH[37] by hybrid distance geometry-dynamical simulated annealing. The NOE energy was introduced as a square well potential

with a force constant of 50 kcal/mol·Å^2 that was kept constant throughout the protocol. The copper−sulfur/oxygen and the zinc−sulfur bonds were introduced using patches within Xplor according to geometries given in Supporting Information, Table S6. Ensembles of 50 initial structures were generated from which low-energy structures were chosen for further analysis that had no NOE violations, deviations from ideal bond lengths of less than 0.05 Å, and bond angle deviations from ideality of less than 5°, with the exception of the metal patches where deviations are specified in Supporting Information, Table S6. Molmol[56] was used to obtain the final ensemble of structures presented in Table 1. Structural analysis, identification of hydrogen bonds, and presentation were done using Chimera.[47]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details, 1D and 2D NMR spectra, NMR structures derived with or without different metal binding constraints, lowest-energy structures calculated with no Zn(II) binding constraints, tables of NOE interaction statistics and constraints used, and MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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