

# Design of a Three-Helix Bundle Capable of Binding Heavy Metals in a Triscysteine Environment\*\*

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An important objective of de novo protein design is the preparation of metalloproteins, as many natural systems contain metals that play crucial roles for the function and/or structural integrity of the biopolymer.<sup>[1,2]</sup> Metalloproteins catalyze some of the most important processes in nature, 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, with no known beneficial effects (e.g., Hg<sup>II</sup> or Pb<sup>II</sup>). Ideally, we would hope to be able to use an approach based on first principles to create both known metalcenters and novel sites, which may lead to exciting new catalytic transformations. However, the design of novel metalloproteins is a challenging and complex task, especially if the aim is to prepare asymmetric metal environments.

Numerous metalloprotein systems have been designed over the past 15 years, typically through the use of unassociated peptides that assemble into three-stranded coiled coils or helix–loop–helix motifs that form antiparallel four-stranded bundles. In terms of metal-ion binding, these systems have been functionalized with heme<sup>[3,4]</sup> and nonheme mononuclear<sup>[5]</sup> and binuclear centers.<sup>[6,7]</sup> It is often difficult to prepare nonsymmetrical metal sites through these strategies owing to the symmetry of the systems, which rely on homooligomerization. Thus, the preparation of a single polypeptide chain capable of controlling a metal-coordination environment is a key objective.

Previously, we designed soft, thiol-rich metal-binding sites involving cysteine and/or penicillamine as the ligating amino

acid residues into the interior of parallel, three-stranded  $\alpha$ -helical coiled coils.<sup>[8,9]</sup> These systems have served as hallmarks for understanding the metallobiochemistry of different heavy metals, such as Cd<sup>II</sup>, Hg<sup>II</sup>, As<sup>III</sup>, and Pb<sup>II</sup>.<sup>[8–11]</sup> We have shown how to control the geometry and coordination number of metals such as Cd<sup>II</sup> and Hg<sup>II</sup> at the protein interior and how to fine-tune the physical properties of the metals, which led to site-selective molecular recognition of Cd<sup>II</sup>.<sup>[12–14]</sup> Although these homotrimeric assemblies have been very useful, the production of heterotrimeric systems in which metal environments could be fine-tuned controllably or a hydrogen bond could be introduced site-specifically has been elusive.<sup>[15]</sup> Therefore, we chose an alternative strategy to satisfy this objective and used 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<sup>-1</sup> for the desired heteromeric versus homomeric assemblies.<sup>[16,17]</sup> However, the energy gap between a hetero- and homomeric assembly often depends critically on ionic strength, the pH value, and other environmental parameters. Moreover, the objective of many studies in de novo protein design is to make the metal ion adopt an energetically suboptimal coordination geometry, 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 the reactivity of a metal-ion cofactor,<sup>[18]</sup> the noncovalently assembled complexes have often been difficult to characterize structurally, possibly owing to small populations of alternatively assembled species. In this case, the inclusion of the active-site residues in a construct with linked helices greatly facilitated structural analysis and catalytic characterization.<sup>[19]</sup>

An attractive starting scaffold to meet our objectives is the de novo designed three-helix bundle  $\alpha_3D$ . The structure of this protein has been determined by NMR spectroscopy, and it has been proven that the helices are oriented in a counterclockwise topology.<sup>[20]</sup> Although the  $\alpha_3D$  protein originated from a coiled coil, its helices were shortened to such an extent that 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  $\alpha_3D$  is similar to that of natural proteins. Thus,  $\alpha_3D$  should be tolerant to mutations, and this protein should serve as an excellent framework for the engineering of specific metal-binding sites. Additionally, with this protein scaffold, we can study the effect of the ligating residue located on the second

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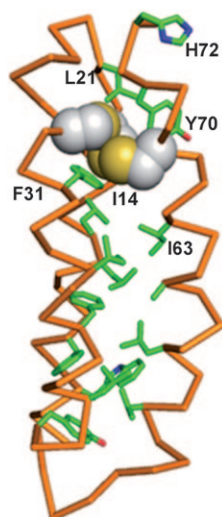
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helix, which is antiparallel to the first and third helices of the bundle.

Before attempting to prepare asymmetric metal-coordination environments or site-specific hydrogen bonds, we felt it was important to first redesign  $\alpha_3$ D with symmetric metal-binding sites involving cysteine residues. This approach would allow us to exploit the extensive body of work defining heavy-metal complexation by the TRI and Coil Ser peptides,<sup>[8,15]</sup> to assess whether a specific metal structure could be created in the modified  $\alpha_3$ D construct. It would also allow us to probe the physical properties of metals such as Cd<sup>II</sup>, Hg<sup>II</sup>, and Pb<sup>II</sup> in a more natural antiparallel helical system. A significant amount of literature exists describing the design of metal-binding sites in existing four-helix bundles and in a mixture of  $\alpha/\beta$  protein structural frameworks.<sup>[21,22]</sup>

However, there are no such examples of the engineering of novel metal-binding sites 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, in structures ranging from helical IgG-binding domains<sup>[23]</sup> to DNA-binding proteins, structural proteins, and enzymes.<sup>[24]</sup> Despite its widespread occurrence in nature, only a few attempts have been made to prepare single-chain three-helix bundles.<sup>[25,26]</sup>

On the basis of visual inspection of the  $\alpha_3$ D structure, four potential sites along the bundle were identified at which three cysteine residues, one from each helix, could be introduced. Out of these four mutants,  $\alpha_3$ DIV (Figure 1), located at the C-terminal end of the bundle, seemed to be optimal in terms of the properties of the starting protein.<sup>[20,27–29]</sup> Previous NMR spectroscopic structural and dynamic investigations showed a gradient in the dynamic behavior and malleability of the protein, whereby the C-terminal end of the bundle was most amenable to amino acid substitutions. The selected location



**Figure 1.** PyMol model of  $\alpha_3$ DIV generated from the structure determined by NMR spectroscopy for  $\alpha_3$ D. 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.

has a well-ordered backbone conformation; however, 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 of the box are formed by the backbone of the helices and the bottom by the apolar side chains of Phe31, Ile14, and Ile63. The aromatic residue of Phe31 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 nonhelical loops, including Leu21, as well as the apolar portion of Tyr70 at the terminus of helix 3. His72, which was entirely disordered in the solution structure determined by NMR spectroscopy, 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<sup>G</sup> atoms formed a nearly equilateral triangle with the side chains well-oriented to form the desired site (inter-S<sup>G</sup> distances: 3.5–4.5 Å). Overall, the location is ideal to explore the effects of hydrophobic sequestration in the present study. The sequence of  $\alpha_3$ DIV is shown in Table 1.

**Table 1:** Sequence of  $\alpha_3$ DIV.<sup>[a]</sup>

Peptide	Sequence
$\alpha_3$ DIV	MGS WAEF K QR LAAIKTR <b>CQAL</b> GG SEAE <b>CAAF</b> E KE IAAFESE LQAY KGKGNPE VEAL R KE AAAIRDE <b>CQAY</b> RHN

[a] Residues in bold are mutations with respect to the WT  $\alpha_3$ D.

After expression of a synthetic gene of  $\alpha_3$ DIV in *Escherichia coli*, followed by purification by HPLC methods (see the Experimental Section), the molecular weight of  $\alpha_3$ DIV was determined by electrospray ionization (ESI) mass spectrometry to be 7945.1 Da, which corresponds to  $\alpha_3$ DIV with the deletion of the N-terminal Met residue (calculated MW: 7946.9 Da). The folding behavior of  $\alpha_3$ DIV was studied in solution by CD and NMR spectroscopy. The CD spectrum of 5  $\mu$ M  $\alpha_3$ DIV showed double minima at 208 and 222 nm at pH 8 with molar ellipticity [ $\theta$ ] values characteristic of a well-folded  $\alpha$ -helical construct (97% folded on the basis of [ $\theta$ ]<sub>222</sub>; see Figure S1A in the Supporting Information). Furthermore,  $\alpha_3$ DIV remained well-folded between pH 3 and 9. The <sup>1</sup>H–<sup>1</sup>H NOESY spectrum of 3 mM  $\alpha_3$ DIV showed chemical-shift dispersions characteristic of a well-folded  $\alpha$ -helical structure at pH 6 (see Figure S1B). The guanidine hydrochloride (GuHCl) induced unfolding of  $\alpha_3$ DIV was studied by monitoring the change in ellipticity at 222 nm as a function of the concentration of GuHCl at pH 8. The resulting titration curve was plotted as the concentration of folded protein versus the concentration of GuHCl (see Figure S2) and was fit to a two-state equilibrium.<sup>[30–32]</sup> In this way, the free energy of unfolding ( $\Delta G_u$ ) was found to be 2.5 kcal mol<sup>-1</sup>, with a degree of cooperativity ( $m$ ) of 1.4 kcal mol<sup>-1</sup>M<sub>GuHCl</sub><sup>-1</sup>. The midpoint of the transition ( $C_m$ ) occurred at a 1.8M GuHCl concentration. These results indicate that the replacement of three Leu residues of wild-type (WT)  $\alpha_3$ D with Cys residues resulted in a loss of unfolding free energy of approximately 2.5 kcal mol<sup>-1</sup>.<sup>[20]</sup>

Having established that  $\alpha_3$ DIV is well-folded and stable in solution, we investigated the complexation of  $\text{Hg}^{\text{II}}$ ,  $\text{Cd}^{\text{II}}$ , and  $\text{Pb}^{\text{II}}$  to this peptide. Metal-ion-binding titrations were performed by adding aliquots of stock solutions of metals to peptide solutions at pH values at which each metal fully coordinated to  $\alpha_3$ DIV (pH 8.6 for  $\text{Hg}^{\text{II}}$ , and pH 8 for  $\text{Cd}^{\text{II}}$  and  $\text{Pb}^{\text{II}}$ ). 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<sup>[15,33,34]</sup> upon the formation of metal–thiolate bonds in the complexes  $[\text{Hg}^{\text{II}}(\alpha_3\text{DIV})]^-$ ,  $[\text{Cd}^{\text{II}}(\alpha_3\text{DIV})]^-$ , and  $[\text{Pb}^{\text{II}}(\alpha_3\text{DIV})]^-$ . The resulting UV/Vis absorption spectra (see Figure S3) and the molar absorption coefficients ( $\Delta\epsilon$ ) at various wavelengths (Table 2) were consistent with the

**Table 2:** Physical parameters of  $\text{Cd}^{\text{II}}$ ,  $\text{Hg}^{\text{II}}$ , and  $\text{Pb}^{\text{II}}$  complexes of  $\alpha_3$ DIV.

Complex	UV/Vis $\lambda$ [nm] ( $\Delta\epsilon$ [ $\text{M}^{-1}\text{cm}^{-1}$ ])	$\delta$ [ppm]		Apparent $\text{p}K_a$	Binding constant ( $K_b$ ) [ $\text{M}^{-1}$ ] <sup>[a]</sup>
		<sup>113</sup> Cd	<sup>199</sup> Hg		
$\text{Cd}(\alpha_3\text{DIV})^-$	232 (18 200)	583 595		$10.6 \pm 0.1$ <sup>[b]</sup>	$2.0 \times 10^7$
$\text{Hg}(\alpha_3\text{DIV})^-$	247 (12 500) 265 (8400) 295 (3900)		-244	$7.1 \pm 0.1$ <sup>[c]</sup>	
$\text{Hg}(\alpha_3\text{DIV})^{\text{[d]}}$	240 (850)		-938 <sup>[e]</sup>		
$\text{Pb}(\alpha_3\text{DIV})^-$	236 (18 000) 260 (14 400) 278 (9100) 346 (3150)			$10.2 \pm 0.1$ <sup>[b]</sup>	$3.1 \times 10^7$

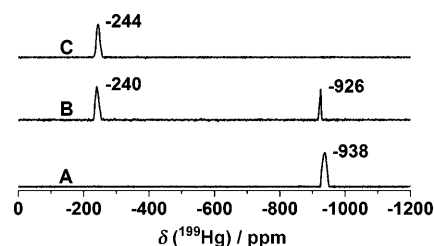
[a] The model used to obtain binding constants is:  $\text{M}^{\text{II}} + (\alpha_3\text{DIV})^{3-} \rightleftharpoons \text{M}^{\text{II}}(\alpha_3\text{DIV})^-$  ( $K_b$ ). These values represent the lower limit of  $K_b$ . [b] The model used to obtain  $\text{p}K_a$  values for  $\text{Cd}^{\text{II}}$  and  $\text{Pb}^{\text{II}}$  is:  $\text{M}^{\text{II}}(\alpha_3\text{DIVS}(\text{SH})_2)^+ \rightleftharpoons \text{M}^{\text{II}}(\alpha_3\text{DIV})^- + 2\text{H}^+$  ( $K_{a2}$ ). [c] The model used to obtain the  $\text{p}K_a$  value for  $\text{Hg}^{\text{II}}$  is:  $\text{Hg}^{\text{II}}(\alpha_3\text{DIVS}_2(\text{SH})) \rightleftharpoons \text{Hg}^{\text{II}}(\alpha_3\text{DIV})^- + \text{H}^+$  ( $K_a$ ). [d] Linear  $\text{HgS}_2$  complex of  $\alpha_3$ DIV. [e] NMR chemical shift of <sup>199</sup>Hg in the linear  $\text{HgS}_2$  complex at pH 5.8.

conclusion that all three Cys thiolate groups of  $\alpha_3$ DIV were incorporated into the first coordination sphere of the metal ions.<sup>[15,33,34]</sup>  $\text{Cd}^{\text{II}}$  and  $\text{Pb}^{\text{II}}$  binding constants were determined to be  $2.0 \times 10^7$  and  $3.1 \times 10^7 \text{ M}^{-1}$ , respectively, from analysis of the titration data.<sup>[35]</sup> Owing to the high-affinity binding, the association constant of  $\text{Hg}^{\text{II}}$  could not be determined. Next, we examined the pH-dependent complexation of these metals to  $\alpha_3$ DIV by monitoring changes in the LMCT band as a function of the pH value. The fitting of titration data of  $\text{Hg}^{\text{II}}$  to the release of one thiol proton upon the formation of a  $\text{HgS}_3$  complex from a linear  $\text{HgS}_2(\text{SH})$  complex of  $\alpha_3$ DIV<sup>[15]</sup> resulted in a  $\text{p}K_a$  value of  $7.1 \pm 0.1$  (Table 2; see also Figure S4). For  $\text{Cd}^{\text{II}}$  and  $\text{Pb}^{\text{II}}$ , the titration curves (see Figure S4) were fit to the simultaneous dissociation of two Cys thiols,<sup>[15,33,34,36]</sup> which yielded a  $\text{p}K_{a2}$  value of  $10.6 \pm 0.1$  and  $10.2 \pm 0.1$ , respectively (Table 2). These  $\text{p}K_a$  values are slightly more acidic than those for TRI peptides.<sup>[15,33,36]</sup> Nonetheless, they are consistent with the coordination modes of  $\text{Hg}^{\text{II}}$  as trigonal  $\text{HgS}_3$ ,  $\text{Cd}^{\text{II}}$  as pseudotetrahedral  $\text{CdS}_3\text{O}$  (in which O belongs to an exogenous water molecule), and  $\text{Pb}^{\text{II}}$  as trigonal-pyramidal  $\text{PbS}_3$  complexes.

<sup>199</sup>Hg NMR and <sup>199m</sup>Hg PAC (perturbed angular correlation) spectroscopy were used to probe the coordination environment around  $\text{Hg}^{\text{II}}$  bound to  $\alpha_3$ DIV at pH 5.8, 8.6, and 7.4. On the basis of the  $\text{p}K_a$  value,  $\text{Hg}^{\text{II}}$  is expected to form a

linear  $\text{HgS}_2$  complex at pH 5.8 and a trigonal  $\text{HgS}_3$  complex at pH 8.6, with a mixture of linear and trigonal complexes at intermediate pH values. The <sup>199</sup>Hg NMR chemical shifts (Figure 2) and <sup>199m</sup>Hg PAC spectral parameters (see Figure S5 and Table S1) of  $\alpha_3$ DIV at pH 5.8, 8.6, and 7.4 confirm that  $\alpha_3$ DIV forms a linear  $\text{HgS}_2$  complex at pH 5.8 and a trigonal  $\text{HgS}_3$  complex at pH 8.6; dithiolate– $\text{Hg}^{\text{II}}$  and trithiolate– $\text{Hg}^{\text{II}}$  complexes are both formed at pH 7.4 with distorted geometries.<sup>[10,37–43]</sup> The <sup>113</sup>Cd NMR spectrum of  $\alpha_3$ DIV has two resonances at  $\delta = 595$  and 583 ppm at pH 8 (see Figure S6), which indicate the presence of two  $\text{Cd}^{\text{II}}$  species with chemical-shift values similar to those observed for four-coordinate pseudotetrahedral  $\text{CdS}_3\text{O}$  species.<sup>[36]</sup> <sup>111m</sup>Cd PAC spectroscopy was used to confirm the coordination environment and

geometry of  $\text{Cd}^{\text{II}}$  complexes of  $\alpha_3$ DIV. The <sup>111m</sup>Cd PAC spectrum of  $\alpha_3$ DIV has three nuclear quadrupole interactions (NQIs), at  $\omega_0 = 0.35$ , 0.27, and 0.17  $\text{rad ns}^{-1}$  at pH 8.1 (see Figure S7 and Table S2). The first two peaks at 0.35 and 0.27  $\text{rad ns}^{-1}$  agree strikingly well with a typical  $\text{CdS}_3\text{O}$  signal in *exo* and *endo* conformations, respectively, as observed for TRI peptides.<sup>[36]</sup> The lowest-frequency NQI at 0.17  $\text{rad ns}^{-1}$  can be best assigned to a  $\text{CdS}_3\text{N}$  species in which N corresponds to His72. Even though the chemical shift of  $\delta = 595$  ppm in the <sup>113</sup>Cd NMR spectrum (see Figure S6) is lower than that reported for a  $\text{CdS}_3\text{N}$



**Figure 2.** <sup>199</sup>Hg NMR spectra of solutions containing  $\alpha_3$ DIV (2.93 mM) and <sup>199</sup>Hg(NO<sub>3</sub>)<sub>2</sub> (0.8 equiv) at pH 5.8 (A), 7.4 (B), and 8.6 (C).

species,<sup>[44]</sup> quantum-chemical calculations show that a change in the Cd–S bond lengths of 0.01 Å can cause a change in chemical shift of about 20 ppm.<sup>[45]</sup> Thus, tentatively, the <sup>113</sup>Cd NMR resonance at  $\delta = 595$  ppm can be best assigned to the  $\text{CdS}_3\text{N}$  species.

In conclusion, we have been successful in engineering metal-binding sites containing cysteine residues in an existing antiparallel three-helix bundle. The resulting protein,  $\alpha_3$ DIV, is well-folded and stable in solution and capable of binding heavy metals with high affinity ( $> 10^7 \text{ M}^{-1}$ ). The spectroscopic properties of  $\text{Hg}^{\text{II}}$ ,  $\text{Cd}^{\text{II}}$ , and  $\text{Pb}^{\text{II}}$  complexes of  $\alpha_3$ DIV are very similar to those of existing parallel three-stranded coiled coils.

Thus, we achieved our objective of preparing a single polypeptide chain capable of binding metal ions with high affinity and predefined coordination geometry. Understanding of the biochemistry of the binding of heavy metals to a single polypeptide chain is potentially useful for the development of peptide-based water-purification systems or sensors for specific heavy-metal ions. Clearly, the use of 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, as well as the effects of the electronic structure of the aromatic residue, Phe31, and second-shell effects through the replacement of any of the surrounding residues and the synthesis of catalytic metalloproteins.

## Experimental Section

Synthetic DNA for  $\alpha_3$ DIV was cloned into the pET-15b vector (Celtek Genes) and expressed in *E. coli* BL21(DE3) competent cells (Stratagene) grown in M9 medium. After sonication and heat denaturation at 55 °C, the lyophilized powder was purified on a C18 preparative reversed-phase HPLC column with a linear gradient of 100% H<sub>2</sub>O/0.1% trifluoroacetic acid (TFA) to 10% H<sub>2</sub>O/90% acetonitrile/0.1% TFA over 50 min. The molecular weight of the pure peptide was determined by ESIMS to be 7945.1 Da, which corresponds to  $\alpha_3$ DIV without the first Met residue (calculated MW: 7946.9 Da). The yield of the pure protein was 17 mg L<sup>-1</sup>. The concentration of the protein was determined on the basis of its absorbance at 280 nm by using the known molar absorbance  $\epsilon_{280} = 8.61 \text{ mM}^{-1}\text{cm}^{-1}$ .<sup>[46]</sup>

CD spectra were collected on an Aviv model 202 CD spectrometer by using rectangular open-top quartz cuvettes at 25 °C. GuHCl titration experiments were carried out by using a MicroLab 500 series syringe-pump automatic titrator controlled by Aviv software. Titrations were carried out by mixing two separate solutions of the peptide (10  $\mu\text{M}$ ) containing GuHCl at concentrations of 0.0 and 7.63 M in 10 mM phosphate buffer at pH 8. The observed ellipticities in millidegrees were converted into molar ellipticities ( $\text{deg cm}^2 \text{ dmol}^{-1} \text{ res}^{-1}$ ), as described previously,<sup>[33]</sup> by using 59 amino acids in the helical region of the protein. GuHCl titration data were fit to an equation derived from a two-state model.<sup>[30–32]</sup> A <sup>1</sup>H–<sup>1</sup>H NOESY experiment was performed according to standard procedures.<sup>[47]</sup>

Cd<sup>II</sup>, Hg<sup>II</sup>, and Pb<sup>II</sup>-binding titrations were performed at room temperature on a Cary 100 Bio UV/Vis spectrometer with anaerobic cuvettes (Starna Inc.) with a 1 cm path length by adding aliquots of stock solutions of different metals. Peptide samples with concentrations of 20–30  $\mu\text{M}$  were prepared in appropriate buffers (2-amino-2-hydroxymethylpropane-1,3-diol (TRIS) for pH 8 and 2-(cyclohexylamino)ethanesulfonic acid (CHES) for pH 8.6; 50 mM) in the presence of tris(2-carboxyethyl)phosphane (TCEP; 40–60  $\mu\text{M}$ ) inside an inert-atmosphere box (Vacuum Atmospheres Co., model OMNILAB). Stock solutions of 8 mM CdCl<sub>2</sub>, 7.37 mM HgCl<sub>2</sub>, and 5.16 mM PbCl<sub>2</sub> were also prepared inside the inert-atmosphere box. In each case, difference spectra were obtained by subtracting the background spectra of samples containing the peptide, the buffer, and TCEP. Direct titration data were analyzed by nonlinear least-squares fits to an equation used previously.<sup>[35]</sup> The difference molar absorbances ( $\Delta\epsilon$ ) were determined on the basis of the total metal concentrations after subtraction of the background spectra.

pH titrations were performed as described previously<sup>[34,36]</sup> by adding small aliquots of KOH to solutions containing  $\alpha_3$ DIV (20–30  $\mu\text{M}$ ) and CdCl<sub>2</sub>, HgCl<sub>2</sub>, or PbCl<sub>2</sub> (1 equiv). In the cases of Cd<sup>II</sup> and

Pb<sup>II</sup>, the titration data were analyzed by using the model of a simultaneous two-proton dissociation, as described previously.<sup>[34,36]</sup> For Hg<sup>II</sup>, the data were analyzed by using the model of the dissociation of one thiol proton of Cys.<sup>[15]</sup>

<sup>113</sup>Cd NMR and <sup>199</sup>Hg NMR spectroscopic experiments were performed according to standard procedures.<sup>[48]</sup> An exponential line broadening of 200 Hz was applied prior to Fourier transformation during processing of the <sup>199</sup>Hg NMR spectroscopic data.

Samples for <sup>111m</sup>Cd PAC measurement contained  $\alpha_3$ DIV (300  $\mu\text{M}$ ), Cd<sup>II</sup> (1/12 equiv), 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.<sup>[36,48]</sup> Samples for <sup>199m</sup>Hg PAC experiments contained  $\alpha_3$ DIV (200  $\mu\text{M}$ ), Hg<sup>II</sup> (80  $\mu\text{M}$ ), and 55% sucrose in an appropriate buffer (phosphate for pH 5.8 and 7.4, and CHES for pH 8.6; 100 mM). Sample preparation and data collection were performed at CERN.<sup>[41]</sup>

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