Communications

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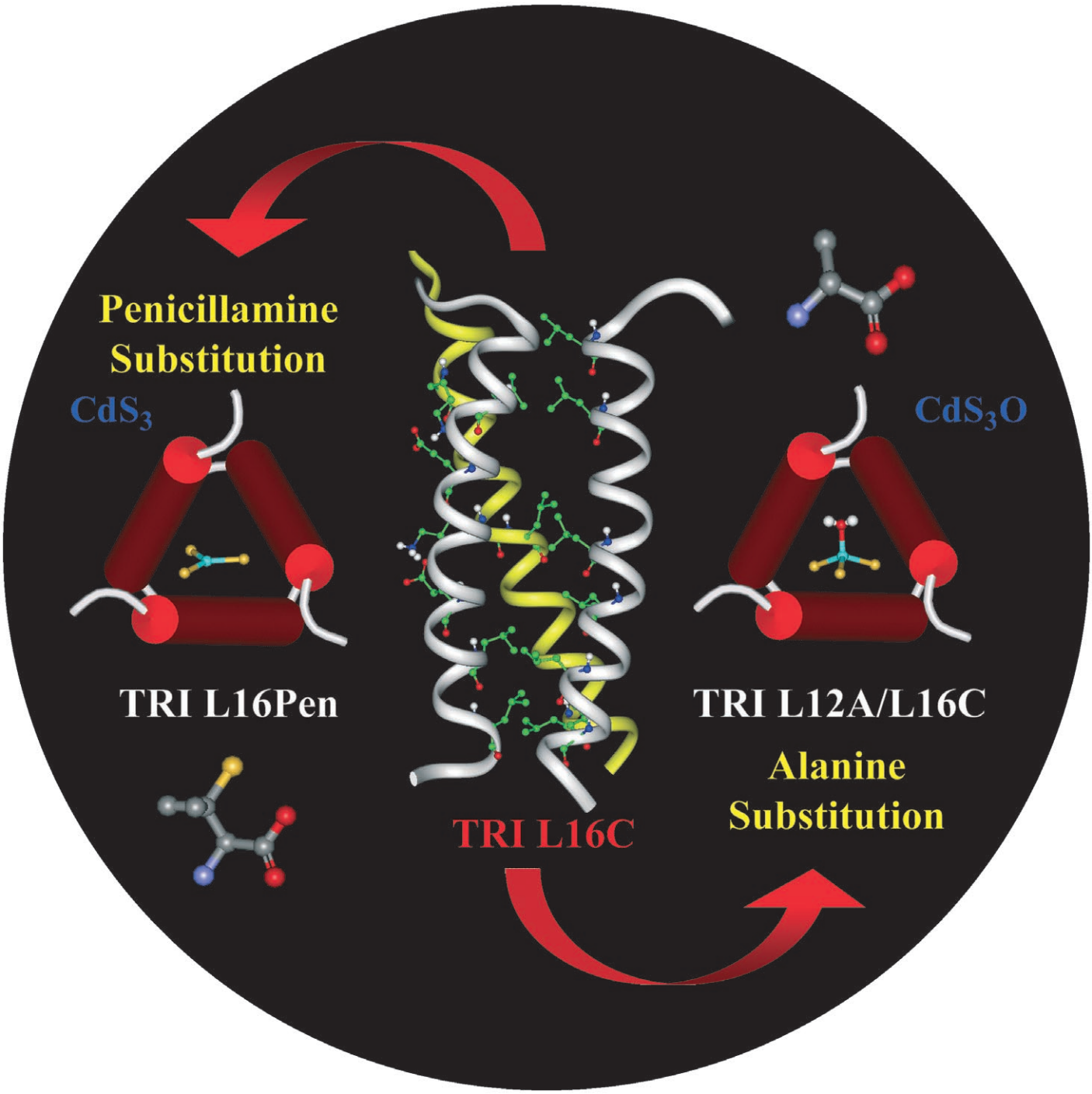

Controlling the coordination geometry of a metal center is the first step in preparing proteins capable of catalysis. Substitution of a single amino acid can shift an equilibrium of 3- and 4-coordinate Cd^{II}–peptide complexes to proteins containing exclusively 3- or 4-coordinate metal sites. For details, see the communication by V. L. Pecoraro and co-workers on the following pages.

Metalloproteins

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Using Nonnatural Amino Acids to Control Metal-Coordination Number in Three-Stranded Coiled Coils**

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Proteins are remarkably adept at controlling the geometry and the type and number of ligands bound to a metal center. A single protein may contain two or more metal ions in very different coordination environments.[1,2] For example, 5-aminolevulinic acid dehydratase, which is found in mammals and yeast and is involved in the biosynthesis of heme, contains two Zn^{II} ions, one bound in a five-coordinate geometry with oxygen and nitrogen atoms as ligands and the other bound in a pseudo-tetrahedral geometry with three cysteine sulfur atoms and an exogenous water molecule.[1,2] Lead toxicity results in part from the displacement of the Zn^{II} ion by Pb^{II} from the tetrahedral site (but not from the pentacoordinate site), thus rendering the enzyme inactive.[3] Such exquisite control of ion recognition by proteins is difficult to reproduce with small molecules under most synthetic conditions.

We have been exploring how subtle changes in small, de novo designed peptides can produce even stronger control of metal-coordination number and ligand type.[4] In particular, we have asked whether the same metal can be bound to structurally related peptides with identical first-coordination-sphere ligands derived from the protein, but in one case form a trigonal structure and in a second case form a four-coordinate pseudo-tetrahedral environment.[4] The relative importance of a metal's geometric preference and the inherent protein structure is fundamental to understanding the folding,[5–8] stability,[9,10] and conformational changes[4,5,11] of metalloproteins, and hence the control of such site discrimination has been an important objective of protein design studies.

The peptides described herein are variants of the TRI family of peptides shown in Table 1. The sequence of the TRI peptide is Ac-G(LKALEEK)$_4$G-NH_2.[12] A leucine residue at the hydrophobic layers is replaced by a cysteine residue at position 16 (TRI L16C) for generating a metal-binding site.[12] According to previous studies of Cd^{II} binding to TRI L16C by ^{113}Cd NMR spectroscopy,[13] ^{111m}Cd perturbed angular correlation (PAC) spectroscopy,[14,15] and X-ray absorption spectroscopy (XAS),[16] two cadmium species were present at pH 8.5.[17] The PAC results on the Cd complex of TRI L16C indicated that 41% of the Cd^{II} centers had a trigonal planar geometry ($\omega_o = 0.438$; $\eta = 0.20$) and 59% the tetrahedral ($\omega_o = 0.337$; $\eta = 0.22$) cadmium structure.[17] In contrast, ^{113}Cd NMR spectroscopy exhibited a single resonance at 625 ppm. These observations indicate that the two structures interconvert rapidly on the NMR timescale (0.01–10 ms), but are in the slow exchange regime on the PAC timescale (0.1–100 ns).[4,17,18] The observed Cd–S bond distance of 2.49 Å for the TRI L16C peptide is in good agreement with an equilibrium distribution of three- and four-coordinate Cd^{II} complexes (CdS_3: Cd–S 2.45 Å; CdS_4: Cd–S 2.54 Å).[17,19–22]

Our objective was to prepare peptides with minimal sequence changes that would bind Cd^{II} exclusively in either three- or four-coordinate geometries. The goal is particularly challenging because three-coordinate Cd^{II} structures have never been observed in biological molecules or in small-molecule complexes in water. Our strategy was to modify steric bulk around the metal-binding site in an effort to either allow or exclude exogenous water ligands from approaching the metal. Hodges had previously estimated that replacing leucine with alanine could allow as many as five water molecules to enter the hydrophobic interior of a four-stranded α-helical bundle.[34] As shown by the data in Figures 1 and 2 and Table 2, decreasing the steric bulk above the metal-binding site by substitution of a leucine with an alanine

Table 1: Sequences of the peptides used in this study.

Peptide	Sequence[a]
TRI L16C	Ac-G LKALEEK LKALEEK **C**KALEEK LKALEEK G-NH_2
TRI L12A/L16C	Ac-G LKALEEK LK**A**AEEK **C**KALEEK LKALEEK G-NH_2
TRI L12Hfl/L16C	Ac-G LKALEEK LK**X$_1$**EEK **C**KALEEK LKALEEK G-NH_2
TRI L16Pen	Ac-G LKALEEK LKALEEK **X$_2$**KALEEK LKALEEK G-NH_2

[a] X_1 = L-5,5,5,5′,5′,5′-hexafluoroleucine, X_2 = Penicillamine.

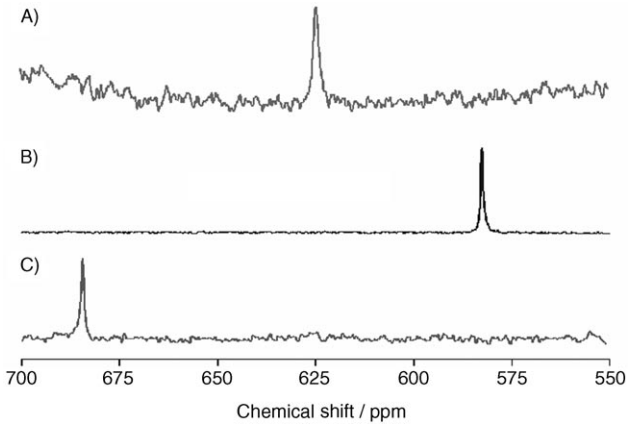

Figure 1. ^{113}Cd NMR spectra of the TRI family of peptides studied. A) $[Cd(TRI\ L16C)_3]^-$; B) $[Cd(TRI\ L12A/L16C)_3]^-$; C) $[Cd(TRI\ L16Pen)_3]^-$.

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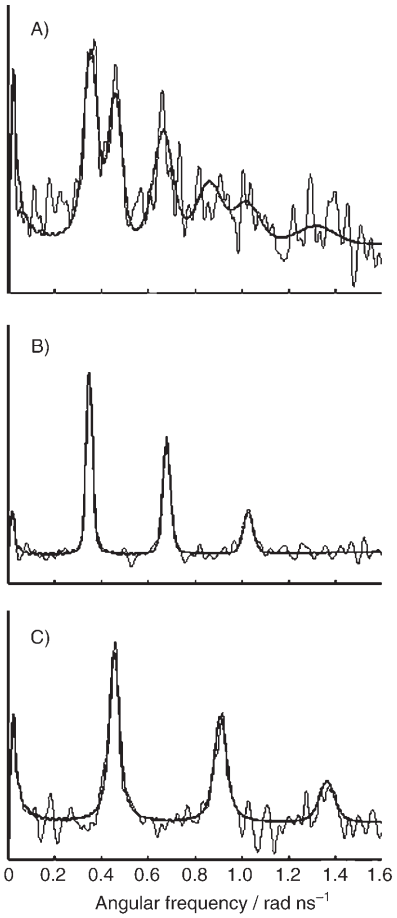

Figure 2. PAC spectra of A) $[Cd(TRI\ L16C)_3]^-$, B) $[Cd(TRI\ L12A/L16C)_3]^-$, and C) $[Cd(TRI\ L16Pen)_3]^-$.

Table 2: Physical parameters for $[Cd^{II}(TRI)_3]^-$ peptides.

Peptide[a]	λ_{max} ($\Delta\varepsilon$)[b]	^{113}Cd NMR[c]	^{111m}Cd PAC (ω_o, η, %)[d]
L16C[17]	232 (22600)	625	0.438(4), 0.20(3), 41% 0.337(2), 0.22(1), 59%
L12A/L16C	231 (21200)	574	0.3405(3), 0.141(4), 100%
L12Hfl/L16C	231 (19400)	607	–
L16Pen	231 (21600)	684	0.4540(9), 0.02(10), 100%

[a] All complexes correspond to $[Cd^{II}(TRI\ derivative)_3]^-$. [b] λ_{max} given in nm and $\Delta\varepsilon$ given as $M^{-1}cm^{-1}$. [c] Given in ppm versus the standard $Cd(ClO_4)_2$ in D_2O. Data collected at pH 8.5. [d] ω_o given in rad ns^{-1}; η is a unitless quantity. Percentage values of species are based on best fit to PAC. Calculated values for pure trigonal planar $Cd^{II}S_3$ are $\omega_o = 0.450$ and $\eta = 0$.

residue in the hydrophobic core provided 100% of the four-coordinate structure $[Cd(TRI\ L12A/L16C)_3]$.[4] Thus, the pure four-coordinate complex could be prepared straightforwardly by hydrating the metal-binding cavity.

By using the ^{113}Cd NMR chemical shifts for $[Cd(TRI\ L16C)_3]^-$ and $[Cd(TRI\ L12A/L16C)_3]^-$ together with the quantification of the percentages of three- and four-coordinate species from PAC spectroscopy, we are now able to calculate the theoretical ^{113}Cd NMR chemical shift of a complete three-coordinate cadmium species ($Cd^{II}S_3$) to be approximately 698 ppm. This value, as well as that of the $Cd^{II}S_3O$ species (574 ppm), differs from previously used[22,23] assignments for three- and four-coordinate Cd^{II} and leads to the reassessment of earlier reports of pure three-coordinate Cd^{II}.[4,23]

The more challenging objective was to dehydrate the Cd^{II} core environment. We reasoned that if decreasing the substituent bulk above the metal site allowed the access of more water, and hence a higher metal-coordination number, then increasing the steric constraints might yield a less hydrated environment that could provide the desired adduct with a lower coordination number. To this end, we synthesized the nonnatural amino acid L-5,5,5,5′,5′,5′-hexafluoroleucine,[24] and incorporated this residue into the layer above the metal-binding site (TRI L12Hfl/L16C) by manual peptide synthesis.[25] To ensure that the aggregation state and metal-binding properties of this peptide were unperturbed by the fluoro groups, we completed guanidinium chloride denaturation titrations of the system as well as metal-binding titrations with Hg^{II} and Cd^{II} solutions. As expected on the basis of previous literature precedent,[26–29] there was a slight stabilization of the three-stranded coiled coil ($\Delta G_{bind} = -0.4$ kcal mol^{-1} per peptide) as a result of the increased hydrophobicity of the hexafluoroleucine side chain. The metal-binding titrations with Hg^{II} and Cd^{II} ions gave similar results to those of other peptide derivatives, and metal-binding association constants were determined which showed that the $[Hg^{II}(TRI\ L12Hfl/L16C)_3]^-$ complex followed the free-energy correlation reported for numerous other derivatives in the TRI peptide family.[30] Based on these results, we concluded that the fluorinated peptides behaved as other members of this series. The ^{113}Cd NMR chemical shift for this peptide corresponds to a mixture of CdS_3 and CdS_3O complexes (Figure 1 and Table 2). Furthermore, relative to TRI L16C, the distribution was shifted away from the desired three-coordinate complex. These observations demonstrate that obtaining the elusive CdS_3 geometry requires more than simply increasing the side-chain bulk above the metal.

An alternative strategy was to provide more steric bulk within the trigonal plane of the Cd^{II} ion. This was achieved by replacement of a cysteine with a penicillamine residue[31] (TRI L16Pen), which has two methyl groups replacing the β-methylene hydrogen atoms of cysteine. We reasoned that these two methyl groups could affect both the conformation of the sulfur donors to Cd, while enhancing the local bulk directly around the metal center. As shown in Figure 1 and Table 2, the ^{113}Cd NMR spectrum of $[Cd(TRI\ L16Pen)_3]^-$ was markedly different from those of all other TRI derivatives. The value of 684 ppm in Table 2 is very close to the predicted fully three-coordinate chemical shift of 698 ppm. Conclusive proof for the assignment of this derivative as a CdS_3 species comes from the PAC measurements shown in Figure 2. Only one species is observed with $\omega_o = 0.4540$ and $\eta = 0.02$. Thus, we have prepared for the first time a water-stable, three-coordinate CdS_3 structure.

In conclusion, we have shown that it is possible to control the coordination number of a metal ion in a model protein of $M_r \approx 10200$ Da by changing only one of the amino acid residues in the primary sequence. By starting with the TRI L16C peptide, simple replacement of the cysteine

residue with penicillamine can shift a 41:59 equilibrium of three- and four-coordinate structures to fully three-coordinate. Alternatively, by substituting an alanine residue for leucine in the layer above the metal, TRI L16C can be converted to a peptide that binds Cd^{II} exclusively as a four-coordinate CdS_3O complex. Clearly, small changes, even in such simple systems, can profoundly alter the energy landscape for a metalloprotein. As an example, further structural studies are required to understand why additional bulk from hexafluoroleucine above the metal leads to more water binding, whereas bulk within the plane of the metal fully excludes water from the center. Furthermore, these studies underscore an advantage of de novo design methods, as this technique allows us to employ nonnatural amino acids in our protein sequence readily. We expect that future studies with these peptides will help to unravel the mechanism of heavy-metal insertion into proteins, and may provide insight into metal trafficking in cells by metallochaperones.

Experimental Section

Peptide synthesis and purification: All the peptides of the TRI family except the fluorinated one were synthesized with Fmoc-protected (Fmoc = 9-fluorenylmethoxycarbonyl) amino acids by standard protocols on an ABI 433A automated synthesizer. Peptides were cleaved from the resin by stirring the mixture of trifluoroacetic acid (TFA), ethanedithiol, thioanisole, and anisole (90:3:5:2) at room temperature for 2 h. Peptide TRI L12Hfl/L16C was synthesized by using *t*Boc-protected (*t*Boc = *t*-butoxycarbonyl) amino acids for Merrifield solid-phase synthesis on 4-methylbenzhydrylamine (MBHA) resin. Coupling reactions were carried out manually with the in situ neutralization/HBTU protocol[25] on a 0.25 mM scale. The peptide was cleaved from the resin under vigorous HF conditions. The nonnatural fluorinated amino acid L-5,5,5,5′,5′,5′-hexafluoroleucine was synthesized as reported previously[23] and converted to *t*Boc-protected derivatives by standard procedures for *t*Boc solid-phase peptide synthesis. Peptides were dissolved in 10% acetic acid and purified by reversed-phase HPLC on a preparative C_{18} column, with a linear gradient from 0 to 90% acetonitrile containing 0.1% TFA. The identity of the peptides was proved by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and the purity was determined by analytical HPLC. The peptide concentrations were determined by Ellman's test.[32]

^{113}Cd NMR spectroscopy: ^{113}Cd NMR spectra were collected on a Varian UnityINOVA 500-MHz NMR spectrophotometer equipped with a 5-mm tunable broadband probe. $Cd(ClO_4)_2$ (0.1M) in D_2O was used as an external reference. All spectra were recorded at room temperature for 3 h. The ^{113}Cd–TRI peptide complexes were prepared by using $^{113}CdCl_2$ (95% enriched, Cambridge Isotopes). The complex solutions ($\approx$6 mM) were prepared in 10% D_2O without using a buffer solution. The pH values of the solutions were adjusted to 8.5 with KOH solution. All the spectra were generated from the free induction decays with the software MestRe-C.[33]

^{111m}Cd PAC spectroscopy: PAC experiments were carried out at a temperature of 1 ± 2 °C. The radioactive ^{111m}Cd solution (10–40 μL) was mixed with nonradioactive cadmium acetate and Tris buffer. The TRI family of peptides was then added to the ^{111m}Cd solution and the sample was left to equilibrate for 10 min to allow metal binding. Sucrose was added to produce a 55% (w/w) solution to reduce the Brownian tumbling of the molecules, and the pH was adjusted to 8.7 with H_2SO_4 and KOH. All buffers were purged with argon and treated to prevent metal contamination. The final volumes of the samples were from 0.05 to 0.5 mL with a concentration of 300 μM peptide and 20 mM Tris and a Cd^{II}/peptide ratio of 1:12. All fits were carried out with 400 data points, but the first three points were disregarded because of systematic errors.

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