De Novo Design of Mercury-Binding Two- and Three-Helical Bundles

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Fundamental to the understanding of metalloprotein structure is the relative importance of the metal's geometric preference vs the inherent protein structure. One approach to studying the interplay between metal and protein conformation is to introduce metal-binding sites into *de novo* designed peptide structures. The use of designed metallopeptides has several potential advantages over small molecule models. First, the peptides can be designed to assume well-defined tertiary structures in solution, thus providing a molecular scaffolding into which biologically relevant metal-binding residues can be introduced. Second, the secondary/tertiary structures of the peptides can be utilized to generate distorted coordination environments which are often difficult to achieve in spontaneously-assembled small model complexes. Third, metallopeptides can be designed to be water soluble.

Few *de novo* designed metallopeptides have been reported to date.[1] Our approach to metallopeptide design has been to introduce a metal-binding site into the hydrophobic interior of an α-helical coiled coil, a folding motif found in many natural proteins[2] and designed peptides.[3] Each ligand is donated by a different α-helix, and the helices are not covalently connected. With this design, we can utilize the noncovalent self-assembly of the peptides to control the coordination environment of the metal. More importantly, by building the binding site in the interior of the bundle, solvent and buffer ligation to the metal can be controlled, thus allowing for the preparation of metal-coordination environments that are difficult to obtain in aqueous solution.

The first site selected to test our approach was $Hg(S\text{-}Cys)_3$.[4] While mercury generally prefers a coordination number of 2,[5] a few well-characterized aliphatic mononuclear trigonal thiolate complexes have been reported to date,[6,7] and these are stable only in the solid state or nonaqueous environments. However, spectroscopic[7−9] and mutagenesis studies[10] suggest that a trigonal cysteine environment is utilized by the metalloregulatory protein MerR to selectively bind Hg(II), activating the transcription of *merTPAD* mercury-resistance genes in prokaryotes.[11] Our goal, therefore, was to design a metallopeptide that could create a water-stable, trigonal cysteine environment for Hg(II).

The three-coordinate site was engineered into a trimeric model of the peptide Tri,[12] a peptide based on CoilSer.[13] Replacement of Leu at position 16 with Cys (an *a* position in the heptad repeat[2]) gave the peptide L16C. In three-helix bundle models, L16C generates a three-coordinate thiolate site in both the parallel and antiparallel (up−up−down) bundle topologies.[14] L16C has been shown by CD to be greater than 80% helical at micromolar concentrations.[15]

CoilSer has been shown to exist in a monomer/dimer/trimer equilibrium, which favors trimers at reasonably high (micromolar) concentrations. Similarly, L16C forms dimers and trimers, with the trimeric state being favored near neutral pH. Sedimentation equilibrium at 150−590 μM showed that L16C is predominantly dimeric and trimeric at pH 2.5 and 5.5, respectively.[16] At pH 8.5, L16C is trimeric both in the absence and presence of 1 equiv of Hg(II) per three-helix bundle.[16]

The stoichiometry of the complexes formed between L16C and Hg(II) illustrates the interplay between metal/ligand and noncovalent peptide/peptide interactions. The L16C complexation of Hg(II) was studied at pH 8.5, where the thiolate form of cysteine should dominate. L16C was titrated into aqueous Hg(II), and the UV absorbance (arising from geometry-dependent ligand-to-metal charge transfer bands) was monitored to determine the stoichiometry of the Hg/peptide complexes formed (Figure 1). At low peptide/Hg(II) ratios, a two-coordinate complex was formed based on the slope of a plot of $\Delta\epsilon$ vs equivalents of L16C per Hg(II) (Figure 1b). The wavelength and extinction coefficient for the major transition are similar to those observed for linear, bis-thiolate Hg(II) complexes[5] (Table 1), suggesting that at these peptide/Hg(II) ratios binding of the metal ion favors formation of a 1:2 Hg/

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(4) Abbreviations used: Ac, acetylated N-terminus; CD, circular dichroism; $\Delta\epsilon$, difference in extinction coefficient; S^{t}Bu, *tert*-butyl thiolate; EXAFS, extended X-ray absorption fine structure; σ, Debye−Waller factor; Cys, cysteine; Leu, leucine.

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(14) In the structure of CoilSer[13b,13c] and other three-stranded coiled coils, the side chains of the residues at *a* and *d* positions segregate into distinct layers. In the parallel orientation, the three-coordinate site would form within a layer. In the antiparallel orientation, the site would form between two layers, with two Cys originating from one layer and the third residue from an adjacent layer.

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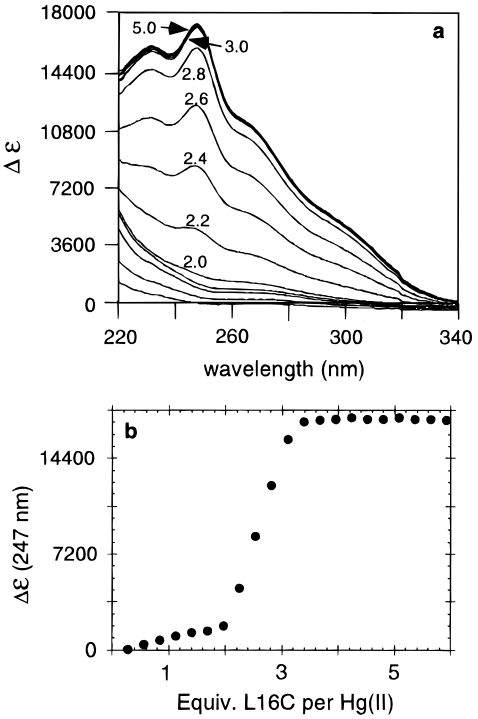

Figure 1. UV titration of L16C added to Hg(II) at pH 8.5 (20 mM phosphate buffer). (a) Spectra shown are difference spectra ($\epsilon_{L16C+Hg} - \epsilon_{L16C}$) and correspond to samples with 0−2.0 equiv of L16C per Hg(II) (every 0.4 equiv of L16C), and 2.0−5.0 equiv of L16C (every 0.2 equiv of L16C). (b) $\Delta\epsilon$ at 247 nm vs equiv of L16C per Hg(II) at pH 8.5.

Table 1. Spectroscopic Values for Hg/MerR and Hg/L16C Complexes

sample	ET[a] (nm) [ϵ (cm^{-1} mM^{-1})]	R_{HgS}[b] (Å)	δ_{obsd}[c] (ppm)
Hg/MerR	242 [19.8][d]	2.43[e]	−106[f]
	260 [14.6][d]		−109[f,g]
	290 [6.45][d]		
1:2 Hg/L16C	240 [2.70]	2.30	−834[h]
			−179
1:3 Hg/L16C	247 [16.8]	2.39	−179
	265 [10.6]		
	295 [5.00]		

[a] Electronic transitions. [b] Average Hg−S bond length. [c] ^{199}Hg NMR chemical shift (relative to $Hg(CH_3)_2$). [d] Reference 7, pH 7.00. [e] Reference 8, pH 7.5. [f] Reference 9, pH 6.02. [g] Hg/MerR complexed with DNA. [h] Major species.

L16C complex. However, as the concentration of peptide is increased beyond the 1:2 Hg/L16C ratio, a new species grows into the spectra with the spectral characteristics expected for a three-coordinate Hg complex. The new transitions at 247, 265, and 295 nm (Figure 1a and Table 1) are similar to those observed for $Hg(S^tBu)_3^-$.[7] The spectrum of the new species, formulated as $Hg(L16C)_3^-$,[16] remained constant upon addition of excess peptide.

The Hg(II)-binding site was further characterized by ^{199}Hg NMR (Table 1). The excellent correlation between the observed ^{199}Hg chemical shift and that of the primary coordination environment of Hg(II) thiolate model complexes has been used to elucidate the Hg-coordination environments of several Hg(II)-substituted proteins.[9,17] The ^{199}Hg NMR of a peptide sample with a 1:3 Hg/L16C stoichiometry at both pH 7.45 and 8.5 exhibited the same ^{199}Hg chemical shift of −179 ppm, relative to neat $Hg(CH_3)_2$. This resonance is shifted slightly upfield from the chemical shift range of −80 to −160 ppm for mononuclear three-coordinate aliphatic thiolate mercury complexes, but far from bis-thiolate Hg(II) complexes which exhibit solution ^{199}Hg shifts ranging from −760 to −990 ppm.[5] At lower peptide concentrations (approximately 1:2 Hg/L16C), an additional signal was observed at −834 ppm, consistent with the conclusion that L16C forms two-coordinate Hg complexes at lower peptide/Hg(II) ratios.

EXAFS further confirmed that L16C bound Hg(II) in a two-coordinate geometry at low peptide/Hg(II) ratios, and in a three-coordinate geometry when at a 1:3 Hg/L16C ratio (Table 1). The EXAFS data for the 1:2 Hg/L16C sample give an average bond length consistent with well-ordered, two-coordinate mercury.[5] The EXAFS oscillations show a marked shift to higher frequency when an additional equivalent of L16C is present at pH 8.5. This shift corresponds to an average Hg−ligand bond length of 2.39 Å, an increase of 0.09 Å, which is consistent with a change from two- to three-coordinate Hg(II).[5,8] The goodness of fit to the 1:3 Hg/L16C (pH 8.5) data did not improve significantly if nitrogen or oxygen ligation was included.[16] A bond length of 2.39 Å is slightly shorter than that expected for a perfectly trigonal Hg−S site.[5] The short bond length, in conjunction with the dramatically lower EXAFS amplitude and higher σ^2 for the 1:3 sample, suggests that either the Hg(II) site is distorted from a trigonal geometry or else the EXAFS sample contained a small amount of the two-coordinate form in addition to the three-coordinate complex; either would be sufficient to give rise to the observed value of σ^2.

Previous work on the *de novo* design of metalloproteins has focused on highly stable and common ligand geometries, which serve to define the fold or dynamics of the protein.[1] The ability to control both the ligand geometry and the aqueous exposure of a metal-binding site is an important step in the ultimate realization of functional *de novo* designed metalloproteins. In the present work, we demonstrate that it is also possible to design molecular assemblies whose tertiary structures define the geometry and stoichiometry of a metal-binding site—giving the common bis-thiolate site in the dimeric assembly or the far less-common three-coordinate environment in the trimer. It is significant to note that $(L16C)_3$ is the first water-soluble and stable model for MerR that supports a three-coordinate Hg(II)-binding site. These results also show how the free energy balance between metal-binding and protein folding can influence the association state of a peptide. At high peptide/Hg(II) ratios, the intrinsic trimeric conformational preference of L16C can enforce an unusual three-coordinate geometry on the Hg(II).

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Supporting Information Available: Experimental procedures, EXAFS, and sedimentation equilibrium results (5 pages). See any current masthead page for ordering and Internet access instructions.

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