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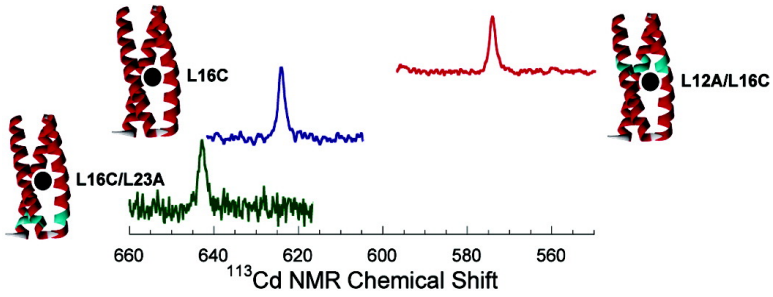

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Control of Metal Coordination Number in de Novo Designed Peptides through Subtle Sequence Modifications

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A major goal of de novo metalloprotein design is the ability to control the coordination number and geometry of bound metal ions.[1−3] We have previously demonstrated that metals may be sequestered into the hydrophobic core of parallel, α-helical, three-stranded coiled coils generated by folding of the TRI peptide series which contains a single cysteine residue in each sequence.[4−7] We designed metal binding sites that were highly sterically hindered, based upon analogy with small-molecule model compounds,[8−10] to maintain a maximum coordination number of three.[11] By using this system, a higher than normal coordination number could be enforced on Hg(II),[4] whereas complexation of Cd(II) led to an equilibrium mixture of three-coordinate trigonal planar species and a second one that was either four-coordinate species or trigonal pyramidal.[12] Previously, Hodges studied the modification of coiled coil structure upon amino acid substitution of the hydrophobic interior.[13] It seemed reasonable that modifying the steric bulk of the hydrophobic layer either above or below the metal binding region should alter the equilibrium between these two cadmium species. In particular, we felt that decreasing the steric bulk in an adjacent layer might be used to develop a cavity[13] that would allow a water molecule to permanently occupy a fourth coordination position on the metal. We have now investigated such substitutions with ^{113}Cd NMR and find, contrary to expectations, that decreasing the steric bulk in layers above or below the metal leads to signals that can best be described as belonging to a species with a decreased metal coordination number. Furthermore, modification of steric bulk at residues located significantly away from the metal binding site can still be used to control metal coordination number and may do so either by lowering or increasing the number of metal-bound ligands.

The peptides used in this work are members of the TRI family shown in Scheme 1. Substitution of a leucine for a cysteine at position 9 (TRI L9C) or 16 (TRI L16C) provides a thiolate for metal binding. Previous studies of Cd(II) binding to TRI L16C using ^{113}Cd NMR, perturbed angular correlation spectroscopy (^{111m}Cd PAC), and X-ray absorption spectroscopy (XAS) demonstrated that two metal species were in equilibrium at pH 8.5.[12] In particular, the metal coordination geometry can be directly probed using ^{113}Cd NMR spectroscopy, a well-established method for determining coordination number and ligand identity, since it is known that Cd(II)S_3 complexes exhibit a resonance in the range 570−575 ppm[14] whereas four-coordinate S_3O and S_3N species appear at ∼650 ppm.[15] If water exchange is fast on the NMR time scale, then a coalesced resonance between 570 and 650 ppm is observed. Cd(II)-(TRI L16C)$_3^-$ has a single ^{113}Cd resonance at 624 ppm which corresponds to a 35 to 65% ratio of three- to four-coordinate species, respectively.[12] The dominant species is composed of a four-coordinate S_3O complex using three cysteine sulfur atoms, one from each peptide, and one exogenously ligated water molecule.[12] The species at a lower proportion was assigned as a trigonal planar S_3 structure.[12]

Scheme 1. Sequences of the Peptides Used in This Study; All the Peptides Are Variants of the Peptide TRI

L9C	Ac-G LKALEEK **C**KALEEK (LKALEEK)$_2$ G-NH_2
L16C	Ac-G (LKALEEK)$_2$ **C**KALEEK LKALEEK G-NH_2
L9A/L16C	Ac-G LKALEEK **A**KALEEK **C**KALEEK LKALEEKG-NH_2
L12A/L16C	Ac-G LKALEEK LKA**A**EEK **C**KALEEK LKALEEKG-NH_2
L12V/L16C	Ac-G LKALEEK LKA**V**EEK **C**KALEEK LKALEEKG-NH_2
L12G/L16C	Ac-G LKALEEK LKA**G**EEK **C**KALEEK LKALEEKG-NH_2
L16C/L19A	Ac-G (LKALEEK)$_2$ **C**KA**A**EEK LKALEEK G-NH_2
L16C/L23A	Ac-G (LKALEEK)$_2$ **C**KALEEK **A**KALEEK G-NH_2

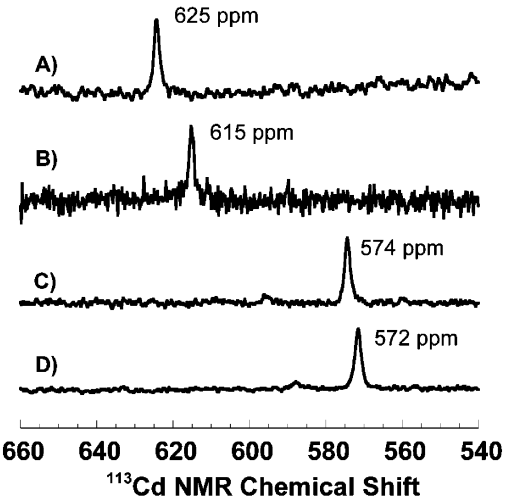

Figure 1. ^{113}Cd NMR spectra of (A) Cd(TRI L16C)$_3^-$, (B) Cd(TRI L12V/L16C)$_3^-$, (C) Cd(TRI L12A/L16C)$_3^-$, and (D) Cd(TRI L12G/L16C)$_3^-$.

We have now examined the substitution of a leucine in the 12 position of TRI L16C by a valine (TRI L12V/L16C), an alanine (TRI L12A/L16C), or a glycine (TRI L12G/L16C). Residues in the 12 position form the hydrophobic layer directly above the Cd(II) binding site. As shown in Figure 1, progressive substitution of sterically less demanding residues leads to an upfield shift of the ^{113}Cd resonance until, at glycine, a signal at 572 ppm is achieved. The simplest explanation for this transition, based on model compounds, is the lowering of the coordination number to three. The shift toward lower coordination upon decreasing the bulk of the adjacent residues is contrary to most design strategies for small molecules and peptides where steric control has been thought to be essential to achieve low coordination number complexes.[8−10] A comparison of the chemical shifts for Cd(TRI L12A/L16C)$_3^-$, 574 ppm, and Cd(TRI L12G/L16C)$_3^-$, 572 ppm, indicates that both species have nearly equivalent proportions of trigonal Cd(II). Therefore, further experiments were completed using the helix-stabilizing alanine substitution. The stability of the peptides was not significantly perturbed by the alanine substitutions as determined from guanidinium denaturation experiments (data not shown).

A similar, but less pronounced effect is observed when a leucine-to-alanine substitution occurs in the layer below the Cd binding

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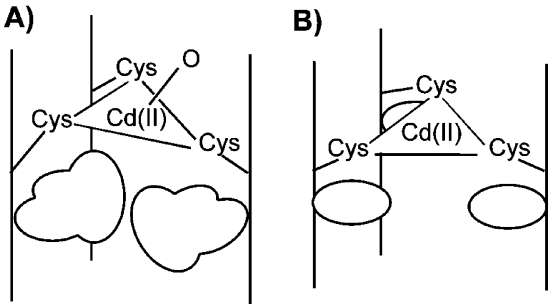

Figure 2. Schematic representation of the effect of bulky hydrophobic ligands on the orientation of the metal plane. (A) A leucine layer causes the metal plane to tilt, exposing it to water. (B) An alanine layer allows for the metal plane to be repositioned perpendicular to the helical axis.

site. In this case, Cd(TRI L16C/L19A)$_3^-$ exhibits a 605 ppm resonance that corresponds to roughly 60% S_3 and 40% S_3O (or S_3N) complexes. It is not surprising that the impact of the substitution at the 12 and the 19 positions is of slightly different magnitude since the layers are not equivalently disposed with respect to the Cd binding site (e.g. substitution at the 12 position is four residues from the 16 position, whereas the 19 layer is only three residues below the metal binding site).

Without detailed crystallographic information, it is difficult to conclusively ascertain the explanation for these effects. However, we believe the most appropriate model to understand these observations lies in the modification of the packing of hydrophobic layers. It is likely that substitution of the smaller alanine for leucine allows for an alteration of the packing of the leucine residues in layers above (TRI L12A/L16C) or below (TRI L16C/L19A) the metal binding layer. In so doing, the cysteine residues may be able to reorient in such a way that the Cd(II) is rotated fully into the hydrophobic interior of the coiled coil. Increasing the steric bulk would tilt the Cd(II) trigonal plane and allow a water molecule or a side chain nitrogen of lysine to insert into the Cd(II) first-coordination sphere through the interhelical interface. This model is shown as Figure 2. However, we cannot exclude an additional model in which the initial cavity design has forced the Cd(II) to adopt an undesirable trigonal pyramidal geometry.

A prediction of our model is that substitution at a hydrophobic layer at even further distances should have an effect on the Cd(II)S_3 and Cd(II)S_3O or Cd(II)S_3N distribution which can be monitored using ^{113}Cd NMR spectroscopy. To test this hypothesis, we prepared TRI L9A/L16C and TRI L16C/L23A which retain the steric bulk adjacent to the metal site but contain a modification to a hydrophobic layer approximately 7 Å from the metal. With TRI L9A/L16C, a major perturbation of the metal site occurs with an observed resonance at 583 ppm corresponding to ~90% formation of the Cd(II)S_3 species. In marked contrast to every other derivative that we examined, TRI L16C/L23A shows a downfield shift to 643 ppm, indicating according to ^{113}Cd NMR that *the site is predominantly four-coordinate*. Because of this observation, we prepared TRI L9C/L12A and TRI L9C/L16A to test whether this was a general phenomenon. Again, TRI L9C/L12A shifted upfield consistent with formation of primarily a trigonal site (605 ppm), whereas TRI L9C/L16A gave a downfield shifted resonance indicative of the four-coordinate structure (643 ppm). These studies with L9C derivatives also demonstrate conclusively that the effect occurs in a parallel three-stranded coiled coil as this is the only orientation that can offer three sulfur atoms to the metal. This is a remarkable demonstration of how a remote modification that neither presents a change in hydrogen bonding to metal ligands nor an additional ligand atom to the metal, or for that matter, does not alter markedly the secondary or quaternary structure of the aggregate can lead to profound changes in the metal coordination sphere. The biological importance of these observations is underscored by the report that remote alteration of residue packing in carbonic anhydrase alters metal selectivity and metal geometry.[16,17]

In conclusion, we have presented a simple designed system that allows exquisite control of metal coordination geometry through the simple expedient of remote modifications to helical packing. While further experiments are necessary to fully explain this very interesting set of observations, those in metalloprotein design and protein folding communities now have available a well-characterized model of how noncoordinating amino acids can markedly alter the resultant structure of a metal center in a protein.

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Supporting Information Available: Difference UV titration data of TRI L12A/L16C with Cd(II) and a NOESY spectrum of Cd(TRI L12V/L16C)$_3^-$. This material is available free of charge via the Internet at http://pubs.acs.org.

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