Apoprotein Structure and Metal Binding Characterization of a *de Novo* Designed Peptide, α_3DIV, that Sequesters Toxic Heavy Metals

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Ⓢ *Supporting Information*

ABSTRACT: *De novo* protein design is a biologically relevant approach that provides a novel process in elucidating protein folding and modeling the metal centers of metalloproteins in a completely unrelated or simplified fold. An integral step in *de novo* protein design is the establishment of a well-folded scaffold with one conformation, which is a fundamental characteristic of many native proteins. Here, we report the NMR solution structure of apo α_3DIV at pH 7.0, a *de novo* designed three-helix bundle peptide containing a triscysteine motif (Cys18, Cys28, and Cys67) that binds toxic heavy metals. The structure comprises 1067 NOE restraints derived from multinuclear multidimensional NOESY, as well as 138 dihedral angles (ψ, φ, and χ_1). The backbone and heavy atoms of the 20 lowest energy structures have a root mean square deviation from the mean structure of 0.79 (0.16) Å and 1.31 (0.15) Å, respectively. When compared to the parent structure α_3D, the substitution of Leu residues to Cys enhanced the α-helical content of α_3DIV while maintaining the same overall topology and fold. In addition, solution studies on the metalated species illustrated metal-induced stability. An increase in the melting temperatures was observed for Hg(II), Pb(II), or Cd(II) bound α_3DIV by 18−24 °C compared to its apo counterpart. Further, the extended X-ray absorption fine structure analysis on Hg(II)-α_3DIV produced an average Hg(II)−S bond length at 2.36 Å, indicating a trigonal T-shaped coordination environment. Overall, the structure of apo α_3DIV reveals an asymmetric distorted triscysteine metal binding site, which offers a model for native metalloregulatory proteins with thiol-rich ligands that function in regulating toxic heavy metals, such as ArsR, CadC, MerR, and PbrR.

T-shaped HgS₃ complex
EXAFS Hg(II)-S(Cys): 2.36 Å
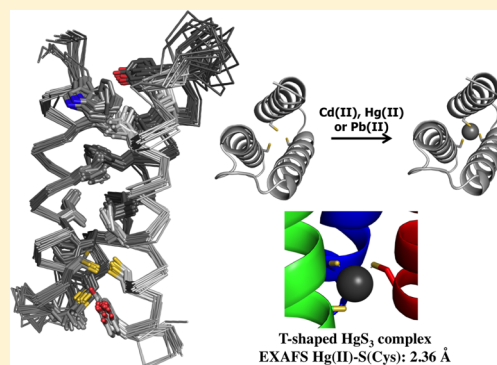
Metalloproteins acquire metal ions to perform the most essential reactions in nature including respiration, photosynthesis, and nitrogen fixation.[1] Metalloregulatory proteins are a subset of metalloproteins used by microorganisms to control the levels of essential transition metal ions (Fe, Cu, Zn, and Mn) or decrease levels of toxic metals (Hg, As, Pb, and Cd) within their cells.[1,2] Toxic heavy metals threaten biological pathways that use proteins with thiol-rich metal binding sites by displacing existing metal ions in proteins that function in catalytic pathways or in the storage or transport of essential metals.[3,4] Understanding the structure−function relationship found in the overall fold and at the metal centers of these metalloproteins is critical to gaining insight into their chemical properties and roles in biological systems. This knowledge can be applied to developing ecological strategies aimed at managing the serious threat that toxic heavy metals in our environment pose to all forms of life.

One biologically relevant approach used to study the concept of the structure−function relationship in native metalloproteins is *de novo* protein design.[5−7] Using what is known about the biophysical and biochemical properties of amino acids, this emerging approach involves designing and synthesizing a polypeptide scaffold from scratch. It allows one to tailor a sequence that is designed to form the proper hydrophobic, electrostatic, and hydrogen bonding interactions that will manifest into a unique and well-defined fold, which is a defining characteristic of native proteins.[5] Therefore, *de novo* protein design provides a novel approach in studying the mechanisms behind protein folding and modeling the active sites of metalloproteins in a simplified or unrelated fold. Many groups have actively used this approach by incorporating the core components of the desired metal center in order to achieve a specific metal−ligand coordination or function, such as catalysis or redox activity.[6,7]

DeGrado and co-workers made a significant impact in the field of *de novo* protein design through the design, preparation, and characterization of α_3D, a 73-residue peptide (Table 1) with a single conformation in solution and a unique native-like fold.[8] A common *de novo* designed peptide uses a heptad repeat sequence that self-assembles into a parallel three-stranded

Table 1. Amino Acid Sequence of *de novo* Designed Peptides[a]

Peptide	Sequence				Reference
	`abcdefg`	`abcdefg`	`abcdefg`	`abcdefg`	
CoilSer	`Ac-E` `WEALEKK`	`LAALESK`	`LQALEKK`	`LEALEHG` `–NH₂`	10
	`abcdefg`	`abcdefg`	`abcdef`	`loop`	
α₃D	`MGSWAEFKQR` `EAELAAFEKE` `NPEVEALRKE`	`LAAIKTR` `IAAFESE` `AAAIRDE`	`LQAL` `LQAY` `LQAYRHN`	`GGS` `KGKG`	8
					23
α₃DIV	`MGSWAEFKQR` `EAE`**C**`AAFEKE` `NPEVEALRKE`	`LAAIKTR` `IAAFESE` `AAAIRDE`	**C**`QAL` `LQAY` **C**`QAYRHN`	`GGS` `KGKG`	
α₃D**H₃**	`MGSWAEFKQR` `EAE`**H**`AAFEKE` `NPEVEALRKE`	`LAAIKTR` `IAAFESE` `AAAIRDE`	**H**`QAL` `LQAY` **H**`QAYR`**VNGSGA**	`GGS` `KGKG`	56

[a]The sequences are prepared in heptads. Residues that are underlined and bolded were changed from the previous design.

coiled coil (3SCC) tertiary structure, such as the TRI[9] and coil-Ser (CS)[10] peptide family. In contrast to the 3SCC analogues, α₃D is a single polypeptide chain that folds into a three-helix bundle, a fold that is ubiquitous in the molecular recognition domain of immunoglobulin G, DNA binding proteins, and various enzymes.[11] Using the sequence of CS as a foundation, DeGrado and co-workers isolated α₃D through an iterative process of modifying helix-capping interactions to dictate the topology of the bundle (clockwise vs counterclockwise), electrostatic (or salt-bridging) interactions to force the desired helix–helix pairing, and hydrophobic interactions to achieve a well-packed core.[8,12] The result of this process led to a counterclockwise three-helix bundle fold that contains every natural amino acid residue except cysteine. It possesses the physical properties similar to native proteins[13−16] and has a solved NMR structure (PDB 2A3D),[8] offering a novel opportunity to add function to this *de novo* designed framework.

We redesigned the sequence of α₃D by introducing a triscysteine motif that is found in the metal binding site of metalloregulatory proteins MerR,[17−19] ArsR/SmtB,[20] and CadC/CmtR.[20−22] Three Leu to Cys mutations (Leu18Cys, Leu28Cys, and Leu67Cys) at the C-terminal end of α₃D were performed to produce α₃DIV.[23] Utilizing several spectroscopic methods, we demonstrated that apo α₃DIV is stable and well folded in solution at a pH range between 5 and 9. The coordination mode of Hg(II), Pb(II), and Cd(II) bound α₃DIV is pH dependent. From a linear mercury complex, [Hg(II)-S₂(SH)], a thiol group is deprotonated to form a trigonal [Hg(II)S₃]⁻, and the thiol group has a pK_a of 7.1 (0.1). Therefore, a pH condition below and above this pK_a value leads to the linear or a trigonal complex, respectively. The formation of a trigonal pyramidal [Pb(II)S₃]⁻ and pseudotetrahedral [Cd(II)S₃(N/O)]⁻ complex was determined to require a simultaneous deprotonation of two Cys thiol groups, yielding pK_{a2} values of 10.6 (0.1) and 10.2 (0.1), respectively. A pH condition greater than 6 results in the formation of a [Pb(II)S₃]⁻ and [Cd(II)S₃(N/O)]⁻ complex. The [Pb(II)S₃]⁻ complex was determined to have a lower limit binding constant of 2.0×10^7 M⁻¹, and the [Cd(II)S₃(N/O)]⁻ complex has the corresponding value of 3.1×10^7 M⁻¹. In this work, we present the solution structure of apo α₃DIV, solved at pH 7.0. This is the first reported structure that incorporates a triscysteine metal binding site in an antiparallel three-helix bundle fold of α₃D

through the modifications of stabilizing core hydrophobic residues to introduce a new function. The solution structure of α₃DIV possesses the same overall topology and counterclockwise bundle as α₃D, and the incorporation of Cys residues increased the helical content of the scaffold. Overall, the α₃DIV structure provides evidence that the framework of α₃D is amenable to mutations that involve removing Leu residues that were thought to be essential in inducing hydrophobic interactions. This structure offers insight into how the protein is preorganized before metal binding, which is essential for utilizing this framework in designing future functional metallopeptides.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. A pET15b recombinant DNA plasmid (Celtek Genes) that contains the gene for α₃DIV was transformed and expressed in *Escherichia coli* BL21(DE3) (Life Technologies). To overexpress ¹⁵N-labeled and ¹⁵N,¹³C- labeled α₃DIV, *E. coli* cells were grown in M9 minimum media that contained 1 g/L ¹⁵NHCl₄ (Cambridge Isotope Laboratories) or 1 g/L ¹⁵NHCl₄ and 2 g/L [U−¹³C]glucose (Cambridge Isotope Laboratories), respectively. The cells were grown at 37 °C to an OD₆₀₀ of 0.6, induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (Life Technologies), and incubated at 18 °C overnight. The cells were lysed by sonication, and the soluble protein was obtained after heat denaturation (50 °C) and lyophilization. The protein powder was redissolved in 10% acetic acid and purified on a reversed-phase C18 HPLC, using a flow rate of 20 mL/min and a linear gradient of 0.1% TFA in water to 0.1% TFA in 9:1 CH₃CN/H₂O over 45 min. Using an electronspray mode on a Micromass LCT Time-of-Flight mass ionization spectrometer, the peptide mass was determined to be 7946.9 Da, which accounts for 72 of the 73 amino acids as Met1 is cleaved post-translation.

NMR Sample Preparation. Stock peptide concentrations were determined from the absorption band of the aromatic residues (Trp4, Tyr45, and Ty70) at 280 nm. Experiments that involved coherence transfer from the backbone amide protons required a 9:1 H₂O-to-²H₂O solution, which contained 1.0 mM ¹⁵N, ¹³C labeled peptide, 100 mM sodium chloride, 0.8 mM tris(2-carboxyethyl) phosphine (Fisher), 0.05 mM phenylmethylsulfonyl fluoride (Fisher), and 0.5% sodium azide (Sigma-Aldrich). Samples for carbon-filtered aromatic to

aliphatic NMR experiments were prepared in a 100% ^{2}H$_2$O solution and incubated overnight, containing the reagents listed above. The pH of the apoprotein samples used in the structure was adjusted at 7. The Pb(II)-^{15}N-α_3**D**IV sample was prepared by adding 1.0 equiv of PbCl$_2$ to a 1 mM apo ^{15}N-α_3**D**IV solution, containing 20 mM BIS-TRIS buffer set at pH 7.0 in a 9:1 H$_2$O-to-^{2}H$_2$O solution. The Hg(II)-^{15}N-α_3**D**IV sample was prepared under similar conditions but contained no buffer, and the pH was adjusted to 8.6 after the addition of 1.0 equiv of HgCl$_2$. Control apo samples were also prepared to match the sample conditions of their metalated counterparts.

NMR Data Collection and Processing. NMR experiments for the structure determination were performed at 25 °C on a Varian (Agilent) INOVA 800 MHz NMR spectrometer, equipped with a triple resonance cold probe. The NMR acquisition parameters for the 800 MHz spectrometer are reported in Supporting Information, Table 1. ^{15}N-HSQC spectra were collected for the metalated α_3**D**IV species and their corresponding apo controls. These experiments were performed on a Bruker Avance 500 MHz with room temperature triple resonance probes and a Varian VNMRS 500 MHz equipped with a switchable probe. The ^{15}N-HSQC experiments for the Pb(II)-^{15}N-α_3**D**IV and its apo control were collected at 25 °C, while the Hg(II)-^{15}N-α_3**D**IV and its apo control were obtained at 9 °C. The ^{15}N-HSQC experiments were also performed at 25 °C for apo ^{15}N-α_3**D**IV with pH conditions of 5.8 and 8.6. The spectra were processed using NMRPIPE[24] and then visualized and analyzed with SPARKY.[25]

Assignments. Backbone assignments were determined from a series of complementary three-dimensional (3D) triple resonance[26] transverse relaxation optimized spectroscopy (TROSY)[27−29] experiments: HNCO/HN(CA)CO,[30] HN-(CO)CA/HNCA,[31] HN(CO)CACB/HNCACB,[32,33] and HN-(CA)HA.[34] Since the sequence of α_3**D**IV is known, the backbone assignments were manually completed and then successively confirmed with an autoassignment program SAGA.[35] Ser24, Thr16, and Ser40 were used as landmarks in the manual assignment because the β-carbons of Ser and Thr residues are downfield of their α-carbons. Next, aliphatic assignments were obtained from the combined inphase/antiphase spectra of 3D HC(C)H-correlation spectroscopy (COSY)[36] and (H)CCH-total correlation spectroscopy (TOCSY)[37,38] experiments. These assignments were further confirmed with 3D (^{1}H, ^{15}N, ^{1}H) nuclear Overhauser effect spectroscopy (NOESY)-TROSY[39] and (^{13}C, ^{15}N, ^{1}H) hetero-nuclear multiple-quantum coherence (HMQC)-TROSY[40] experiments, providing a through-space validation of the COSY and TOCSY assignments. Chemical shift assignments for residues 3−73 were then compiled and used to assign sequential-intra and inter-residue NOE upper distance limits (upl).

NOESY Experiments. In order to attain structural distance restraints, several 3D-^{13}C-edited and -resolved NOESY experiments were completed. Aliphatic−aliphatic NOEs were acquired from (^{13}C-edited) HC(C)H-NOESY (inphase/antiphase), which were collected at 60 and 200 ms mixing times. Aromatic to aliphatic NOEs were obtained from a 3D NOESY ^{13}CHSQC experiment, while aromatic to amide NOEs were collected from 3D NOESY-^{13}C(resolved)-TROSY and the 3D NOESY (^{13}C resolved)-TROSY. Further, non-intraresidue amide proton NOEs were acquired from 3D (^{1}H, ^{15}N, ^{1}H) NOESY-TROSY and (^{13}C, ^{15}N, ^{1}H) HMQC-NOESY-TROSY experiments. The upper-limit NOE restraints, which contain intraresidue, intrahelical, and interhelical NOEs, were then determined from the manual chemical shift assignments made on the COSY and TOCSY spectra.

Dihedral Angles and Hydrogen Bonds Restraints. TALOS-N[41] was used to generate φ, ψ, and $\chi1$ dihedral angle restraints from ^{1}H, ^{1}H$_\alpha$, ^{14}N, ^{13}C, ^{13}C$_\alpha$, and ^{13}C$_\beta$ chemical shift assignments. Dihedral φ and ψ angles that were classified as "strong" (residues 2−20, 25−45, and 45, 48, 51−70) were included in all the structure calculations, and $\chi1$ angles for selected aliphatic residues were then included in the final rounds of calculations. TALOS-N also predicted the secondary structure and order parameters for each amino acid residue, which provided the structured regions: residues 5−20, 25−44, and 51−70. Furthermore, backbone hydrogen bonds in most of the structured regions were later incorporated in the upl restraints, excluding residues in the loop-turn regions. Upper limit distance restraints of 2.0 and 3.0 Å were given to O$_i$ to H$_{i+4}$ and O$_i$ to N$_{i+4}$, respectively.

Structure Calculation. CYANA 2.1[42] was used in the structure calculations and the input files comprised of the upper limit restraints obtained from 3D ^{15}N- and ^{13}C-edited/resolved-NOESY spectra, dihedral angle restraints (φ, ψ, and $\chi1$), and backbone hydrogen bond restraints in the structured regions of the sequence. In the initial rounds of structure calculations, φ and ψ dihedral angle restraints were used along with the NOE upper limit distances, which were set at 3 or 5 Å. These values were then adjusted in the later rounds of calculations. That is, intraresidue upper limit distances were modified to have a 3−7 Å range, increasing the restraints between pseudoatom−pseudoatom contacts. For sequential−intrahelical distances, a 3−4.5 Å range was applied following Wuethrich's ^{1}H−^{1}H short-to-medium-range distances for an α-helical secondary structure.[43] Long sequential−intrahelical distances, ^{1}H$_i$ to ^{1}H$_{i+4}$, were given a 5−7 Å range. After, interhelical upl distances were adjusted to a 4−7 Å range, where the lower ends distances were based off the intensity in the NOESY spectra, and higher ends were set to again compensate for pseudoatom−pseudoatom correlations. In the final rounds of calculations, $\chi1$ dihedral angle restraints were added and were followed by the incorporation of backbone hydrogen bonds. No lower limit distances were used. Of the 100 calculated structures, only the 20 lowest structures are reported here.

Circular Dichroism Experiments. Circular dichorism (CD) spectra were collected on an AVIV CD spectrometer at 25 °C, and each sample was scanned between 260 and 195 nm. CD samples contained 10 μM peptide and 10 mM potassium phosphate buffer. The metalated samples were prepared by adding 1.0 equiv of HgCl$_2$, CdCl$_2$, or PbCl$_2$ to an apo solution. The pH was adjusted at 8.2 for the apo, Cd(II)-, Pb(II)-, and Hg(II)-α_3**D**IV. The pH conditions were chosen so that the metalated samples formed the appropriate complex: [Hg(II)S$_3$]$^-$, [Pb(II)S$_3$]$^-$, and [Cd(II)S$_3$(N/O)]$^-$. All the samples were purged with Ar$_{(g)}$ and prepared in triplicates. The mean residue ellipticities (MRE) were determined using equation:

$$[\theta] = \theta_{\text{obs}}/10lcn$$

where θ_{obs} is the measured ellipticity in millidegrees, l is the cell path length in centimeters, c is the concentration in M, and n is the number of residues in the structured regions. Fifty-five and 59 residues were used in the MRE calculation for α_3**D**IV (apo and metalated species) and α_3**D**, respectively.

Thermal Denaturation Experiments. Thermal denaturation studies were performed on a nano differential scanning calorimetry (N-DSC TA Instruments model 602001). The samples contained 0.13 mM peptide (1.0 mg/mL), 30 mM MOPS or HEPES buffer solution, and 100 mM NaCl. The pH for the samples containing MOPS was adjusted at 7.0, while the HEPES solution had a pH of 8.2. The metalated samples were prepared by adding 1.0 equiv of $HgCl_2$, $CdCl_2$, or $PbCl_2$ to an apoprotein solution. All the samples were prepared in an anaerobic environment and were degassed prior to injection. In each experiment, 300 μL of a peptide-buffer solution and its corresponding buffer solution were injected in the sample cell and reference cell, accordingly. Experiments were performed in duplicates or triplicates. The experimental methods involved a heating cycle that originated at 25 °C and ended at 110 °C, using a 1 or 2 °C/min scan rate, and spectra were collected in an anaerobic Coy Box environment. Thermograms were blanked with the appropriate control (buffer or metal buffer) scans, baseline corrected, and normalized with protein concentration. NanoAnalyze Data Analysis (version 2.4.1 by TA Instruments) was used to fit the thermograms to determine the melting temperatures and thermodynamic parameters (Supporting Information, Figure 1).

X-ray Absorption Spectroscopy (XAS). Samples for XAS were prepared with final concentrations of 2.5 mM $HgCl_2$, 2.7 mM α_3DIV, 30 mM TRIS buffer, and 40% glycerol at pH 8.7. Samples were then loaded into lucite XAS sample cells wrapped in Kapton tape, flash frozen in liquid N_2, and stored at 77 K until data collection. XAS data were collected at the National Synchrotron Light Source (NSLS) on beamline X3-A. This beamline utilized a Si[220] single crystal monochromator equipped with a harmonic rejection mirror. During data collection, samples were maintained at 24° K using a He Displex Cryostat. Protein fluorescence excitation spectra were collected using a 13-element solid-state Ge detector with a Ga fluorescence filter (0.3 μM in width) placed between the cryostat and detector to remove lower energy photons. XAS spectra were measured in 5 eV increments in the pre-edge regions (12200−12270 eV), 0.25 eV increments in the edge regions (12270−12350 eV), and 0.05 Å^{-1} increments in the extended X-ray absorption fine structure (EXAFS) region (to k = 14 Å^{-1}), integrating from 1s to 25 s in a k^3 weighted manner for a total scan length of approximately 50 min. X-ray energies were individually calibrated by collecting Au foil absorption spectra simultaneously with protein data. The first inflection point of the Au foil spectrum was assigned to 11 919 eV. Each fluorescence channel of each scan was examined for spectral anomalies, and data represent the average of 20 scans.

XAS data were processed using the Macintosh OS X version of the EXAFSPAK program suite[44] integrated with the Feff v8 software[45] for theoretical model generation. Data reduction utilized a Gaussian function in the pre-edge region and a three-region cubic spline throughout the EXAFS region. Data were converted to k-space using a mercury E_0 value of 12 284 eV. The k cubed weighted EXAFS was truncated at 1.0 and 12 Å^{-1} for filtering purposes. This k range corresponds to a spectral resolution of ca. 0.14 Å for all mercury−ligand interactions; therefore, only independent scattering environments >0.14 Å were considered resolvable in the EXAFS fitting analysis.[46] EXAFS simulation analysis was performed on filtered and then on raw/unfiltered data; results listed in Supporting Information, Table 2 were from simulating unfiltered data. EXAFS data were fit using single scattering amplitude and phase functions

calculated with the program Feff v8. Single scattering theoretical models were calculated for Hg-oxygen and Hg-sulfur coordination to simulate mercury nearest-neighbor ligand environments. Scale factors (Sc) and E_0 values used during the simulations were calibrated by fitting crystallographically characterized Hg models; specific values include a scale factor of 0.95, and E_0 values for O and S of 0 and 1.5 eV, respectively. During the simulation, only the bond lengths and Debye−Waller factors were allowed to freely vary, adjusting coordination number values during the simulations in a nonvaried incremental fashion. Criteria for judging the best-fit simulation utilized both the lowest mean square deviation between data and fit (F′), corrected for the number of degrees of freedom and a reasonable Debye−Waller factor.[46,47]

■ RESULT

Structure of Apo α_3DIV. *Overall Structure.* Apo α_3DIV has an α-helical fold as indicated by the pattern in the chemical shift dispersion in the ^{15}N-TROSY spectrum (Figure 1A) and sequential NOE correlations (Figure 1B). The ^{15}N-TROSY spectrum of apo α_3DIV at pH 7.0 exhibit core residues with well-dispersed chemical shifts and a pattern that is typical for a well-folded α-helical protein.[48,49] Chemicals shift assignments for 69 of the 72 residues, determined from triple resonance NMR experiments, are indicated in Figure 1A, and the total number of peaks validate the amino acid sequence of α_3DIV. Resonances for residues Met1, Gly2, and Ser3 are not observed in the spectrum. Met1 is cleaved post-translation, Gly2 and Ser3 are in the dynamic region of the structure. Even after three Leu residues in α_3D were substituted for Cys, the ^{15}N-TROSY spectrum contains single peaks for every residue, indicating that α_3DIV exhibits a single conformation in solution or an ensemble of conformations interconverting at a sub-microsecond time scale.

An ensemble of the 20 lowest energy structures is visualized in Figure 2A using PYMOL.[50] The structure of α_3DIV encompasses 1067 NOE restraints, which include 395 intra-residue, 367 short-to-medium, and 305 long-range NOEs (Table 2). There are ∼15 NOE restraints per residue for 70 residues. In addition, 138 dihedral angles restraints were utilized in the structure, which were derived from the chemical shifts of ^{1}H, ^{1}H$_\alpha$, ^{14}N, ^{13}C$_\alpha$, and ^{13}C$_\beta$ atoms for residues 4−73 using TALOS-N. These restraints included 60 φ and 61 ψ angle restraints, as well as 17 χ_1 angles. The majority of the χ_1 restraints (10 restraints) were designated to core aliphatic and aromatic residues. The rotamers largely had a trans or gauche(−) conformation, characteristic rotamers of nonpolar-aliphatic residues. Finally, 39 hydrogen bond restraints (total of 78) were added in the structured regions. The ensemble has an averaged CYANA energy function of 1.9 (0.4) kcal mol^{-1}. Two distance restraints were greater than the 0.35 Å cut off; however, no dihedral angles >5° or van der Waals contacts >0.35 Å were violated. The Ramachandran statistics for the 20 structures show that 90.1% of the backbone stereochemistry is in the favored regions (Supporting Information, Figure 2) and agrees well with the Ramachandran analysis by Protein Structure Validation Suite[51] (PSVS) (Supporting Information, Table 3). There are 55 residues in the structured regions (residues 5−20, 25−44, and 51−70) of α_3DIV. The backbone (N, C$^\alpha$, C) root mean square deviation (RMSD) of the 20 structures for residues 3−73 and the structured regions is 0.79 (0.16) and 0.49 (0.12) Å, respectively. Likewise, these RMSD value corroborates with the structure quality analysis by PSVS
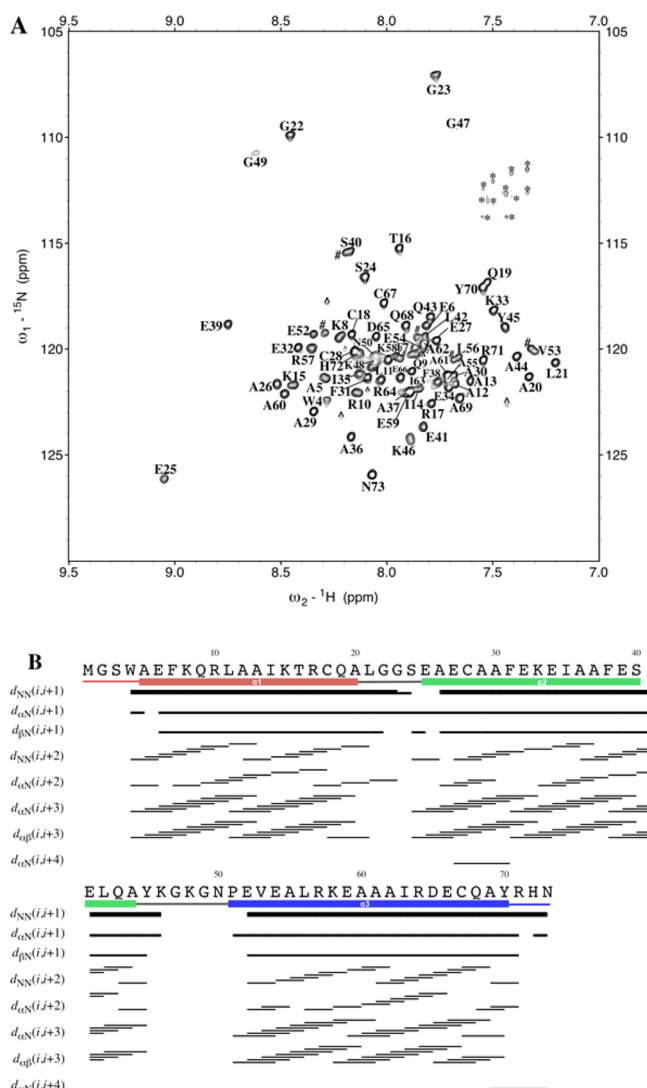
Figure 1. (A) ^{15}N-TROSY spectrum of ^{15}N-labeled α_3DIV. The assignments are adjacent to their corresponding peaks. Of the 73 residues, 69 were assigned, with residues 1−3 and Pro51 not observed in the spectrum. The residual Gln and Asn side-chain peaks were not assigned and are marked with an asterisk. Further, aliased and noise peaks are respectively given a pound and circumflex symbol. (B) Summary of sequential NOEs for apo α_3DIV, which were determined from 3D ^{15}N- NOESY-TROSY and ^{13}C-NOESY-HSQC spectra.

(see Supporting Information, Table 4 for the global quality scores).

The lowest energy structure of α_3DIV is illustrated in Figure 2B and has a CYANA energy function of 1.2 kcal mol^{-1}. The three-helix bundle adopts a counterclockwise orientation, which is in complete agreement with α_3D. The interhelical-tilt angles, which were calculated using QHELIX,[52] further confirmed this counterclockwise topology: $\Omega_{1,2} = -149°$, $\Omega_{1,3} = 21°$, and $\Omega_{2,3} = -156$. These angles decreased by 16°, 7°, and 15° from the $\Omega_{1,2}$, $\Omega_{1,3}$, and $\Omega_{2,3}$ values reported for α_3D, respectively. However, when the same QHELIX analysis was performed on α_3D and compared again to α_3DIV, a much more modest change was observed. The tilt angles of α_3D reduced by 11° ($\Omega_{1,2}$), 8° ($\Omega_{1,3}$), and 8° ($\Omega_{2,3}$).

The triscysteine metal binding site has S$_\gamma$−S$_\gamma$ distances of 6.7, 5.5, and 3.6 Å between Cys18−Cys28, Cys28−Cys68, and Cys18−Cys67, respectively (Figure 2C). It should be noted

that these distances were not restrained in the calculations. This hydrophobic plane has an area of 9.82 Å^2 and can accommodate large heavy metals like Cd(II), Hg(II), and Pb(II). In addition, the χ_1 dihedral angles (N−C$_\alpha$−C$_\beta$−S$_\gamma$) for the Cys residues are 168°, −52.7°, and −68.1° for Cys18, Cys28, and Cys67, respectively. Both Cys28 and Cys67 have χ_1 angles that are close to the most common rotamer for Cys (−65°).[53]

Analysis of Metalated α_3DIV. *^{15}N-HSQC Spectra of Metalated α_3DIV.* Our subsequent objective was to determine the chemical shift perturbation in the ^{15}N-HSQC spectrum of α_3DIV after the addition of Hg(II) and Pb(II). These results provide a qualitative assessment of the change in the peptide fold in the presence of a metal−ligand complex, as well as the opportunity of collecting further 3D NMR experiments in order to solve a metalated structure of α_3DIV. However, the chemical shift peak dispersions in the ^{15}N-HSQC spectrum of Pb(II)- and Hg(II)-α_3DIV were observed to be significantly perturbed.

In Figure 3, a superimposition of the ^{15}N-HSQC spectrum of an apo control and Pb(II)-α_3DIV shows a significant decrease in number of cross peaks for the Pb(II) spectrum. The pH of the apo and metalated species was set at 7.0 in order to achieve a trigonal pyramidal lead complex.[23] The apo spectrum displays 68 of the 69 expected peaks (Trp4 not observed), again demonstrating a well-defined conformation of the apo-peptide. Upon the addition of 1.0 equiv of Pb(II), only about 57 peaks could be observed in the Pb(II)-α_3DIV spectrum, and they were compared to corresponding peaks in the assigned apo spectrum. Of these identified peaks, several core residues, including Cys18, Phe38, Val53, and Ile63 overlay well with or slightly deviate by about ±0.2 ppm in ^{1}H from their corresponding apo peaks. The peaks for other residues, such as Leu21, Phe31, Leu56, and Cys67, are either broadened beyond detection or have significantly shifted, resulting to an incomplete assignment of the chemical shifts for Pb(II)-α_3DIV. Dramatic perturbation was also observed for the ^{15}N-HSQC spectrum of Hg-α_3DIV at pH 8.6 (Supporting Information, Figure 3), which forms a trigonal complex at this condition. The ^{15}N-HSQC spectrum of Hg-α_3DIV contained only 47 observable peaks, while the spectrum of its apo counterpart only has 55 of the 68 expected resonance peaks at the same pH. The resonances in the Hg-α_3DIV spectrum were not assigned.

CD Analysis of Metalated α_3DIV. The CD spectra of Hg(II)-, Pb(II)-, and Cd(II)-α_3DIV at pH 8.2 are compared to apo α_3DIV, as well as to α_3D in Supporting Information, Figure 4A. Like its apo counterpart and α_3D (both at pH 8.2), the metalated spectra display double minima at 208 and 222 nm, displaying a CD profile of a well-folded α-helical system. This is further supported by the large negative molar ellipticity values (Table 3). Apo α_3DIV has the largest $-[\theta]_{222\ nm}$ value of 29 231 (672) deg cm^2 dmol^{-1} res^{-1}, while Hg(II)-α_3DIV, Pb(II)-α_3DIV, and Cd(II)-α_3DIV had values of 27 589 (421), 26 940 (2420) and 27 600 (487) deg cm^2 dmol^{-1} res^{-1}, respectively. α_3D had the lowest $-[\theta]_{222\ nm}$ value of 25 213 (306) deg cm^2 dmol^{-1} res^{-1}, indicating that α_3DIV has a higher helical content than its parent structure. Furthermore, the $[\theta_{222}]/[\theta_{208}]$ ratio for all the samples were ~1.0, which is representative of a bundled or coiled-coil tertiary structure.[54] These results show that binding heavy metal ions to α_3DIV does not lead to the unfolding or destabilization of its overall structure; instead, α_3DIV still retains an α-helical fold.
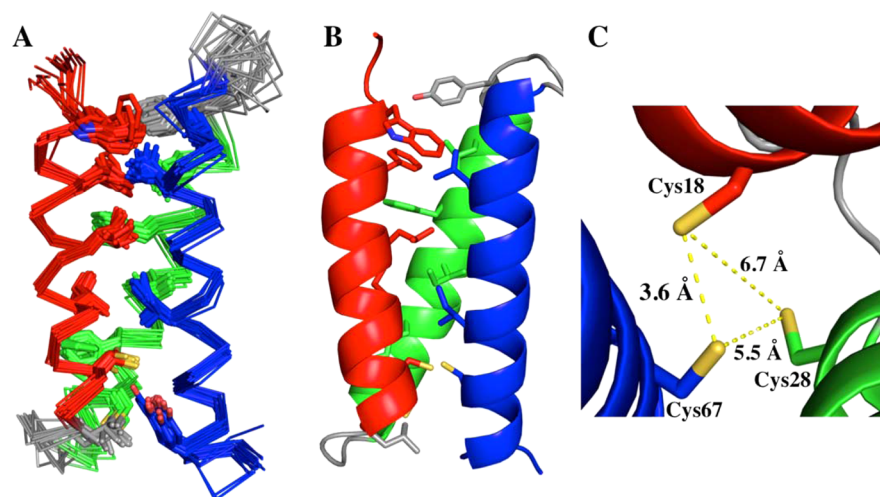
Figure 2. (A) An overlay of the 20 lowest-energy structures of apo α_3DIV, calculated with CYANA 2.1 and visualized with PYMOL.[50] Showing residues 1−73 (red: helix1, green: helix 2, blue: helix 3 and gray: loops 1 and 2). The structures were calculated from 1067 experimental NOE experimental restraints, 138 dihedral angles generated from the chemical shift index, and 78 backbone hydrogen bonds, added after the initial structure was obtained. The backbone and heavy atom RMSD values for residues 3−73 are 0.79 (0.16) Å and 1.31 (0.15) Å, respectively. For the structured regions residues 5−20, 25−44, and 51−70, the backbone and heavy atom RMSD values are 0.49 (0.12) Å and 0.97 (0.11) Å, respectively. (B) Lowest energy structure, where the side chains of core residues are visualized. (C) Top down view of the metal binding site: Cys18, Cys28, and Cys67.

DSC Studies of Metalated α_3DIV. The thermograms were collected on apo, Hg(II)-α_3DIV, Pb(II)-α_3DIV, Cd(II)-α_3DIV, and α_3D with a differential scanning calorimetry (DSC) apparatus. The thermal denaturation profile of α_3D, apo samples (at pH 7.0 and 8.2), and metalated α_3DIV are overlaid in Supporting Information, Figure 4B and their respective thermodynamic parameters are listed in Table 3. Apo α_3DIV at pH 7.0 displays a broad melting curve, which is resolved in the thermogram of apo α_3DIV at pH 8.2. The pH 7.0 thermograms fitted well to a two-peak model and the higher pH to a three-peak model. The first peak was identified to be the primary melting temperature of α_3DIV. The primary peak at pH 7.0 has a melting temperature of 64.4 (0.6) °C, while the melting maxima at pH 8.2 decreased to 60.2 (0.1) °C, exhibiting a pH-dependence on the thermal-induced denaturation. Both species yielded similar ΔH_{cal} (excess heat capacity) values of 50.0 (0.2) and 49.9 (4.6) kcal mol^{-1} for pH 7.0 and 8.2, respectively. The $\Delta H_{van't\ Hoff}$ was determined (Supporting Information Table 4), and the $\Delta H_{cal}/\Delta H_{van't\ Hoff}$ ratio is listed in Table 3. A value of 1 is indicative of a two-state unfolding model, while a value above or below 1 signifies self-association (e.g., as dimer, trimer, etc.) or unfolding through one or more intermediate states, respectively. This ratio for the parent structure, α_3D, is 1.2. Apo α_3DIV has value of 2.4 and 1.9 at pH 7.0 and 8.2, respectively. The metalated structures have $\Delta H_{cal}/\Delta H_{van't\ Hoff}$ ratio values >2.0.

A significant shift in the melting temperatures was observed for the metalated species of α_3DIV, demonstrating that the metal−ligand complex provides extra stability against thermal denaturation. The melting curve of Hg(II)-α_3DIV and Cd(II)-α_3DIV fitted well to a one-peak model, whereas Pb(II)-α_3DIV was fitted with two peaks. The T_m values for the metalated species increased by a range of 18−24 °C, with Hg(II)-α_3DIV exhibiting the largest T_m. This trend was also present in the enthalpy values, where an 8−20 kcal mol^{-1} growth was observed. In addition, the thermogram of α_3D at pH 8.2 was collected and compared with α_3DIV. The T_m value for α_3D was determined to be 89.6 (0.3), which is consistent with previously

reported values. This T_m is ∼30 °C higher than apo α_3DIV but only about 6−12 °C greater than the metalated species. Nevertheless, the ΔH_{cal} values for apo α_3DIV and α_3D are within a similar range and, most importantly, the metalated α_3DIV generated values that were 3−15 kcal mol^{-1} greater than α_3D. Overall, these stability studies using CD and DSC signify that binding a metal ion in the triscysteine binding site of α_3DIV does not destabilize or unfold its structure. In fact, from the DSC analysis, we illustrate that metal binding further stabilizes the structure of α_3DIV.

XAS Analysis of Hg-α_3DIV. XAS was utilized to determine the metrical parameters for mercury bound to α_3DIV. Several spectra were collected on independent reproducible samples and spectra were averaged independently to verify reproducibility. The spectrum presented in Figure 4 represents an average of the data for optimal signal/noise ratio. The X-ray absorption near edge structure (XANES) spectrum in Figure 4 is consistent with those typically observed for Hg(II)-S complexes.[55] The Hg EXAFS could only be fit with a single Hg−S scattering environment constructed by a coordination number between 2.0 and 3.0 ($\pm$0.5) and centered at a bond length of 2.36 Å (Supporting Information, Figure 5). There is no evidence for Hg−O/N scattering in this area. Long-range scattering could not be deconvoluted from noise signals in that region of the data. The averaged mercury−sulfur bond length in Hg-α_3DIV is compared to relevant Hg(II)-S complexes, including protein systems and model compounds in Table 4.

■ DISCUSSION

Peptide α_3DIV was prepared in our effort to functionalize the framework of α_3D by incorporating a metal binding site.[23] NMR and stability studies that examined the dynamic behavior and malleability of α_3D revealed that the C-terminal end was the most amenable to mutations. In addition, this region of the bundle contained apolar residues that formed a hydrophobic "box" region, which offered a suitable location for a metal binding site. For these reasons, we converted Leu to Cys residues to produce a triscysteine motif and demonstrated its
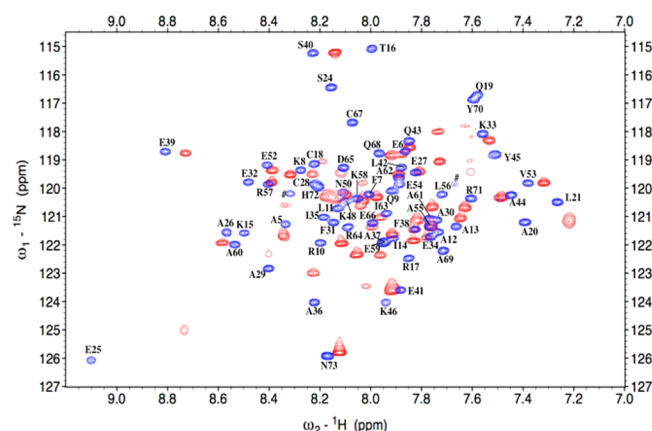
Table 2. Structural Statistics for apo α_3DIV[a]

Restraints		
Total NOE	1067	
intraresidue	395	
short-to-medium range ($1 < |i − j| < 5$)	367	
long range ($|i − j| \geq 5$)	305	
Total dihedral angles[b]	138	
Φ	60	
Ψ	61	
χ^1	17	
hydrogen bonds[c]	39 × 2	
Average CYANA target function (kcal mol^{-1})	1.9 (0.4)	
Residual distance restraint violations[d]		
average no. of violations >0.35 Å	0.00 (0.00)	
average of maximal violations (Å)	0.24 (0.12)	
Residual dihedral angle restraint violations[d]		
average no. of violations >5.0°	0.00 (0.00)	
average of maximal violations (deg)	1.94 (0.67)	
van der Waals violations[d]		
average no. of violations >0.35 Å	0.00 (0.00)	
average of sum violations (Å)	0.28 (0.07)	
Ramachandran statistics	CYANA[e]	PSVS[f]
favored (%)	90.1	90.1
allowed (%)	7.8	7.8
generously allowed (%)	2.0	2.0
disallowed (%)	0.1	0.1
RMSD from the mean structure (Å)	CYANA	PSVS
backbone (N, C$_\alpha$, C)	0.79 (0.16)[g]	0.8[h]
heavy atoms (all non-H atoms)	1.31 (0.15)[g]	1.3[h]
ordered backbone	0.49 (0.12)[i]	0.6[j]
ordered heavy atoms	0.97 (0.11)[i]	1.0[j]
Close Contacts and Deviations from Ideal Geometry (PDB validation software)		PSVS
number of close contacts within 1.6 Å for H atoms and 2.2 Å for heavy atoms		0
RMS deviation for bond angles (deg)		0.2
RMS deviation for bond lengths (Å)		0.001

[a]Summary from CYANA[42] structure calculation. The ensemble of structures did not exhibit distance violations of >0.60 Å or dihedral angle violations >5°. [b]Dihedral angle restraints were obtained from a TALOS-N[41] analysis. [c]Upper-limit hydrogen bond distance restraints were used in the ordered regions of the sequence. [d]Violations were obtained from CYANA. [e]Ramachandran plot summary of Procheck-style analysis on CYANA, 1−73 residues. [f]Ramachandran plot summary for residues 1−73 from PSVS[51] analysis. [g]Residues 3−73. [h]Residues 1−73. [i]Structured regions: residues 5−20, 25−44, and 51−70. [j]Structured regions from PSVS analysis: residues 4−20, 25−42, and 51−70.

Figure 3. Enlargement of the ^{15}N-HSQC spectra of apo α_3DIV (blue) and Pb(II)-α_3DIV (red), both at pH 7.0. Spectra were collected on a 500 MHz Bruker Avance NMR spectrometer at 25 °C. The assignments in the apo spectrum are adjacent to their corresponding peak. In this view, the apo spectrum displays 63 of 68 assigned peaks, while the Pb spectrum α_3DIV shows 54 of its 68 expected peaks.

binding properties to the heavy metal ions Hg(II), Pb(II), and Cd(II). Subsequently, we prepared an α_3DIV related construct that substituted the same leucine residues with histidine in order to form a ZnN$_3$(H$_2$O) that served as an excellent analogue of carbonic anhydrase (Table 1).[56] Despite these successes, we felt it was critical to assess how amino acid substitution at this important position impacted the structure of the apo protein (e.g., was a stable, preformed metal binding site realized by these changes).

Comparison to the Structural Statistics of α_3D. The structural statistics comparison of α_3DIV and its parent structure, α_3D, are found in Table 5. There are notable differences in the NOE and dihedral angle restraints used in the structure calculation of both structures and the empirical origins of these restraints. The solution structure of α_3D was

based on 1143 NOE restraints,[8] incorporating 70 more NOE restraints than the structure of α_3DIV. The majority of its NOEs for α_3D were classified in the short-to-medium range (45%), whereas both structures have roughly the same percentage (~30%) of short-to-medium and long-range restraints. Unlike the structure of α_3D, α_3DIV included φ, ψ, and χ1 dihedral angle restraints that were generated from a TALOS-N analysis, which employed the chemical shift assignments of ^{1}H, ^{1}H$_\alpha$, ^{14}N, ^{13}C, ^{13}C$_\alpha$, and ^{13}C$_\beta$ atoms to predict dihedral angle restraints. α_3D only contained φ angle restraints that were derived from a triple resonance HNHA experiment, as well as χ1 angles, which were determined from NOE patterns. Moreover, the comparison of the backbone and heavy atoms RMSD from the mean structure, for the same residue range and number of calculated structures, illustrated that the ensemble of the 13 lowest energy structures for α_3DIV is lower than α_3D. The RMSD for α_3DIV is ~0.3 Å lower than the 13 structures reported for α_3D, for both the backbone and heavy atoms in the structured regions and the backbone atoms for residues 1−73.

The α-helical regions for the two structures slightly differ, with α_3D (residues 4−21, 24−45, and 51−70) covering two more residues than α_3DIV (5−20, 25−44, and 51−70) in helix 1 and 2. The structure of α_3D incorporated Trp4, Leu21, Ser24, and Tyr45 in its structured regions, but these residues, from a TALOS-N analysis, were designated to have a coiled (Trp4) or loop (Leu21, Ser24, and Tyr45) secondary structure.

Comparison of the 13 Lowest Energy Structures. The 13 lowest energy structures of α_3DIV were calculated in order to properly compare the minimized model to the reported structural statistics of α_3D,[8] and we found that our overall ensemble is better ordered. The backbone (N, C$_\alpha$, C$_O$) and heavy atom RMSD values for the 13 structures of α_3DIV were observed to be lower by ~0.3 Å, even though the α_3D contained more intraresidue and short-to-medium range NOE restraints (Table 5). We attributed this difference to the dihedral angle and NOE sequential restraints used in the structure calculations. Specifically, the α_3DIV structure contains φ and ψ backbone dihedral angles, while α_3D only incorporated φ angle restraints. The presence of both dihedral angles limits the range of interaction between N$_i$-N$_{i+1}$ and C$_i$-C$_{i+1}$ atoms in

Table 3. Circular Dichroism and Thermal Denaturation Parameters[a]

sample	$-[\theta]_{222\ nm}$ (deg cm^2 dmol^{-1} res^{-1})	$[\theta]_{222\ nm}/[\theta]_{208\ nm}$	T_m (°C)	ΔH_{cal}[b] (kcal mol^{-1})	$\Delta H_{cal}/\Delta H_{\text{van't Hoff}}$
α_3D[c]	25213 (306)	1.01	89.6 (0.3)	49.9 (4.6)	1.2 (0.3)
α_3DIV	29231 (672)[c]	1.03	64.4 (0.6)[d]	50.0 (0.2)	2.4 (0.4)
			60.2 (0.1)[c]	44.9 (1.2)	1.9 (0.2)
Hg-α_3DIV[c]	27589 (421)	1.51	84.0 (1.7)	65.1 (3.5)	6.7 (2.4)
Pb-α_3DIV[c]	26940 (2420)	1.01	83.4 (3.3)	59.2 (6.9)	4.5 (2.3)
Cd-α_3DIV[c]	27600 (487)	1.07	78.1 (0.7)	52.7 (4.3)	4.2 (0.5)

[a]All the averaged values were determined from triplicate or duplicate experiments. [b]Calculated excess heat capacity. [c]pH 8.2. [d]pH 7.0.
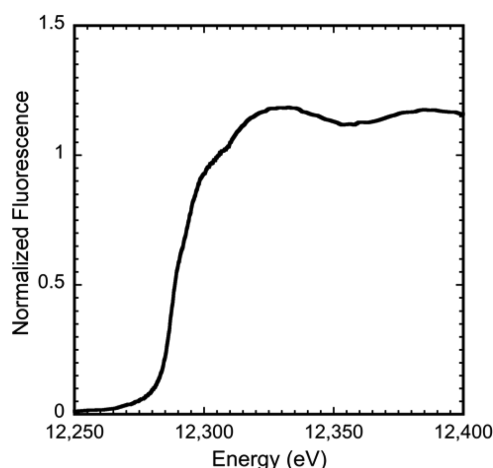
Figure 4. Normalized XANES spectrum for average of all Hg-α_3DIV spectra.

the structured regions, thereby eliminating unfavorable sterics and could result in better ordered α-helical chains. This was tested in an analysis that involved removing ψ angle restraints in the structure calculation of α_3DIV. It was determined that the RMSD from the mean structure values of the backbone and heavy atoms for residues 1−73 in α_3DIV increased to 1.08 (0.31) from 0.78 (0.15) Å and to 1.58 (0.32) from 1.25 (0.13), respectively, which are almost equal to the same parameters determined for α_3D. However, for the structured regions, a very modest increase of 0.02 Å was observed, which is well within the statistical error ($\sim \pm 0.10$ Å).

In addition, the increased unity in the 13 structures of α_3DIV can be further justified by the amount of sequential NOE restraints between ^{1}H backbone atoms used in the calculations. The α_3D structure has a proper extent of H^N_i-H^N_{i+1} and H^{α}_i-H^{β}_{i+3} sequential NOE correlations in all three helices and strong H^{β}_i-H^N_{i+1} NOE correlations in helix 3 for residues 50−70. Nevertheless, it significantly lacked H^{α}-H^N_{i+1}, H^{α}-H^N_{i+3}, and H^{β}_i-H^N_{i+1} in helix 1 and 2 correlations, NOEs that are typically observed in α-helical structures since the distances between these ^{1}H atoms are within the experimental limit of 5 Å. On the other hand, in the structured regions, α_3DIV contained most of the NOE correlations mentioned above in a sequential manner. Additionally, the sequential NOE pattern in the structure of α_3DIV can be viewed as compensating restraints since only a modest increase in the RMSD values were observed in the structured regions after ψ dihedral angle restraints were removed.

Comparison to the Lowest Energy Structure of α_3D. The lowest energy structure of α_3DIV and α_3D are super-imposed in Figure 5A. The two structures were aligned via backbone (N, C$^{\alpha}$, C, O) atoms of residues 1−73, and this alignment yielded an overall heavy atom RMS value of 1.75 Å. From the overlay, α_3DIV possesses the same overall topology as α_3D, with a counterclockwise bundle that was expected in the design process of α_3D. Figure 5B is an overlay of the Leu-to-Cys mutation site, positions 18, 28 and 67, of both structures. The C$_{\beta}$ distances between the Leu−Leu and Cys−Cys were measured, and the triangular plane that forms between these residues has an area of 15.5 Å^2 in α_3D and 14.1 Å^2 in α_3DIV. This slight deviation demonstrates that the fold at the C-terminal end was not significantly affected even after removing Leu residues that provide stabilizing and packing interactions in α_3D. Regardless, incorporating Cys residues with polar, uncharged thiol groups to form a metal-binding site in α_3DIV led to two significant structural differences as a result of a more packed core.

First, the α_3DIV structure has lower $\Omega_{1,2}$, $\Omega_{2,3}$, and $\Omega_{1,3}$ angles by 16°, 15°, and 7°, respectively. This deviation from α_3D could stem from better packing of the apolar layers above

Table 4. Hg EXAFS Fitting Analysis of α_3DIV Compared to Hg−S Bonds of Relevant Model Compounds and Proteins

complex	CN	geometry	Hg−S R (Å)	$\sigma \times 10^3$ (Å^2)	F	pH	ref
Hg-α_3DIV[a]	2		2.36	3.54	2.24	8.7	this work
	2.5		2.36	4.97	2.21		
	3		2.36	6.20	2.21		
Hg-TRIL16C[b]	2		2.324			5.5	71
	3		2.443			9.5	
Hg-MerR[b]	3		2.43			7.5	19
average of five models[c]	2	linear	2.348				72
average of 11 models[c]	3	trigonal	2.462				72
average of three models[c]	3	trigonal T-shaped	2.497 avg two shortest bonds: 2.372				72

[a]Data fit over a k range of 1 to 12 Å^{-1}. Independent metal−ligand scattering environment at R < 3.0 Å. Scattering atoms: S (sulfur). F = number of degrees of freedom weighted mean square deviation between data and fit. [b]Hg(II)−S bond lengths determined from EXAFS. [c]Hg(II)−S bond lengths determined from an X-ray crystal structure (see Supporting Information).

Table 5. Structural Statistics of α₃DIV Compared to α₃D

RMSD from the mean structure (Å)	α₃D	α₃DIV	
backbone (residues 1−73, N, C$_\alpha$, C)	1.06[a]	0.78 (0.15)[a]	1.08 (0.31)[b]
backbone (residues 4−21, 24−45, and 51−70, N, C$_\alpha$, C)	0.75[a]	0.48 (0.11)[a]	0.53 (0.15)[b]
heavy atoms (residues 1−73)	1.61[a]	1.25 (0.13)[a]	1.58 (0.32)[b]
heavy atoms (residues 4−21, 24−45, and 51−70)		0.92 (0.10)[a]	0.99 (0.10)[b]

[a]Thirteen structures.[8] [b]ψ angles excluded from the structure calculation.
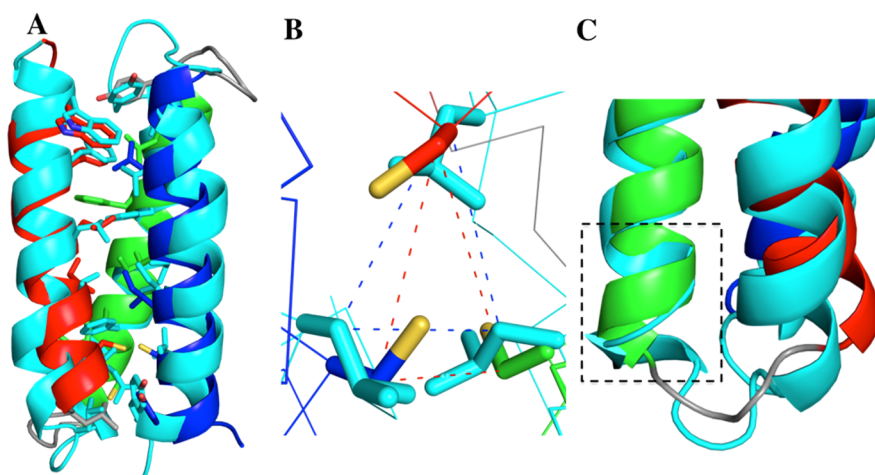
Figure 5. (A) An overlay of α₃DIV (helix 1: red, helix 2: green, and helix 3: blue) and α₃D (cyan). The backbone (N, C$_\alpha$, C,O) rms was determined on PYMOL to be 1.75 Å. (B). Top-down view of the mutation site (18, 28, and 67), displaying superimposed Cys/Leu residues. (C) Gain of helical content in helix 2 for residues 26−28.

the triscysteine site (Supporting Information, Figure 6). The first layer, which is composed of a plane between the C$_\beta$ atoms of Ile14, Phe31, and Ile63, has an area of 12.9 Å² in α₃DIV, whereas in α₃D this plane is 9.0 Å² greater. This trend is also observed in the two subsequent layers 2 (Leu11, Ile35, and Ala60) and 3 (Phe7, Phe38, and Leu56), which has an area of 7.8 Å² (16.1 Å²) and 7.3 Å² (17.7 Å²) lower than α₃D.

Next, we found that the incorporation of Cys residues did not disrupt the overall α-helical framework of α₃D, as demonstrated in the ¹⁵N-TROSY and CD studies. Unexpectedly, the alignment of α₃D and α₃DIV revealed that the addition of a Cys residue in the 28th position in helix 2 improved its helical content (Figure 5C). In α₃D, the helicity in the second strand breaks after Ala26 and becomes continuous again at Ala29. Residues Glu27 and Leu28 appear to have a more coil-like secondary structure. In contrast, this disruption in the helicity between residues 26−28 is not observed in the structure of α₃DIV, which demonstrates an increase in the α-helical content. Additionally, the analysis from two web-based structural determination programs, TALOS-N[41] and volume area dihedral angle reporter (VADAR),[57] as well as the CD comparison of apo α₃DIV with α₃D supports this observation. TALOS-N uses a database of 580 proteins with almost complete backbone NMR chemical shifts (¹H, ¹H$_\alpha$, ¹⁴N, ¹³C, ¹³C$_\alpha$, and ¹³C$_\beta$) and an additional database of 9523 high-resolution X-ray crystal structures (without experimental chemical shifts) to predict dihedral angles φ, ψ, and $\chi 1$, as well as the secondary structures from experimental NMR chemical shifts. Similar to the dihedral angle prediction, the TALOS-N secondary structure classification system involves a two-system artificial neural network (ANN) and categorized residues 26−28 into an α-helical secondary structure, with a probability score of ∼0.96. VADAR analysis on the PDB

coordinates of the lowest energy structure of α₃DIV structure also characterized residues 26−28 to be in an α-helical structure, while the same analysis for α₃D resulted in a coiled secondary structure. Furthermore, the molar ellipticity value for apo α₃DIV is about −4200 deg cm² dmol⁻¹ res⁻¹ greater than α₃D. This again indicates that the replacement of Leu residues with a Cys at the 28th position may have increased the overall helical content of the α₃D framework.

Preorganization of the Triscysteine Site. Native metalloproteins have evolved to possess metal coordination environments that are unique to their function, and two limiting extremes of metal binding sites have been described. The first group is typified by zinc finger proteins, which adopt a stable fold only after a metal has bound to the endogenous ligands of the sequence.[58] In this case, metal binding defines protein structure. Less extreme, but still within this category, are cases where metal binding leads to important protein conformational changes that define function. Examples of such behavior can be seen with metalloregulatory proteins such as MerR or ArsR.[1,2] The second group is illustrated by the cupredoxins that have a well-defined, preorganized binding site. The concept of a racked-induced bonding or entatic state model has been proposed for such systems to elucidate the unique properties of these electron transfer (ET) proteins.[59] The rack-induced bonding model explicates that the ligand environment (CysHis₂ site and one or two axial weakly bound axial ligands) in a cupredoxin fold forces the copper ion, regardless of the oxidation state, into a strict distorted-tetrahedral geometry. This feature in cupredoxins and redox-active metalloproteins was discovered to be one of the fundamental driving forces in attaining efficient electron transfer activity. Ultimately, it is of great importance in the de novo design of metallopeptides to be able to predict accurately the extent of metal binding site
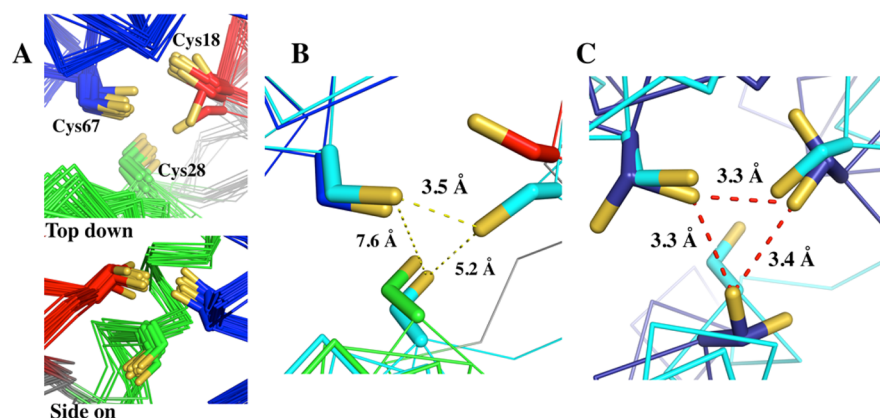
Figure 6. (A) Preorganization of the triscysteine metal binding site of the 20 lowest energy structures (Cys18: red, Cys28: green and Cys67: blue). (B) A partially preformed trigonal site for metal binding in structure 16 (cyan) of 20 aligned with the lowest energy structure, structure 1. The Sγ–Sγ distances are measured for structure 16 of α_3DIV. (C) Structure 16 (cyan) aligned with the Cys "a" sites of CSL9C (PDB: 3LJM) (blue), which contain two conformations.[61] The Sγ–Sγ distances are measured for CSL9C.

preorganization prior to the introduction of a metal ion in order to achieve the desired affinity, selectivity and function.

The sequence of α_3D was designed to contain diverse sets of heptad repeats (defined as "abcdefg"), where nonpolar residues are incorporated at the "a" and "d" positions to provide core stabilizing interactions and charged residues at the "e" and "g" to form salt-bridges between helices.[9] Our previous work on *parallel* three-stranded coiled-coil (TRI and coil-Ser) systems showed that the subtle difference between the "a" and "d" positions can produce distinctive outcomes in heavy metal binding affinity and geometry, which can be attributed to the preorganization of the sulfur ligands prior to metal binding.[9,60] We found that the substitution of Leu to Cys residues in "a" sites tend to favor the formation of a trigonal metal binding site, where three Sγ atoms point into the core (Supporting Information, Figure 7A). For instance, apo CSL9C (PDB 3LJM)[61] comprises "a" site Sγ atoms with distances of 3.3 and 3.4 Å. Conversely, "d" site substitutions orient Sγ atoms away from the core and toward the helical interface forming a much larger cavity (Supporting Information, Figure 7B). Apo CSL19C (PDB 2X6P)[61] contains "d" site Cys residues with Sγ–Sγ distances of 3.4 and 4.6 Å. Initial examination of the α_3D NMR structure indicated that a preorganized metal binding site could be carved into a hydrophobic box region of the protein. Therefore, it was for these structural reasons that leucines in the "a" positions of α_3D were targeted for modification; however, one should recognize that the side chain orientations are expected to differ in this antiparallel α_3DIV framework.

In α_3DIV, we incorporated "a" site Cys residues and expected the Sγ atom of Cys18 and Cys67 to point into the core to initiate the generation of a preformed trigonal plane of sulfurs starting with these two ligands. Cys28 was intended to provide a more adaptable ligand, which was predicted to orient toward the C-terminal end since this residue is on the antiparallel strand. Our objective with the design of α_3DIV was to create a single-polypeptide scaffold that produces a more asymmetric or a distorted triscysteine metal binding site than the symmetric Cys sites found in TRI or coil-Ser analogues. As a result, the more native-like properties of α_3DIV could provide a better model for native metalloregulatory proteins or metallochaperones that sequester toxic heavy metals in nature.

The triscysteine metal binding site of the 20 lowest energy structures of apo α_3DIV is superimposed in Figure 6A. Overall,

the 20 conformations of the three Cys residues illustrate a well-defined metal binding site. For instance, the C$_\beta$ and Sγ atoms of Cys28 are uniformly oriented toward the C-terminal end of the bundle (Figure 6A, side-on view), while the S$_\gamma$ atoms of Cys18 are pointing to the interhelical interface between helix 1 and 3 in 18 of the 20 structures. It should be pointed out that the Cys18 χ1 dihedral angle restraint was not constrained, but this corresponding angle was restrained in Cys28 and Cys67. Despite this definition of the average structure, we believe the site is not rigid, because the amide proton ^{15}N-TROSY signals for the three cysteine residues and their sequential neighbors are ~30% more intense than the average, indicating motion on the nanosecond time scale for these residues. With this caveat, we further describe the (time) averaged structure of the metal binding site in the following.

The sulfur atoms of Cys28 and Cys67 are observed to have set orientations, while Cys18 contains two conformations that diverge from its ensemble. The C$_\beta$ and Sγ atoms of Cys28 on the antiparallel strand are directed toward the C-terminal end of the bundle (Figure 6A, side-on view). This orientation was also predicted to occur for the C$_\beta$ atoms of noncoded D-amino acids, such as D-Pen or D-Leu, in 3SCC analogues.[62,63] The change in chirality in these two D derivatives were expected to direct their respective thiol and isopropyl moiety toward the C-terminal end. Consequently, we show here in our α_3DIV structure that an L-Cys in an antiparallel strand provides the same effect as a D-amino acid. For Cys67, its Sγ atoms are oriented inside the core of the bundle, which was predicted for this "a" site substitution. Lastly, the collective Sγ atoms of Cys18 result in a more "d" site-like orientation, where the Sγ atoms are directed at the interhelical interface between helix 1 and 3. Cys18 possesses two structures (16 and 18) that deviate from the ensemble. In structure 14, the "d" site conformation of the Sγ atom of Cys18 is oriented toward the opposite direction, at helix 1 and 2. Structure 16 contains a Cys18 Sγ atom that points inside the core, providing a structure that presents a metal binding site with two "a" site ligands at Cys18 and Cys67 (Figure 6B). When compared to apo CSL9C (Figure 6C), which contain "a" site residues (with two conformations), the C$_\beta$ and Sγ atoms of Cys18 and Cys67 in structure 16 overlay well with two Cys9 residues. As a result, structure 16 demonstrates a partially preformed trigonal cavity for metal

ions that coordinate in a 3 or 4 coordination environment, such as Hg(II), Pb(II), and Cd(II).

Implications on Metal Binding. With a solution structure in hand of apo α_3DIV, one may next consider the consequence of metal binding to the system. The apo structure allowed us to investigate the global, as well as local structural changes that occur when a protein scaffold is functionalized with Cys residues to form a metal binding site. A metalated structure would afford a concise comparison with the apo structure to assess the extent of preorganization of the site, which could provide a mechanistic model for metal ion acquisition in native systems. In terms of *de novo* protein design, both structures offer a base for future design of a metal binding site with mixed residues/ligands or with identical residues that require secondary interactions (such as hydrogen bonding) in able to achieve redox or catalytic activity with transition metals Fe(II/III), Cu(I/II), or Zn(II).

The spectroscopic properties of Hg(II), Pb(II), and Cd(II) bound α_3DIV were previously reported[23] to form the predicted complexes but a much closer inspection of the solution stability of metalated α_3DIV was necessary to illustrate that the addition of a metal ion does not have a unfavorable effect on its fold. We collected ^{15}N-HSQC spectra of metalated α_3DIV and performed both circular dichroism and thermal denaturation studies to illustrate that heavy metal binding reinforces and improves the solution stability of α_3DIV. In addition, the Hg-α_3DIV species was analyzed by XAS to obtain structural metrics on a metalated species.

Circular dichroism studies have been a central tool in investigating and confirming metal-induced (transition and/or heavy metal ions) folding in native proteins, such as zinc finger proteins[64] and calmodulin,[65] as well as in *de novo* designed peptides including isoleucine zipper[66] and BABY[67] 3SCC peptides. The CD fingerprint of α-helical proteins and peptides exhibit two minima at 208 and 222 nm, which arise from $\pi-\pi^*$ and n$-\pi^*$ transitions in the amide atoms.[68] When compared to apo α_3DIV, the CD spectra of the metalated species also contain a double band profile at 208 and 222 nm (Supporting Information, Figure 4A). These two absorption bands are a characteristic feature in α-helical folds. The calculated molar ellipticity values ($[\theta_{222}]$) for the metalated species fall within the range of the apo, and these large negative $[\theta]$ values are typical for a well-folded α-helical fold. Furthermore, the CD results show that the apo form has a high α-helical content, which was the intended target in the original design of α_3D. In this peptide system, metal binding to the C-terminal end of the bundle was not necessarily predicted to increase the helicity of the peptide because the overall fold is highly α-helical. Metal binding was expected to perturb that region of the peptide (positions 18, 28, and 67 at the C-terminal end), resulting in a slight decrease in the helicity of the metalated species. This corroborates well with DSC results (which is discussed subsequently) because, although a loss in helicity was observed, metal binding provides M-thiolate bonds that enhance the tertiary stability of the structure, thus much higher melting temperatures when compared to the apo. In addition, the replacement of Leu to Cys residues, to build a metal binding site, removes the stabilizing hydrophobic interaction within the α_3D fold, and binding a metal ion into this site counters this loss of stability.

A thermal denaturation analysis was performed to further assess the stability of metalated α_3DIV and to explore the concept that metal binding to proteins could lead to greater stability. This rise in thermal stability has been previously observed in ligand-binding studies on native proteins. For example, the melting temperature (T_m) of a copper metal-lochaperone BsSco was determined to increase by 23 °C when bound to Cu(II).[69] Ca(II) and Zn(II) ion binding to coagulation factors IX binding protein increased its melting temperature by 4−5 °C and calculated enthalpic parameter (ΔH_{cal}) by more than 100 kcal mol^{-1}.[70] The DSC results illustrate that metal bound α_3DIV is more stable than its apo counterpart in thermally induced denaturations. The metalated species have much higher melting temperatures, by a range of 18−24 °C, and the calculated enthalpic parameters (ΔH_{cal}) are greater by 8−20 kcal mol^{-1} (Table 3). The enhancement of both thermodynamic values shows that the metal−thiolate bonds (Cd−S, Hg−S, or Pb−S) supplements the weak-nonbonding forces (hydrophobic interactions, hydrogen bonds, and salt-bridges) that are essential in protein folding and disruptive during the denaturation process. In addition, the T_m value of parent structure α_3D is still greater than apo but slightly higher than the metalated α_3DIV species, which indicates that the incorporation of a triscysteine site in its apo or metal-bound form cannot entirely reproduce the hydrophobic interactions that are provided by Leu residues in a protein fold. This finding is further supported by a chemical denaturation performed on apo α_3DIV, where we observed a 2.5 kcal mol^{-1} decrease in the ΔG_{UF} from the reported value of α_3D (5.0 kcal mol^{-1}).[23] Moreover, the $\Delta H_{cal}/\Delta H_{van't\ Hoff}$ ratios (Table 3) reveal the thermal-induced denaturation process and a value of 1 indicates a two-state model. α_3D has a ratio closest to this model of 1.2, which matches well with the reported value.[8] Apo and metalated α_3DIV have values >2.0, and the denaturation curve of these species are much broader than the corresponding curve of α_3D, demonstrating a self-associating unfolding process. This result was not unexpected as the metal binding site contains three thiolate ligands that could induce the formation of external disulfide bonds at higher temperatures.

The transition from a two-coordinate (2 C. N.) [Hg(II)-S$_2$(SH)] to a three-coordinate (3 C. N.) [Hg(II)S$_3$]$^-$ complex in α_3DIV has an apparent pK_a of 7.1,[23] and this transition was examined by ^{199}Hg-NMR and UV−vis. We found that α_3DIV forms a linear [Hg(II)S$_2$(SH)] 2 C.N. below pH 6.0 and a three-coordinate trigonal [Hg(II)S$_3$]$^-$ complex above pH 8.5 with a single chemical shift at −938 and −244 ppm, respectively. Further, under intermediate pH conditions (pH ≈ 7.5), two chemical shifts were observed at −240 and −926 ppm, which demonstrate a mixture of both species. The three-coordinate Hg-α_3DIV species (at pH 8.7) was further analyzed with XAS to obtain structural parameters on metalated α_3DIV, as well as to confirm previous findings. The EXAFS analysis on the Hg(II)-α_3DIV is dominated by nearest-neighbor scattering typical of sulfur ligation (Supporting Information, Figure 5) and yielded an average Hg(II)−S bond length at 2.36 Å and a 2.5 C. N. This bond length falls in between reported values for 2 C. N. and 3 C. N. mercury−sulfur complexes. Therefore, in order to correlate this EXAFS analysis to the coordination environment of the [Hg(II)S$_3$]$^-$ complex of α_3DIV, we compared our result to the Hg(II)−S distances of protein and *de novo* designed peptide systems derived from EXAFS analysis and model compounds measured from X-ray crystal structures in Table 4 and Supporting Information, Table 5. The native protein, MerR, which was determined to form a trigonal mercury complex, has an EXAFS derived Hg(II)−S bond

length at 2.43 Å.[19] Similarly, the EXAFS analysis on Hg(II)-TRIL16C provides Hg(II)−S distances of 2.32 and 2.44 Å for the corresponding 2 and 3 coordinate species.[71] Table 4 also compares our EXAFS result to 2 C. N. and 3 C. N. compounds from the Cambridge Structural Database.[72] The five model compounds with a 2 C. N. have an average Hg(II)−S bond length of 2.348 (0.023) Å. The three-coordinate compounds (11 total) produced an average Hg(II)−-S distance of 2.462 (0.044) Å. Three of these compounds exhibit a T-shaped coordination environment, which consists of two short bonds with an average bond length of 2.372 (0.01) Å and a single longer bond at 2.497 (0.071) Å. These structure based Hg(II)−S bond lengths corroborate well with the EXAFS analysis of Hg(II)−TRIL16C and Hg(II)−MerR.

The two most straightforward interpretations of the observed 2.36 Å value for Hg-α_3DIV is either an approximately equimolar mixture of two- and three-coordinate mercury sites or a single, distorted T-shaped structure similar to those of small molecule models. The mixed speciation behavior was previously observed in our EXAFS analysis of Hg(II)−TRIL16C. A mixture of 2 and 3 C. N. species has a 2.37 Å Hg(II)−S bond length. However, this mixture of two- and three-coordinate Hg(II)-TRIL16C simultaneously show [199]Hg-NMR resonance peaks at ~−180 ppm and ~−830 ppm[73] indicative of both species, whereas Hg-α_3DIV has a single resonance at −244 ppm. Furthermore, the apparent pK_a for the formation of Hg(II)(TRIL16C)$_3$ was calculated to be 7.8, and a 9.5 pH condition was used to obtain a pure 3 C. N species in the EXAFS analysis. This pK_a value is nearly 1 log unit higher than what was determined for the [Hg(II)S$_3$]$^-$ complex of Hg(II)-α_3DIV. Thereby, our pH condition of 8.7, which is 1.6 log unit higher than its apparent pK_a, favors a 3 C. N. species instead of a mixture with a 2 C. N. complex as observed in Hg(II)-TRIL16C. Finally, the absorbance values in the ultraviolet between 2−4 coordinate mercury−sulfur chromophores are distinctive, where Hg(SR)$_2$ complexes have a λ_{max} band <220 nm and Hg(SR)$_3$ and Hg(SR)$_4$ compounds exhibit absorption λ_{max} bands between 230−340 nm.[74] The absorption characteristics of Hg(II)-α_3DIV at pH 8.6 is more consistent with a 3 C. N. complex. For these reasons, we believe that the best interpretation of the XAS data collectively with the [199]Hg NMR and UV−vis is to assign a T-shaped geometry with two short Hg(II)−S bonds and one longer (~2.8−3.0 Å) bond.

Together, the results from the CD and DSC studies demonstrate that metal binding did not disrupt the overall secondary structure of α_3DIV; instead it provides further stability to the framework. In addition, the EXAFS analysis validates Hg(II) binding to the triscysteine site and provides structural metrics on the [HgS$_3$]$^-$ complex. Therefore, the loss of cross peaks in the ^{15}N-HSQC spectrum of Pb(II)- and Hg(II)-α_3DIV is not due to an unfolding of α_3DIV. Instead, it indicates that metal binding quenches the more rapid dynamics of the metal-site (see above) and selects conformations that interconvert on a milli-microsecond time scale, leading to line-broadening and loss of the corresponding ^{15}N-HSQC NMR cross peaks. Nonetheless, in the case of Pb(II)-α_3DIV, we were still able to identify 57 of the 68 resonance peaks and the perturbation of many residues, such as K8, Cys18, Phe38, Ala44, and Val53, can be identified. The missing peaks are due to residues localized near the metal binding site or at the C-terminal end, which was already determined to be more dynamic than the rest of the fold in α_3D. For example, the chemical shift change for residues that are located at this end of the bundle, involving Gln19, Ser24, Ala29, Gln68, and Tyr70, cannot be easily assigned suggesting severe line broadening or overlap with a neighboring signal. For Hg(II)-α_3DIV, the ^{15}N-HSQC spectrum has seven fewer peaks than Pb(II)-α_3DIV and has eight less than its apo counterpart. In contrast to the Pb spectrum, the loss in peaks seems to be global and cannot be assigned to any specific region of the sequence. Like the Pb spectrum, we can attribute the some of peak loss to Hg(II) binding, but the more dramatic effect could be largely associated with the pH environment. As pH increases from 6 to 9, the average lifetime of the exchangeable hydrogen groups, including backbone amide protons, increases from ms to μs range,[75] thereby decreasing the observable resonance peaks in a ^{15}N-HSQC spectrum. This pH effect is exhibited in the comparison of the ^{15}N-HSQC spectra of apo α_3DIV at pH 5.8 and 8.6 (Supporting Information, Figure 8). The higher pH spectrum contains 13 less peaks than the lower pH, demonstrating that the significant loss in cross peaks in the Hg(II)-α_3DIV spectrum is mainly due to the pH condition.

An alternative explanation for the significant change in ^{15}N-HSQC results for the metalated species could stem from the asymmetric nature of the triscysteine site and leading to structural dynamics on micro-milli second time scale resulting in line broadening and peak loss. The side-on view of the triscysteine site of structure 1 in Figure 6A shows that the C$_\beta$ and S$_\gamma$ atoms of Cys28 points toward the C-terminal end of the bundle. Upon metal binding, Cys28 requires a rotation at the N−C$_\alpha$−C$_\beta$ bonds of about 56° toward the N-terminal end in order to achieve the proper trigonal plane for Hg(II) or Pb(II). In the same manner, the "d" site-like conformation of Cys18 will have to adopt an "a" site conformation as seen in structure 16 of α_3DIV or translate toward the core (Figure 6B). To further illustrate this process, apo α_3DIV was overlaid to the X-ray crystal structures of metalated de novo designed peptides, As(III)(CSL9C)$_3$ (at 1.81 Å resolution)[76] and [Hg(II)]$_S$[Zn-(II)(H$_2$O/OH$^-$)]$_N$(CSL9PenL23H)$_3$[n+] (at 2.20 Å resolution)[77] in Figure 7.
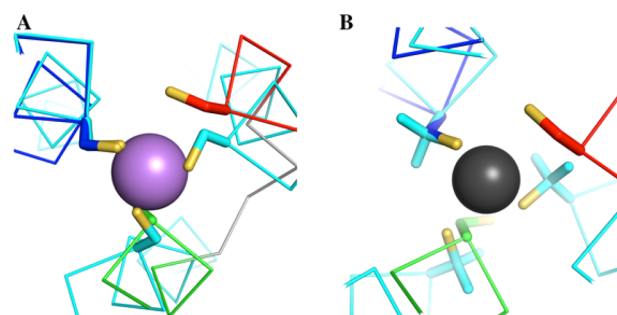
Figure 7. (A) Structure 1 of α_3DIV superimposed over the As-Cys$_3$ site of As(CSL9C)$_3$ (PDB 2JGO at 1.81 Å resolution).[76] The C$^\alpha$ and C$^\beta$ of Cys67 was visually aligned with a Cys residue (B) Structure 1 of α_3DIV superimposed over the Hg-Penicillamine$_3$ site of [Hg(II)]$_S$-[Zn(II)(H$_2$O/OH$^-$)]$_N$(CSL9PenL23H)$_3$[n+] (PDB 3PBJ at 2.20 Å resolution).[77] The C$^\alpha$ and C$^\beta$ of Cys67 were also visually aligned with a penicillamine residue.

Similar to Pb(II), As(III) has a sterochemically active lone pair of electrons and binds to a triscysteine environment in a trigonal pyramidal geometry. There are several possible orientations for the As(III) ion, with the most symmetric being endo and exo.[78] In the energetically preferred endo conformation, the As(III) ion and all three C$_\beta$ atoms are on the

same side of the plane of S atoms. In the *exo* conformation, As(III) and the C_β atoms are on opposite sides of this plane. It is also possible to have a mixed conformation, where one or two C_β atoms are on the same side and one or two are on the opposite side. The crystal structure of As(III)(CSL9C)$_3$ reveals an *endo* conformation (Figure 7A). For Pb(II)-α_3DIV in solution, it is possible that the Pb atom forms a distorted, asymmetric triscysteine site that undergoes a exchange between these conformations; thereby, influencing the chemical shift dispersion in the ^{15}N-HSQC spectrum. In addition, the ^{207}Pb-NMR spectrum of ^{207}Pb(II)-α_3DIV contained no resonances in the range observed for the more symmetric Pb(II)-S$_3$ complexes in coil-Ser, BABY, and GRAND parallel three-stranded coiled coils.[79] UV−vis studies confirm that Pb(II) does bind in a trigonal pyramidal geometry,[23] so the absence of the ^{207}Pb NMR signal supports the idea that Pb(II) is in a very dynamic environment. ^{207}Pb-NMR is much more sensitive to the motion in the coordination environment than UV−vis spectroscopy and can provide insight into the dynamic behavior of a metal−ligand complex.

Hg(II) can form two- and three-coordinate sulfur complexes depending on the pH. In the 3 C. N. complex, two geometries are possible: a trigonal planar complex with equal Hg−S$_\gamma$ bond lengths (∼2 Å), and a T-shaped complex with two short Hg−S$_\gamma$ bonds (2.1−2.3 Å) and one long (∼3 Å) bond. Our EXAFS analysis on the 3 C. N. complex of Hg(II)-α_3DIV illustrates a T-shaped coordination environment; therefore, the triscysteine site of α_3DIV was aligned with the X-ray crystal structure of the T-shaped Hg(II)-trispenicillamine of [Hg(II)]$_S$[Zn(II)(H$_2$O/OH$^-$)]$_N$(CSL9PenL23H)$_3{}^{n+}$ (Figure 7B). This alignment shows that the S$_\gamma$ atom of Cys67 has the proper orientation to form a short Hg−S$_\gamma$ bond; however Cys18 and Cys28 would require ample conformational changes to align with this T-shaped mercury-complex in [Hg(II)]$_S$[Zn(II)(H$_2$O/OH$^-$)]$_N$-(CSL9PenL23H)$_3{}^{n+}$. Thus, like the Pb(II) complex, the distorted T-shaped geometry of Hg(II)-α_3DIV could form different conformations, where the one long bond can alternate between Cys18 and Cys28. The resonance peak in the ^{199}Hg-NMR spectrum of ^{199}Hg-α_3DIV can elucidate this dynamic process. This nucleus is exceptionally sensitive to a slight perturbation in the Hg-ligand bond length and angle. A 0.01 Å deviation in a Hg−Cl bond length and 10° change in Cl−Hg−Cl bond angle was calculated to change their corresponding chemical shift values by ∼50 and 100 ppm.[80] For Hg(II)-α_3DIV in a 3 C. N. complex, its ^{199}Hg-NMR chemical shift value was experimentally determined to be −240 ppm. This value falls in between the ^{199}Hg-NMR signals reported for symmetric Cys "a" and "d" sites in 3SCC peptides with chemical shifts of ∼−185 and ∼−316 ppm, respectively. This deviation by +55 ppm from an "a" and −76 ppm from a "d" site could be representative of a distorted trigonal thiolato [HgS$_3$]$^-$ T-shaped complex of Hg(II)-α_3DIV with metal−ligand bond lengths and angles that deviate from its 3SCC counterparts.

Implications on Future Designs. *De novo* protein design allows for an iterative approach in modifying a stable peptide framework. The structure of apo α_3DIV demonstrates that by mutating packing Leu to Cys residues to achieve a thiol-rich metal binding site we are still able to retain a well-folded peptide construct. With this at hand, we can now prepare new constructs that will result in a more preformed, more symmetric triscysteine metal binding site in an antiparallel three-helix bundle. In the ensemble of structures, the S$_\gamma$ of Cys28 consistently orients toward the C-terminal end forming a

skewed S$_3$ plane. The first iteration involves moving the Cys28 one layer toward the N-terminal end by performing Cys28Phe and Phe31Cys mutations (Figure 8A). Placing a Cys in the 31st
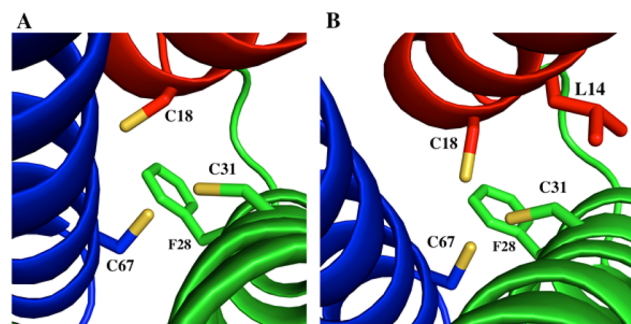
Figure 8. (A) Model of α_3DIV C28F, F31C, where the Cys residue on helix 2 is moved one layer above its original position in order to form a triscysteine plane that is more perpendicular with the bundle. (B) Model of α_3DIV I14L, C28F, F31C, where a bulky Ile is replaced with a Leu residue in able generating space for the Cys residues to ultimately form a preformed metal binding site.

position produces a S$_3$ plane that is perpendicular with the helical bundle and a Phe in the 28th provides a capping and directing force for Cys31, which yields a preformed site. The Cys28Phe/Phe31Cys α_3DIV construct can then be further modified with an iteration that directs the S$_\gamma$ of Cys18 to form more of an "a" orientation (Figure 8B). This can be achieved with an Ile14Leu mutation, where a smaller Leu residue affords space to form for the thiol group of Cys18 to orient toward the core.

■ CONCLUSIONS

Toxic heavy metals, such as Hg, Pb, As, and Cd, threaten all forms of life. By understanding the mechanism by which bacteria detoxify heavy metal through metalloregulatory proteins, one can develop strategies to combat this environmental crisis. *De novo* protein design is an emerging and biologically relevant approach in studying the metal binding sites of metalloregulatory proteins, as well as metalloproteins. It offers a novel approach of modeling metal centers using simple peptide scaffolds. Here, we report the solution structure of apo α_3DIV, a *de novo* designed three-helix bundle peptide with a triscysteine metal binding that sequesters heavy metals Hg(II), Pb(II), and Cd(II). This thiol-rich environment is a ubiquitous motif in the metal binding site of metalloregulatory proteins, including ArsR, MerR, and CadC. We show that α_3DIV has the same overall topology as and aligns well with its parent structure α_3D. We found that the incorporating Cys in place of Leu residues, which provide stabilizing packing interactions in the core, increased the helical content of the α_3D framework and resulted in a more packed core. In addition, heavy metal binding to α_3DIV induces further stability. Ultimately, this structure provides a stable framework for designing future metallopeptides that could perform specific catalytic or redox reactions.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

This section contains figures of thermal denaturation curves, ^{15}N-TROSY spectrum of Hg(II)-α_3DIV overlaid with an apo control, Ramachandran plot of the 20 lowest structures, CD

plots, EXAFS fit, ^{15}N-TROSY spectra of α_3DIV at pH 5.8 and 8.6 and so on. In addition, tables that contain the NMR acquisition parameters for the structural determination of apo α_3DIV, EXAFS metrical parameters, Ramachandran plot summary, PSVS global quality scores for α_3DIV and α_3D, thermal denaturation parameters and metrical parameters for mercury−sulfur model compounds are located in this section. Atomic coordinates, structural restraints (distance and dihedral angle restraints), and NMR chemical shift assignments for α_3DIV have been deposited via ADIT-NMR system (http://bmrb.wisc.edu). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00064.

Accession Codes

Apo α_3DIV has PDB code 2MTQ, and BMRB ID is 25177 and both depositions will be released upon publication.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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