Published in final edited form as:
Angew Chem Int Ed Engl. 2011 February 25; 50(9): 2049–2053. doi:10.1002/anie.201006413.

Realization of a Designed Three-Helix Bundle Capable of Binding Heavy Metals in a Tris(Cysteine) Environment**

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An important objective of *de novo* protein design is the preparation of metalloproteins, as many natural systems contain metals which play crucial roles for the function and/or structural integrity of the biopolymer.[1,2] Metalloproteins catalyze some of the most important processes in nature, ranging from energy generation and transduction to complex chemical transformations. At the same time, metals in excess can be deleterious to cells and some ions are purely toxic, having no known beneficial effects (e.g., Hg^{II} or Pb^{II}). One hopes to use a first principles approach for realizing both known metallocenters and also to prepare novel sites which may lead to exciting new catalytic transformations. However, designing novel metalloproteins is a challenging and complex task, especially if one desires to prepare asymmetric metal environments.

Numerous metalloprotein systems have been designed over the past fifteen years, typically using unassociated peptides that assemble into three- stranded coiled coils or helix-loop-helix motifs that form anti-parallel four- stranded bundles. In terms of metal ion binding these systems have been functionalized with heme,[3,4] and non-heme mononuclear,[5] and binuclear centers.[6,7] It is often difficult to prepare non-symmetrical metal sites through these strategies due to the symmetry of the systems relying on homo-oligomerization. Thus, the preparation of a single polypeptide chain capable of controlling a metal coordination environment is a key objective.

Previously, we have designed soft, thiol-rich metal binding sites into the interior of parallel, three-stranded α-helical coiled coils involving cysteine and/or penicillamine as the ligating amino acid residues.[8,9] These systems have served as hallmarks for understanding the metallobiochemistry of different heavy metals such as Cd^{II}, Hg^{II}, As^{III}, and Pb^{II}.[8-11] We have shown how to control the geometry and coordination number of metals such as Cd^{II}

**V.L.P. thanks the National Institute of Health for support of this research (ES012236), P.W.T. and L.H. thank ISOLDE collaboration at CERN for the ^{199m}Hg beam time grant (experiment IS488) and The Danish Council for Independent Research | Natural Sciences.

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and Hg^{II} at the protein interior as well as fine-tuning the physical properties of the metals, which has led to site-selective molecular recognition of Cd^{II}.[12-14] While these homotrimeric assemblies have been very useful, the production of heterotrimeric systems that are capable of fine tuning metal environments controllably or introduction of a site specific H-bond has been elusive.[15] Therefore, we have chosen an alternative strategy to satisfy this objective using a single polypeptide chain instead of multiple self associating peptides.

Existing designed heteromeric helical bundles and coiled-coils show energetic preferences of several kcal/mol for the desired heteromeric versus homomeric assemblies.[16,17] However, the energy gap between a hetero and homomeric assembly often depends critically on ionic strength, pH, and other environmental parameters. Moreover, the objective of many studies in *de novo* protein design is to impart an energetically sub-optimal coordination geometry on the metal ion, and the degree to which this strategy will be successful depends on the size of the energy gap between the desired heteromeric assembly and other homomeric or misfolded states. Also, even when heterooligomeric bundles have been used to successfully identify specific environmental effects that influence substrate binding or metal ion cofactor reactivity,[18] the non-covalently assembled complexes have often been difficult to characterize structurally, possibly due to small populations of alternately assembled species. In this case, combining the active site residues into a construct with linked helices greatly facilitated structural analysis and catalytic characterization.[19]

An attractive starting scaffold to meet our objectives is the *de novo* designed three-helix bundle α_3D. The NMR structure of this protein is known and it has been proven that the helices are oriented in a counter-clockwise topology.[20] Although the α_3D protein originated from a coiled-coil, its helices were shortened to the point where it might be better considered as a globular protein whose repetitive structure makes each of the heptads very similar to one another (in the absence of end-effects). The stability of α_3D is similar to that of natural proteins and thus α_3D should be tolerant to mutations, allowing this protein to serve as an excellent framework to engineer specific metal binding sites. Additionally, with this protein scaffold we can study the effect of the ligating residue located on the second helix which is antiparallel to the first and third helices of the bundle.

Before attempting to prepare asymmetric metal coordination environments or site specific H-bonds, we felt it was important to first redesign α_3D so as to introduce symmetric metal binding sites involving cysteine residues. This approach would allow us to exploit the extensive body of work defining heavy metal complexation using the TRI and Coil Ser peptides,[8,15] to assess whether a specific metal structure could be achieved in the modified α_3D construct. It would also allow us to probe the physical properties of metals such as Cd^{II}, Hg^{II} and Pb^{II} in a more natural antiparallel helical system. A significant amount of literature exists where metal binding sites have been designed in existing four-helix bundles and in a mixture of α/β protein structural frameworks.[21,22] However, there are no such examples where novel metal binding site(s) have been engineered within an antiparallel single chain three-helix bundle by rational design. The three-helix bundle occurs ubiquitously in nature as a versatile and robust scaffold ranging from the helical IgG-binding domains,[23] to DNA-binding proteins, structural proteins, and enzymes..[24] Despite its widespread occurrence in nature only a few attempts have been made to prepare single chain three-helix bundles. [25,26]

Based on visual inspection of the α_3D structure, four potential sites along the bundle were identified where three cysteines, one from each helix, could be introduced. Out of these four mutants, α_3D**IV** (Figure 1), located at the C-terminal end of the bundle, seemed to be optimal based on the properties of the starting protein.[20,27-29] Previous NMR structural

and dynamic investigations showed a gradient in the dynamic behavior and malleability of the protein, with the C-terminal end of the bundle being most amenable to amino acid substitutions. The selected location has a well-ordered backbone conformation, but the side chains of the residues to be mutated are less well ordered than residues in other locations of the bundle. The 3-Cys site, which is largely sequestered from solvent, occupies a “box”; the sides are formed by the backbone of the helices and the bottom by the apolar side chains of Phe 31, Ile 14, and Ile 63. The aromatic residue of Phe 31 lies directly over the predicted metal-binding site and lines most of the bottom of the box. The top is formed by the main-chain and side chains of residues in the non-helical loops including Leu 21, as well as the apolar portion of Tyr 70 at the terminus of helix 3. His 72, which was entirely disordered in the NMR solution structure, also lies proximal to the site. Moreover, after introduction of the Cys side chains in one of the two preferred rotamers for Cys, the thiol S^G atoms formed a nearly equilateral triangle with the side chains well oriented to form the desired site (inter S^G distances = 3.5 − 4.5 Å). Overall, the location is ideal to explore the effects of hydrophobic sequestration in the present work. The sequence of α_3D**IV** is shown in Table 1. After expression of a synthetic gene of α_3D**IV** in *E. coli* followed by purification by HPLC (see Materials and Methods) the MW of α_3D**IV** was determined to be 7945.1 Da by Electrospray Ionization Mass Spectrometry (ESI), which corresponds to α_3D**IV** with the deletion of the N-terminal Met (calc MW = 7946.9 Da). The folding behavior of α_3D**IV** was studied in solution by CD and NMR spectroscopy. The CD spectrum of 5 μM α_3D**IV** has double minima at 208 and 222 nm at pH 8 with the molar ellipticity [θ] values characteristic of a well-folded α-helical construct (97% folded based on $[\theta]_{222}$) (Figure S1A). Furthermore, α_3D**IV** remained well-folded between pH 3 and 9. The ^{1}H-^{1}H NOESY spectrum of 3 mM α_3D**IV** shows chemical shift dispersions characteristic of a well-folded α-helical structure at pH 6 (Figure S1B). GuHCl-induced unfolding of α_3D**IV** was performed by monitoring the change in ellipticity at 222 nm as a function of GuHCl concentration at pH 8. The resulting titration curve was plotted as concentration of folded protein vs. GuHCl concentration (Figure S2) and was fit to a two-state equilibrium,[30-32] yielding the free energy of unfolding (ΔG_u) to be 2.5 kcal mole^{-1}, with the degree of cooperativity (m) being 1.4 kcal mole^{-1} M_{GuHCl}^{-1}. The midpoint of the transition (C_m) occurred at a 1.8 M GuHCl concentration. These results indicate that replacing three Leu residues of the WT α_3D with cysteines resulted in a loss of unfolding free energy of ~ 2.5 kcal mole^{-1}.[20]

Having established that α_3D**IV** is well folded and stable in solution, we investigated the complexation of Hg^{II}, Cd^{II}, and Pb^{II} to this peptide. Metal ion binding titrations were performed by adding aliquots of stock solutions of metals to peptide solutions at pH values where each metal fully coordinated to α_3D**IV** (pH 8.6 for Hg^{II}, and pH 8 for Cd^{II} and Pb^{II}). The progress of the titrations was monitored by the appearance of characteristic absorption bands due to Ligand to Metal Charge Transfer (LMCT) transitions at characteristic wavelengths,[15,33,34] upon formation of metal-thiolate bonds in the complexes $Hg^{II}(\alpha_3D\mathbf{IV})^-$, $Cd^{II}(\alpha_3D\mathbf{IV})^-$, and $Pb^{II}(\alpha_3D\mathbf{IV})^-$. The resulting UV/Vis absorption spectra (Figure S3) and the molar absorption coefficients (Δε), at various wavelengths (Table 2) are consistent with the assignment that all three Cys thiolates of α_3D**IV** are incorporated into the first coordination sphere of the metal ions.[15,33,34] Cd^{II} and Pb^{II} binding constants were determined to be 2.0×10^7 M^{-1} and 3.1×10^7 M^{-1}, respectively, from the analysis of the titration data.[35] Due to the high affinity binding, the association constant of Hg^{II} could not be determined. Next, we examined pH dependent complexation of these metals to α_3D**IV** by monitoring changes in the LMCT band as a function of pH. Titration data of Hg^{II} were fit to the release of one thiol proton upon forming a HgS_3 complex from linear $HgS_2(SH)$ complex of α_3D**IV**,[15] resulting in a pK_a of 7.1±0.1 (Table 2,Figure S4). For Cd^{II} and Pb^{II} the titration curves (Figure S4) were fit to simultaneous dissociation of two Cys thiols, [15,33,34,36] yielding pK_{a2} of 10.6±0.1 and 10.2±0.1 (Table 2), respectively. These pK_a values are slightly more acidic than TRI peptides.[15,33,36] Nonetheless, these pK_a values

are consistent with the coordination modes of Hg^{II} as trigonal HgS_3, Cd^{II} as pseudotetrahedral CdS_3O (O being an exogenous water molecule), and Pb^{II} as trigonal pyramidal PbS_3 complexes.

^{199}Hg NMR and ^{199m}Hg PAC (Perturbed Angular Correlation) spectroscopies were used to probe the coordination environment around Hg^{II} bound to α_3D**IV** at pH 5.8, 8.6, and 7.4. Based on the pK_a value, Hg^{II} is expected to form a linear HgS_2 complex at pH 5.8 and a trigonal HgS_3 complex at pH 8.6, with a mixture of linear and trigonal complexes at the intermediate pH. ^{199}Hg NMR chemical shifts (Figure 2) and ^{199m}Hg PAC spectral parameters (Figure S5, Table S1) of α_3D**IV** at pH 5.8, 8.6, and 7.4 confirm that α_3D**IV** forms a linear HgS_2 complex at pH 5.8 and a trigonal HgS_3 complex at pH 8.6, with the formation of both dithiolate- and trithiolate-Hg^{II} complexes at pH 7.4 in distorted geometries.[10,37-43] ^{113}Cd NMR spectrum of α_3D**IV** has two resonances at δ = 595 and 583 ppm at pH 8 (Figure S6) indicating the presence of two Cd^{II} species with chemical shift values similar to what has been observed for 4-coordinate pseudotetrahedral CdS_3O species.[36] ^{111m}Cd PAC spectroscopy was used to confirm the coordination environment and geometry of Cd^{II} complexes of α_3D**IV**. ^{111m}Cd PAC spectrum of α_3D**IV** has three Nuclear Quadrupole Interactions (NQIs), at ω_0 = 0.35, 0.27 and 0.17 rad/ns (Figure S7, Table S2) at pH 8.1. The first two peaks at 0.35 rad/ns and 0.27 rad/ns agree strikingly well with a typical CdS_3O signal in exo and endo conformations, respectively, as observed for TRI peptides.[36] The lowest frequency NQI at 0.17 rad/ns can be best assigned to a CdS_3N species where N is His 72. Even though the chemical shift of 595 ppm in the ^{113}Cd NMR spectrum (Figure S6) is lower than what has been reported for a CdS_3N species,[44] Quantum chemical calculations show that a change in Cd-S bond length of 0.01 Å can cause a change in chemical shift of ~20 ppm (Hemmingsen *et.al.* unpublished results). Thus, tentatively, the ^{113}Cd NMR resonance at δ = 595 ppm can be best assigned to the CdS_3N species.

In conclusion, we have been successful in engineering metal binding sites containing cysteine residues in an existing *de novo* antiparallel three-helix bundle, yielding α_3D**IV**, a protein which is well folded and stable in solution, and capable of binding heavy metals with high affinity ($>10^7$ M^{-1}). Spectroscopic properties of Hg^{II}, Cd^{II} and Pb^{II} complexes of α_3D**IV** are very similar to existing parallel three-stranded coiled coils, showing that we have achieved our objective of preparing a single polypeptide chain capable of binding metal ions with high affinity and predefined coordination geometry. Understanding the biochemistry of binding of heavy metals to a single polypeptide chain can be potentially useful for developing peptide-based water purification systems or specific heavy metal ion sensors. Clearly, having a single peptide chain rather than self-associating helical peptides makes these goals more achievable. Further studies will explore the possibilities of preparing similar constructs containing asymmetric metal binding sites such as those in Type I blue copper proteins, and to explore the effects of the electronic structure of the aromatic residue, Phe 31, or second-shell effects by replacing any of the surrounding residues as well as making catalytic metalloproteins.

Experimental Section

A synthetic DNA of α_3D**IV** was cloned into pET-15b (Celtek Genes) vector and expressed in *E. coli* BL21(DE3) competent cells (Stratagene) grown in M9 media. After sonication and heat denaturation at 55°C the lyophilized powder was purified on a C18 preparative reverse phase HPLC column over a linear gradient of 100% H_2O/0.1% TFA to 10% H_2O/90% Acetonitrile/0.1% TFA over 50 minutes. The MW of the pure peptide was determined to be 7945.1 Da by ESI which corresponds to α_3D**IV** without the first Met (calc MW 7946.9 Da). Yield of the pure protein was 17 mg/L. Concentration of the protein was determined by A_{280nm} using the known extinction coefficient ε_{280} = 8.61 mM^{-1}.[45]

CD spectra were collected on an Aviv model 202 CD spectrometer using rectangular open top quartz cuvettes at 25°C. GuHCl titration experiments were carried out using a Microlab 500 series syringe pump automatic titrator controlled by Aviv software. Titrations were carried out by mixing two separate solutions of 10 μM peptide containing 0.0 and 7.63 M GuHCl in 10 mM phosphate buffer at pH 8. Observed ellipticities in millidegree were converted to molar ellipticities (deg cm^2 $dmole^{-1}$ res^{-1}) as described previously,[33] using 59 amino acids in the helical region of the protein. GuHCl titration data were fit to an equation derived based on a two-state model. [30-32] A ^1H-^1H NOESY experiment was performed using standard procedures.[46]

Cd^{II}, Hg^{II}, and Pb^{II} binding titrations were performed at room temperature on a Cary 100 Bio UV/Vis spectrometer using anaerobic cuvettes (Starna Inc.) of 1-cm path length by adding aliquots of stock solutions of different metals. Peptide samples of 20 – 30 μM were prepared in 50 mM of appropriate buffers (TRIS for pH 8 and CHES for pH 8.6) and 40 – 60 μM TCEP inside an inert atmosphere box (Vacuum Atmospheres Co., model OMNI-LAB). Stock solutions of 8 mM $CdCl_2$, 7.37 mM $HgCl_2$, and 5.16 mM $PbCl_2$ were also prepared inside the inert atmosphere box. In each case, difference spectra were obtained by subtracting the background spectra of samples containing peptide, buffer, and TCEP. Direct titration data were analyzed by non-linear least squares fits to an equation used previously. [47] The difference molar extinction coefficients (Δε) were determined based on the total metal concentrations after subtracting the background spectra.

pH titrations were performed as described previously,[34,36] by adding small aliquots of KOH to solutions containing 20 – 30 μM of α_3D**IV** and 1 equivalent of $CdCl_2$, $HgCl_{2'}$ or $PbCl_2$. In the cases of Cd^{II} and Pb^{II}, the titration data were analyzed using the model: simultaneous two proton dissociation as described previously.[34,36] For Hg^{II}, the data were analyzed using the model: dissociation of one thiol proton of Cys.[15]

^{113}Cd NMR and ^{199}Hg NMR experiments were performed according to standard procedures.[48] An exponential line broadening of 200 Hz was applied prior to Fourier transformation while processing ^{199}Hg NMR data.

Samples for ^{111m}Cd PAC measurement contained 300 μM α_3D**IV**, 1/12 equivalent of Cd^{II}, and 55% sucrose (w/w) in 20 mM TRIS buffer at pH 8.1. Sample preparation and data collection were performed at the University of Copenhagen.[36,48] Samples for ^{199m}Hg PAC experiments contained 200 μM α_3D**IV**, 80 μM Hg^{II}, and 55% sucrose in 100 mM of an appropriate buffer (phosphate for pH 5.8, 7.4 and CHES for pH 8.6). Sample preparation and data collection were performed at CERN.[41]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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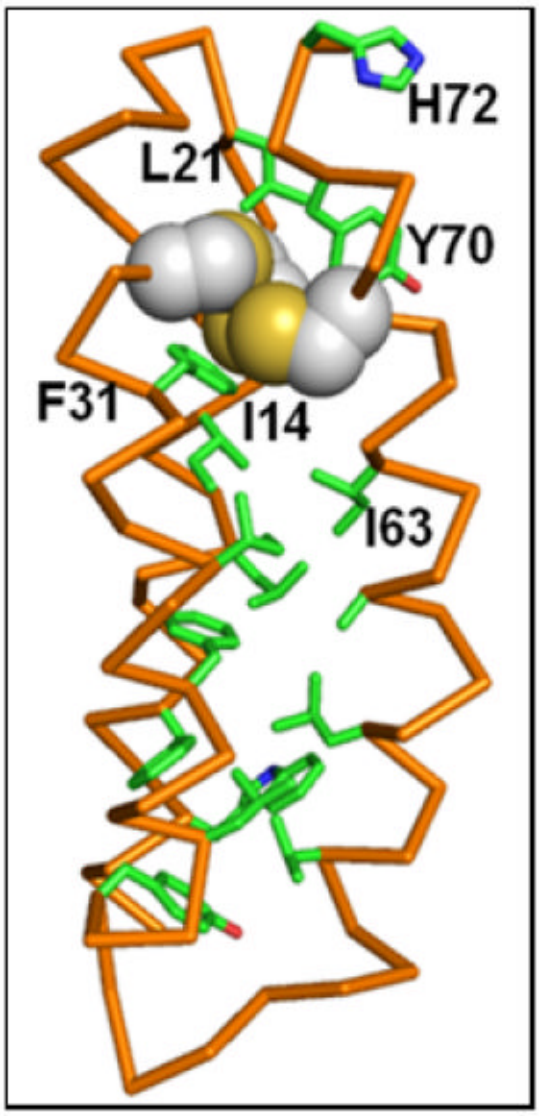

Figure 1.

PyMol model of α_3D**IV** generated from the NMR structure of α_3D. Cys residues, located at the C-terminal end of the bundle are shown as spheres. The protein backbone is shown in orange. The Cys site can be considered to be located in a hydrophobic "box" formed by the hydrophobic residues F31, I14, I63, L21, and Y70, shown as sticks.

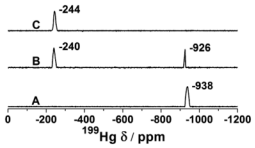

Figure 2.

^{199}Hg NMR spectra of solutions containing 2.93 mM α_3D**IV** and 0.8 equivalent of $^{199}Hg(NO_3)_2$ at pH 5.8 (A), 7.4 (B), and 8.6 (C).

Table 1

Sequence of α_3DIV.[a]

Peptide	Sequence
α_3D**IV**	MGS WAEF K QR LAAIKTR **C**QAL GG SEAE**C**AAF E KE IAAFESE LQAY KGKGNPE
	V E AL R KE AAAIRDE **C**QAY RHN

[a]Residues in red represent mutations to the WT α3D

Table 2

Physical Parameters of Cd^{II}, Hg^{II} and Pb^{II} complexes of α_3DIV.

Complex	UV/Vis λ_{nm} ($\Delta\varepsilon$ $M^{-1}cm^{-1}$)	NMR δ (ppm) ^{113}Cd	NMR δ (ppm) ^{199}Hg	Apparent pK_a	Binding Constant (K_b) (M^{-1})[a]
Cd(α_3D**IV**)$^-$	232 (18, 200)	583		10.6±0.1[b]	2.0×10^7
		595			
Hg(α_3D**IV**)$^-$	247 (12,500)		−244	7.1±0.1[c]	
	265 (8,400)				
	295 (3,900)				
Hg(α_3D**IV**)[d]	240 (850)		−938[e]		
Pb(α_3D**IV**)$^-$	236 (18,000)			10.2±0.1[b]	3.1×10^7
	260 (14,400)				
	278 (9,100)				
	346 (3,150)				

[a] Model used to obtain binding constants is $M^{II} + (\alpha_3D\mathbf{IV})^{3-} \rightleftharpoons M^{II}(\alpha_3D\mathbf{IV})^-$ (K_b). These values represent the lower limit of K_b.

[b] Model used to obtain pK_{a2} values for Cd^{II} and Pb^{II} is $M^{II}(\alpha_3D\mathbf{IV}S(SH)_2)^+ \rightleftharpoons M^{II}(\alpha_3D\mathbf{IV})^- + 2H^+$ (K_{a2}).

[c] Model used to obtain the pK_a for Hg^{II} is $Hg^{II}(\alpha_3D\mathbf{IV}S_2(SH)) \rightleftharpoons Hg^{II}(\alpha_3D\mathbf{IV})^- + H^+$ (K_a).

[d] Linear HgS_2 complex of α_3D**IV**.

[e] ^{199}Hg NMR δ of linear HgS_2 complex at pH 5.8.