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De Novo Protein Design as a Methodology for Synthetic Bioinorganic Chemistry

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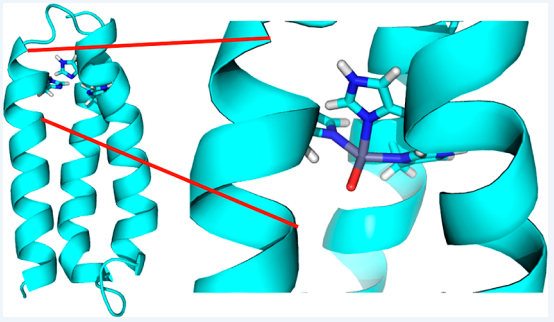
CONSPECTUS: The major advances in molecular and structural biology and automated peptide and DNA synthesis of the 1970s and 1980s generated fertile conditions in the 1990s for the exploration of designed proteins as a new approach for inorganic chemists to generate biomolecular mimics of metalloproteins. This Account follows the development of the TRI peptide family of three-stranded coiled coils (3SCC) and α_3D family of three-helix bundles (3HB) as scaffolds for the preparation of metal binding sites within *de novo* designed constructs. The 3SCC were developed using the concept of a heptad repeat (*abcdefg*) putting hydrophobes in the *a* and *d* positions. The TRI peptides contain four heptads with capping glycines. Via substitution of leucine hydrophobes, metal ligands can be introduced into the *a* or *d* sites in order to bind metals.



First, the ability to use cysteine-substituted 3SCC aggregates to impose higher or lower coordination numbers on Hg(II) and Cd(II) or matching the coordination preferences of As(III) and Pb(II) is discussed. Then, methods to develop dual site peptides capable of discriminating metals based on their type (e.g., Cd(II) vs Pb(II)), their preference for *a* vs *d* sites, and then their coordination number is described.

Once these principles of metal site differentiation are described, we shift to building dual site peptides using both cysteine and histidine metal binding sites. This approach provides a construct with both a Hg(II) structural and a Zn(II) hydrolytic center, the latter of which is capable of hydrating CO_2. With these Zn(II) proteins, we consider the relative importance of the location of the catalytic center along the primary sequence of the peptide and show that only minor perturbations in catalytic efficiencies are observed based on metal location. We then assess the feasibility of preparing enzymes competent to reduce nitrite with copper centers in a histidine-rich environment. As part of this discussion, we examine the influence of surface residues on catalyst reduction potentials and catalytic efficiencies.

We end describing approaches to prepare asymmetric proteins that can incorporate acid−base catalysts or water channels. In this respect, we highlight modifications of a helix−turn−helix−turn−helix motif called α3D and show how this 3HB can be modified to bind heavy metals or to make Zn(II) centers, which are active hydrolytic catalysts. A comparison is made to the comparable parallel 3SCC.

INTRODUCTION: A NEW SYNTHETIC APPROACH FOR BIOINORGANIC CHEMISTRY

While metalloproteins have been known for nearly a century, it was only in the 1960s that inorganic chemists attempted to synthesize these fascinating biological cofactors. This successful approach often utilizes multidentate ligands containing relevant heteroatoms to bind metals in appropriate oxidation states and coordination numbers. A major strategic advantage was that small molecules were easy to crystallize, allowing well-defined complexes to be used as models for spectroscopy and reactivity of enigmatic metalloprotein sites. Through these studies, the mysteries of many interesting bioinorganic systems were revealed (*vide infra*).

While this chemical methodology was being practiced, major advances in biology were realized. This included the ability to isolate and manipulate genomes or individual strands of DNA, achieving selective mutations of proteins, crystallizing and structurally interrogating metalloproteins, and developing automated synthesis, at both peptide and DNA levels, which allowed for the preparation of newly minted proteins. This ease of synthetic access to real proteins with defined metal binding sites, which could now be defined at the same high resolution as small molecules, ushered in the field of *de novo* metalloprotein design. No longer was one restricted to ligands that were analogues of amino acids, no longer was chemistry often required to be completed in organic solvents to mimic a protein environment or confer stability on the metal site, no longer would one need to complete multistep and tedious chemical syntheses to develop chirality around the metal center or probe second or third coordination sphere effects on spectroscopy and catalysis. In essence, bioinorganic chemistry was ready to

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Table 1. Peptide Sequences

Peptide	Sequence
	abcdefg
BABY	Ac-GLKALEEKLKALEEKLKALEEKG-NH_2
TRI	Ac-GLKALEEKLKALEEKLKALEEKLKALEEKG-NH_2
GRAND	Ac-GLKALEEKLKALEEKLKALEEKLKALEEKLKALEEKG-NH_2
TRIL16C	Ac-GLKALEEKLKALEEK***C***KALEEKLKALEEKG-NH_2
CoilSer	Ac-EWEALEKKLAALESKLQALEKKLEALEHG-NH_2
α_3D	MGSWAEFKQRLAAIKTRLQALGGSEAELAAFEKEIAAFESELQA
	YKGKGNPEVELRKEAAAIRDELQAYRHN
α_3D**IV**	MGSWAEFKQRLAAIKTR***C***QALGGSEAE***C***AAFEKEIAAFESELQA
	YKGKGNPEVELRKEAAAIRDE***C***QAYRHN
α_3DH_3	MGSWAEFKQRLAAIKTR***H***QALGGSEAE***H***AAFEKEIAAFESELQA
	YKGKGNPEVELRKEAAAIRDE***H***QAYRHN***GSGA***

be launched into the world of synthetic biology. As defined by the European Union in a Report of a New and Emerging Science and Technology (NEST) High-Level Expert Group:

> *Synthetic biology is the engineering of biology: the synthesis of complex, biologically based (or inspired) systems which display functions that do not exist in nature. This engineering perspective may be applied at all levels of the hierarchy of biological structures—from individual molecules to whole cells, tissues and organisms. In essence, synthetic biology will enable the design of "biological systems" in a rational and systematic way.*[1]

At the most reductionist level, *de novo* protein design, the process of using peptide sequences that are not existent in, but indeed inspired by nature, allows for the building of new function. We have used these designed systems to generate well-defined secondary and tertiary protein structures capable of constraining metals into native-like structures or functions. Significant advances in understanding have allowed for enhancement of the specificity and selectivity of metal binding. Designed three-helix bundles (3HB) are now being tested for practical applications as bioimaging agents (MRI contrast and luminescent probes) or for removal of uranyl ions from ocean water.[2,3]

A SYNTHETIC BIOLOGY APPROACH USING METALATED 3SCC

Many reviews have covered metalloprotein design and redesign with respect to inclusion and alterations of metal binding sites to obtain functional metalloproteins.[4−9] Our lab utilizes *de novo* designed three-stranded α-helical coiled coils (3SCC) and α-helical bundles to build, study, and manipulate metal binding sites in pseudo-3-fold symmetric assemblies. Herein, we discuss how these peptides can impose uncommon coordination geometries and differentially recognize either the same metal in different geometries or different metals in different regions of the same scaffold and then discuss how these insights can be used to generate catalytic systems capable of hydrolytic or redox transformations.

In 1992, we initiated a collaboration with Bill DeGrado to study metal binding sites in *de novo* designed peptides. We developed the TRI family of 3SCC that exploits a heptad repeat, a seven-amino acid sequence (*abcdefg*),[10] containing hydrophobic residues (*a* and *d*) and charged residues capable of H-bonding and salt bridging (*e* and *g*) and filling the remaining locations (*b*, *c*, and *f*) with either helix-inducing or water-solubilizing residues. Acetylated or amidated terminal glycine residues also induce the helical structure. Hydrophobic collapse drives the formation of 3SCC forming layers of leucine residues (alternating between *a* and *d* positions) along the interior of the coiled coil that may be substituted with metal binding residues. Moving from N to C termini, there are three residues above (sequence *d*XXX*a*) and two below (*a*XX*d*) an *a* layer within the α-helical interior. This layer separation is reversed from the *d* site perspective, resulting in changes of the side chain orientations between *a* and *d* site metal environments (Figure 2A, B). The pattern between adjacent leucine layers becomes *a*XX*d* above and *d*XXX*a* below the *d* site. The TRI peptides have four heptad repeats with the sequence given in Table 1. This sequence can be truncated to three heptads (BABY) or elongated to five heptads (GRAND).

Extensive studies demonstrated that these three-stranded coiled coils adopted native folds in aqueous solution rather than molten globules.[11] Analytical ultracentrifugation, HPLC analysis, and diffusion based NMR techniques demonstrated that at pH $\geq$ 5.5 the TRI peptides form parallel 3SCC. Folding free energies indicated that these 3SCC were highly stable constructs that only denatured in the presence of multimolar guandinium chloride (i.e., ∼2−4 mM GuHCl).[11,12] Subsequently, numerous structures of apo CoilSer derivatives, a related sequence used in crystallographic studies, have appeared that confirm the parallel 3SCC assignment (Table 1 and Figure 1).

Polyhedra for inorganic ions binding to proteinacious ligands provide coordination numbers from three to six and are generated through triangular structures. Trigonal environments are achieved by introducing a metal binding ligand into an *a* or *d* site, resulting in a 3-fold symmetric binding site within the 3SCC (Figure 2). Metal ion binding into the plane, or layer, of the three ligands provides a trigonal planar geometry, whereas displacing the metal up or down from such a plane generates trigonal pyramidal geometry. Pseudo-tetrahedral and trigonal bipyramidal polyhedra are obtained by placing one or two ligands above (and below) trigonal planes, respectively. Octahedral structures can be realized by placing a second trigonal plane at a 60° angle above the first. The 3SCC motif was selected precisely because the pseudo-C_{3v} symmetry accesses all these polyhedra.

Designing a metal binding site into the TRI peptides is easily accomplished by substituting a leucine residue for a coordinating amino acid such as cysteine (e.g., substitution of leucine to cysteine in the 16th position gives **TRI**L16C, Table 1). While removing a leucine layer is destabilizing, subsequent metal binding provides sufficient stability to afford well-folded metalloproteins. This cysteine substitution allows binding of

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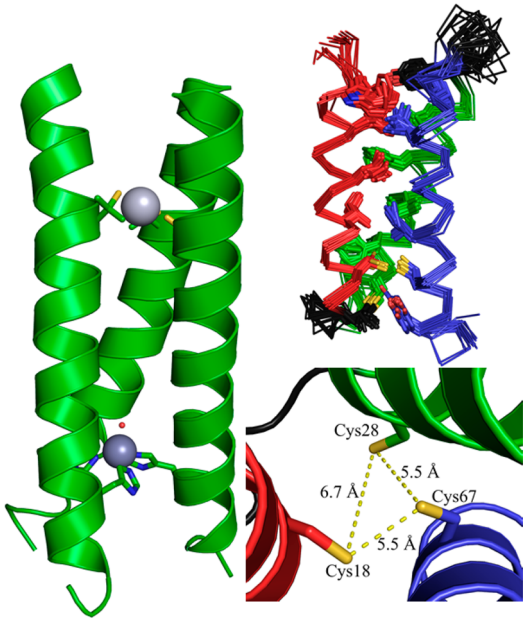

Figure 1. Structures of designed peptides: (left) X-ray structure of $Hg(II)_5Zn(II)(OH_2)_{N/O}$(**CS**L9PenL23H)$_3^+$, a 3SCC, containing Hg(II) at the top and Zn(II) at the bottom; (top right) The 20 lowest energy NMR structures of α_3D**IV**, a single polypeptide chain 3HB; (bottom right) close up of metal tris-cysteine binding site in α_3D**IV**.

heavy metals such as Hg(II), Pb(II), As(III), Cd(II), and Bi(III) (*vide infra*, in these cases, the counterion does not effect metal binding or assembly).[13]

We chose cysteine ligands for the hydrophobic interior because this amino acid is small and neutral and the metal ion preferences are distinctly different from the exterior carboxylate residues. Beyond the obvious objective (selective metal binding within a 3SCC), we wanted to constrain metals in less common coordination geometries. MerR, a metalloregulatory protein, was proposed to bind Hg(II) as a trigonal plane rather than the nearly ubiquitous linear Hg(II) adducts observed in most proteins.[14−17] While trigonal Hg(II) thiolate complexes had been crystallized from nonaqueous solution,[15,18,19] such structures were unstable in water, especially at the nanomolar concentrations where Hg(II) was recognized by MerR. Thus, our first objective was to impose a third sulfur ligand onto Hg(II) within a protein environment in aqueous solution.

A tris-cysteine site was designed in TRI in *a* positions, generating **TRI**L9C and **TRI**L16C. Studies determined that both linear and trigonal Hg(II)−thiol coordination can be achieved. At pH < 4.5, where the apo-peptide predominately forms a dimer, or conditions of 1:0.67 ratios of Hg(II)/trimer (pH > 5.5), spectroscopic features consistent with two-coordinate linear Hg(II) complexes were observed (Table 2).[11,20,21] Between pH 5.5 and 8, with an excess of trimer to Hg(II), a 3SCC exists with a linear Hg(II) and the third thiol uncoordinated. At pH > 8, a three-coordinate, trigonal geometry was obtained with markedly different spectral features, closely matching Hg(II)MerR (Table 2).[20,21] Thus, under conditions sufficient to deprotonate all three thiols, a trigonal planar geometry resulted. Subsequent X-ray crystallographic analysis proved the trigonal binding site when penicillamine was used as a ligand (Figure 2C)[22] and a Hg(II)MerR structure that has just appeared confirms a trigonal thiolato binding site in the native metalloregulatory protein.[23]

Trigonal $Hg(II)S_3$ sites are easily obtained when the thiol ligands are at *a* positions. We learned that higher pH values (pH ≥ 9) were required to obtain trigonal Hg(II) *d* sites as found in **TRI**L12C.[24] X-ray analysis of apo-**CS**L9C and **CS**L19C revealed that the preferred cysteine rotamers in *a* site peptides (e.g., **CS**L9C) positioned thiol groups into the helical interior, whereas cysteine sulfur atoms in **CS**L19C were oriented toward the helical interface (Figure 2A, B), suggesting a significant rearrangement energy to form $Hg(II)S_3$ at the *d* site.[11,25]

Cysteine substituted GRAND and BABY also bind Hg(II) in an unprecedented trigonal geometry in water (experimental parameters are consistent with comparable TRI peptides).[24,26] The more stable GRAND peptides form trigonal Hg(II) complexes at subnanomolar concentrations putting it on par with MerR. Studies with **BABY**L9C demonstrated how metals can nucleate protein folding.[27] Ten micromolar **BABY**L9C at pH 8.5 is unfolded. Addition of Hg(II) associates the peptides forming Hg(II)(**BABY**L9C)$_2$, matching the Hg(II) linear structural preference. Because this system was designed to form a 3SCC, a third **BABY**L9C is recruited generating Hg(II)(**BABY**L9C)$_3^-$, providing a three-coordinate Hg(II) ion.[27] Thus, initially the metal induces its preferred structure, forcing an incorrect protein aggregation that ultimately rearranges to the desired 3SCC constraining the Hg(II) into an uncommon geometry.

The SmtB/ArsR families of metalloregulators represent an alternative set of multithiolate containing proteins that are responsible for controlling detoxification of heavy metals (e.g., Cd(II), As(III), and Pb(II) in bacteria). In ArsR, As(III) has three As−S bonds of 2.25 Å in a proposed trigonal pyramidal geometry; however, structures of the proteins are unavailable.[28] The crystal structure of As(III)(**CS**L9C)$_3$ (1.8 Å resolution,[29] Figure 2D) revealed 2.28 Å As−S bond distances, in close agreement to As(III)ArsR.[28] This first crystallographic report of $As(III)S_3$ in a biologically relevant scaffold confirmed a trigonal pyramidal polyhedron, but in an unanticipated endo isomer orienting the stereochemically active lone pair toward the C-terminus. Prior descriptions had assumed an exo structure.[29]

Lead(II) can also form hemidirected trigonal pyramidal geometries in proteins such as aminolevulinic acid dehydratase.[30] Because Pb(II) is larger than As(III), one might expect different coordination preferences within the TRI peptides. EXAFS analysis of Pb(II)−TRI peptides yielded three 2.63 Å metal−sulfur distances.[31] Computational methods and experiments demonstrated that Pb(II) prefers binding to *d* site cysteines, which provide more space in the interior of the 3SCC in comparison to the *a* site cysteines (Figure 2), and the pK_a of **TRI**L16C is higher (pK_a = 6.3) than that of **TRI**L12C (pK_a= 6.0).[13] This trend is in contrast to Cd(II) (*vide infra*) and Hg(II). Thus, one can conceive of distinguishing metal sites based on differential cysteine substitution at the *a* or *d* layers. The first report of a ^{207}Pb NMR chemical shift for the $Pb(II)(SR)_3$ chromophore was achieved using Pb(II)−TRI peptides. This technique is astoundingly sensitive because changing the cysteine residue from *a* to *d* sites or altering the sterics around the Pb(II) results in NMR shifts of >200 ppm.[32] Current studies are utilizing the combination of Pb(II) and steric matching of surrounding hydrophobic residues to obtain asymmetric 3SCC (Mocny, Manuscript in preparation).

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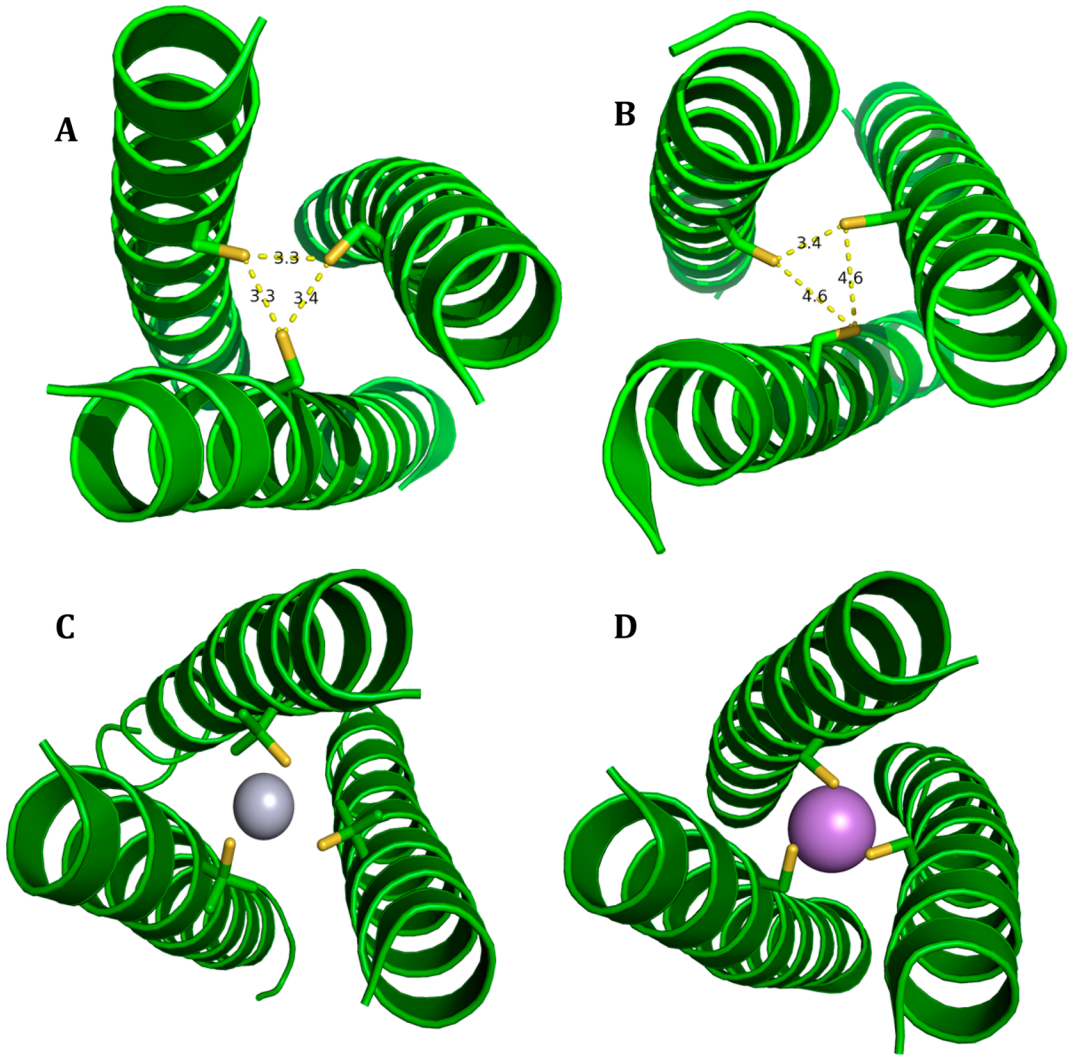

Figure 2. View down the 3-fold axis of X-ray structures of (A) *a* site $(\mathbf{CS}L9C)_3$, with S−S distances of 3.3, 3.3, and 3.4 Å. (B) *d* site $(\mathbf{CS}L19C)_3$, an enlarged metal binding site with S−S distances of 3.4, 4.6, and 4.6 Å. (C) Hg(II) site in $Hg(II)_S Zn(II)(OH_2)_{N/O}(\mathbf{CS}L9PenL23H)_3^+$ (*a* site cysteine). (D) $As(III)_S(\mathbf{CS}L9C)_3$, (*a* site cysteine).

Table 2. Comparison of Hg(II)-Bound MerR and TRI Peptides

sample[a]	δ[b]	λ ($\Delta\varepsilon$)[c]	Å[d]	ω (η)[e]
Hg(II)MerR	−106[f]	242 (19.8)[g]	2.43[h]	1.19 (0.25)[i]
	−109 (DNA)[f]	260 (14.6)[g]		
		290 (6.45)[g]		
Hg(II) $(\mathbf{TRI}LXC)_2$	−834[j]	240 (2.7)[j]	2.30[j]	1.47 (0.13)[k]
Hg(II) $(\mathbf{TRI}LXC)_3^-$	−179[j]	247 (19.2)[j]	2.41[j]	1.17 (0.25)[k,l]
		265 (11.9)[j]		
		295 (5.8)[j]		
$Hg(Cys)_4^{2-}$	−300 to −500[f,m]	280 (NR)[g]	2.54[h]	0.33 (0.0)[n]

[a]**TRI**LXC: X = 9 or 16, both *a* site cysteine modifications. [b]δ given in ppm for ^{199}Hg NMR. [c]λ given in nm, and $\Delta\varepsilon$ as $mM^{-1}\ cm^{-1}$. [d]Average Hg−S EXAFS bond lengths. [e]ω is given in rad/ns, and η is a unitless quantity for ^{199m}Hg PAC. [f]Reference 12, pH 6.02. [g]Reference 14, pH 7.0. [h]Reference 13, pH 7.5. [i]Reference 15, pH NR. [j]Reference 18, pH 8.5. [k]Reference 19, pH 8.1, [l]pH 8.7. [m]Varied pH. [n]Reference 16, pH NR.

CadC is a regulator for Cd(II) detoxification (*Staphylococcus aureus*).[33] Unlike ArsR, which forms trigonal pyramidal structures, CadC has four cysteines that may sequester the metal.[34] Whether Cd(II) binds to CadC as a tetrathiolate S_4 structure or as an S_3O site with an exogenously bound water has been discussed, providing another synthetic target for protein design. Furthermore, the Cu metalloregulator CmtR might bind Cd(II) in an unprecedented trigonal planar structure[35] providing our next objective, imposing a lower than desired coordination number.

Cd(II) binding to **TRI**L16C and **TRI**L12C at pH 8.5 was explored via EXAFS, UV−vis, ^{113}Cd NMR, and ^{113m}Cd PAC spectroscopies. Similar complexes were formed with Cd(II) in either *a* or *d* sites and, as was seen for Hg(II), the pK_a's for binding the third cysteine are different at 7.2 and 6.8 (**TRI**L12C and **TRI**L16C, respectively).[36] Because of the *a* and *d* site similarity, we will focus our discussion on the *a* site peptides. ^{113}Cd NMR and ^{113m}Cd PAC spectroscopies are the most informative techniques for clarifying this system. One ^{113}Cd NMR signal was observed at 625 ppm for **TRI**L16C,[36] which is within the range of previously reported CdS_3 and CdS_3O shifts. However, it was found using ^{113m}Cd PAC spectroscopy (Figure 3) that there were two Cd(II) species present simultaneously, a CdS_3O complex (60%, $\omega_0 \approx 0.350$

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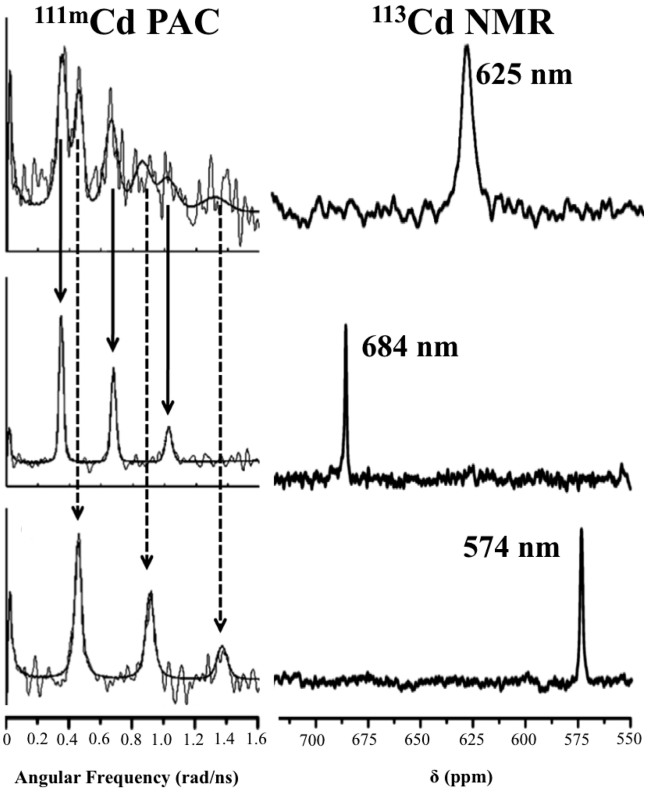

Figure 3. ^{111m}Cd PAC and ^{113}Cd NMR spectra of (top) Cd(II)-(**CS**L16C)$_3^-$, (middle) Cd(II)(**CS**L12AL16C)$_3^-$, and (bottom) Cd(II)(**CS**L16Pen)$_3^-$. Lines correlate the CdS_3O (solid) and CdS_3 (dashed) species.

rad/ns) and a CdS_3 species (40%, $\omega_0 \approx 0.450$ rad/ns). The apparently contradictory speciation is a consequence of different experimental time scales. ^{113}Cd NMR probes the solution at millisecond intervals, whereas ^{113m}Cd PAC interrogates the center on the nanosecond regime. Thus, we conclude that the CdS_3O and CdS_3 forms interconvert rapidly on the NMR, but not the PAC, time scales. Further examination of numerous derivatives, some of which are described below, led to a correlation using ^{113}Cd NMR to quantify the proportion of CdS_3O and CdS_3 in a sample.[37] For *a* site peptides, Cd(II) chemical shifts at ~575 ppm correspond to pure CdS_3O, whereas CdS_3 exhibits a single resonance at ~680−700 ppm. This ^{113}Cd NMR correlation allows us to assess whether we prepared pure CdS_3O or CdS_3 species or a mixture.

We reasoned that a pure CdS_3O center would result by removing the sterically constraining leucines in the layer above the cysteines (**TRI**L12AL16C). The alanine substitution allowed water to enter the cavity above the metal, leading to a compound with 100% $\omega_0 \approx 0.340$ rad/ns and 574 ppm.[38]

Synthesizing the first water-soluble CdS_3 species was more challenging. Ultimately, two different strategies were successful. The first exploited the utilization of a penicillamine ligand, which is bulkier than cysteine, having methyl groups rather than protons at the C-β position (**TRI**L16Pen). We hypothesized that the methyl groups would block water from the hydrophobic region above the metal. Addition of Cd(II) at pH 8.5 to **TRI**L16Pen gave Cd(II)(**TRI**L16Pen)$_3^-$, a 100% CdS_3 complex ($\omega_0 \approx 0.454$ rad/ns, 684 ppm).[38] Crystallographic analysis of apo-(**TRI**L16Pen)$_3$ revealed that the geminal methyls on the β-C would not block the bound water.[39] Neither did the packing of the leucines in the 12 layer become perturbed, so other factors must lead to the desired structure.

Pure CdS_3 was also achieved by exploiting D-amino acids using **TRI**L12$_D$LL16C. The reasoning here was that the leucine side chain orientation shifted toward the metal binding site, blocking water access. Indeed, this hypothesis was correct forming a pure CdS_3 complex ($\omega_0 \approx 0.456$ rad/ns, 697 ppm).[40] Not only do these two examples provide the only known synthetic cases where CdS_3 can be prepared in water, but this was also the first application of using alternative chirality to modify the coordination geometry of a metal in a protein.

Having successfully designed both CdS_3O and CdS_3 binding sites, we designed a single peptide with two distinct cysteine binding sites capable of complexing Cd(II) with different affinities or geometries. The relative affinities of Cd(II) binding to peptides containing either *a* or *d* cysteine sites predicted that the metal could be bound selectively first to the *a* position and then *d*. Preparation of **TRI**L9CL19C confirmed this supposition since 1 equiv of Cd(II) bound exclusively to the L9C (*a* site), while a second bound to the *d* site.[41] Further success was realized when mixtures of Cd(II) and Pb(II) were shown to place Pb(II) in the preferred *d* site while Cd(II) occupied the *a* site.[42] From the perspective of molecular recognition and the development of multicenter designed proteins, these observations were major advancements to the field.

Peptides that could distinguish metals based on coordination number were the next target. A sequence was designed that contained both an alanine/cysteine site and a penicillamine site in a peptide containing an extra heptad in order to retain sufficient aggregate stability. Addition of two equivalents of Cd(II) to **GR**L16PenL26AL30C led to two ^{113}Cd NMR signals at 687 and 588 ppm correlating to the previously characterized CdS_3 and CdS_3O sites, respectively.[43] The first equivalent of Cd(II) bound exclusively to the pseudotetrahedral S_3O site, and occupation of the trigonal S_3 site only occurred with a second equivalent of Cd(II). Therefore, a 3SCC capable of sequestering Cd(II) simultaneously in two different geometries was designed exhibiting different affinities and physical properties.

Clearly, significant understanding of factors that define protein−metal interactions was achieved using the TRI peptides. Metal ions can be placed within environments that match their coordination preferences (Pb(II), As(III)) or that impose higher (Hg(II)) or lower (Cd(II)) coordination numbers. Furthermore, one can now differentiate binding of metals at sites within a protein based on coordination preference, coordination number, or ion type. These developments opened the way for functional multimetallic structures.

SYNTHESIS OF CATALYTIC SCAFFOLDS USING 3SCC

After achieving control of heavy metal coordination chemistry, we embarked on defining the structure and environment surrounding transition metals with the intention of generating new catalysis and redox centers. Many non-heme metal centers in proteins utilize imidazole ligands. This presented a new concern for designing catalytic centers. Could far larger residues, such as histidine, be incorporated within the hydrophobic interior of 3SCC without disrupting the assembly? Our first system, **TRI**L9CL23H, exploited our ability to incorporate metals into 3SCC site selectively. At the N-

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terminal end of the construct, we designed a high affinity binding site for Hg(II) using the existing cysteine methodology to confer additional aggregate stability. Guanidinium denaturation titrations demonstrated that $Hg(II)Cys_3$ served this function.[11] A second Leu23 substitution with histidine yielded a His_3 catalytic binding site offering a structural model of carbonic anhydrase (CA). The 23rd position was chosen since it was an *a* site that was located away from the center of the sequence to minimize steric disruption of the 3SCC, but not at the ends of the sequence to obtain a more structured binding site. CA has a His_3 coordination sphere in a β-sheet-rich domain that binds Zn(II) in a pseudotetrahedral environment with a fourth exogenous water ligand.[44]

The construct, $Hg(II)_SZn(II)_{N/O}(OH_2)$-$(\mathbf{TRI}L16PenL23H)_3^+$, allowed us to address two fundamental issues in bioinorganic chemistry. First, could a catalytic metal center found in a β-sheet structure be constructed within an entirely α-helical domain? Second, if such a structural match occurred, would such a center be competent to catalyze the native reaction? Spectroscopic studies (^{199}Hg NMR, UV–vis) proved that a trigonal Hg(II) was correctly formed. Ultimately, a crystal structure of $Hg(II)_SZn(II)$-$(OH_2)_{N/O}(\mathbf{CS}L9PenL23H)_3^+$ was obtained and refined to 2.2 Å resolution (Figure 1). The model showed excellent overlay in the primary coordination sphere to the crystal structure of CA, with Zn–N and Zn–O distances within experimental error (Figure 4, left panel).[22] Therefore, imidazole is well

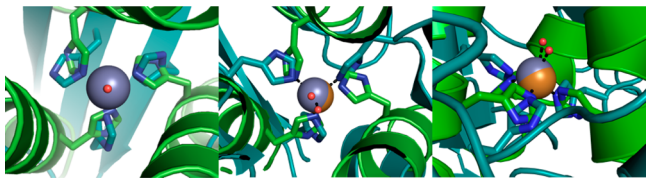

Figure 4. (**A**) Overlay of the Zn(II) active sites of human CAII (PDB:2CBA) and $Hg(II)_SZn(II)_{N/O}(OH_2)(\mathbf{TRI}L16PenL23H)_3^+$ (PDB:3PBJ). Top down (**Center**) and side views (**Right**) of an overlay of $Hg(II)_SZn(II)_{N/O}(OH_2)(\mathbf{TRI}L16PenL23H)_3^+$ and the $Cu(I/II)His_3$ binding site of CuNiR (PDB:2DY2).

accommodated within the 3SCC, Zn(II) and Hg(II) bind exclusively to their intended binding sites, Hg(II) behaved as a structural site providing additional stability against denaturation, and a CA like $ZnHis_3$/water site was viable in a drastically different protein fold.

The reaction catalyzed by CA is essentially diffusion limited hydration of CO_2 to bicarbonate and water.[45,46] Ester hydrolysis, via a similar mechanism as CO_2 hydration (Zn(II)–OH nucelophilic attack), is often analyzed due to the significantly slower reaction times. When reported, our $Hg(II)_SZn(II)(OH)_{N/O}(\mathbf{TRI}L9CL23H)_3$ mimic was the fastest CA mimic of *p*-nitrophenolacetate (*p*NPA) hydrolysis with a k_{cat}/K_m of 23.3 M^{-1} s^{-1} at pH 9.5, only ~100-fold slower than native CAII and a 550-fold increase over other small molecule mimics.[22,46,47] We view access of small molecule substrates to be via breathing modes of the protein scaffold at the helical interface.[48] The pK_a = 8.8 was two log units higher than that reported for CAII (pK_a = 6.8).[49,50] Two other designed Zn(II) enzymes have since been reported that have faster *p*NPA hydrolysis rates.[51,52] However, to date *$Hg(II)_SZn(II)$-$(OH)_{N/O}(\mathbf{TRI}L9CL23H)_3$ is still the fastest reported CA mimic for the native CO_2 hydration reaction with a rate of 1.8 × 10^5 M^{-1} s^{-1} at pH 9.5*. This system is 430-fold faster than the best previously reported small molecule mimic reacting solely in water and only ~370-fold slower than the fastest CA, CAII.[45,46,53] Thus, $Hg(II)_SZn(II)(OH)_{N/O}(\mathbf{TRI}L9CL23H)_3$ was a fantastic structural model of CA and the first reported *de novo* designed peptide that rivaled the rates of catalysis of a native enzyme. This first generation construct sought to mimic only the first coordination sphere of CAII and did not provide residues to facilitate acid–base catalysis. Interestingly, CA mutants that delete a specific active site threonine have catalytic efficiencies 100-fold lower and a water $pK_a \approx 8.8$.[54,55] Our work clearly shows that, within 2 log units, the native protein fold is not essential for catalytic activity and that including an acid–base catalyst could confer the missing activity and altered pH dependence.

We determined whether the catalytic efficiency of our CA mimic depended on the position of the Zn(II) site within the 3SCC. We first inverted **TRI**L9CL23H to **TRI**L9HL23C, which yielded a *p*NPA activity of 15.5 M^{-1} s^{-1}, 67% of the $Hg(II)_SZn(II)(OH)_{N/O}(\mathbf{TRI}L9CL23H)_3$ activity.[48] We then examined how changing from an *a* to a *d* His_3 site would affect the rates. The second peptide left the L9C position untouched but moved the His_3 to the 19th position (**TRI**L9CL19H). This peptide showed slightly less activity, 13.9 M^{-1} s^{-1}, 60% in comparison to $Hg(II)_SZn(II)(OH)_{N/O}(\mathbf{TRI}L9CL23H)_3$. Finally, a peptide only containing Zn(II) was prepared (**TRI**L23H), which was stable and showed that the Hg(II) did not influence enzymatic activity. Additionally the kinetic pK_a's of these constructs were 9.2, a slight increase over the parent peptide (pK_a = 9.0). These studies show that Hg(II) could be removed from the design and that the His_3 site can be relocated across the peptide scaffold without diminishing activity or altering pK_a's. Improvement on the current activity will require the design of second coordination sphere modifications, such as acid–base catalysts.

Some of the most interesting bioinorganic systems complete redox catalysis. From a synthetic perspective, such a scaffold is far more difficult to prepare than for hydrolytic chemistry because one must control the coordination environment of the metal in at least two oxidation states. Copper nitrate reductase, CuNiR, contains a type 2 copper binding site where copper, not unlike Zn(II) in CA, is coordinated to three histidine residues and an exogenous water molecule.[56] Once the crystal structure of $Hg(II)_SZn(II)(OH_2)_{N/O}(\mathbf{CS}L9PenL23H)_3^+$ was obtained, we hypothesized that Cu(I/II) may bind in a similar fashion to offer a structural and functional model of CuNiR (Figure 4, center and right panels). Due to the potential redox sensitivity of thiols, we used **TRI**L23H since we had learned that $Hg(II)S_3$ was not required for stability and did not influence catalysis. Spectroscopic examination supports a five-coordinate Cu(II) structure with three bound imidazoles.[57] The reduced Cu(I) state has been interrogated by ^{1}H NMR and XAS, indicating that a trigonal planar Cu(I) structure forms from pH 5.8 to 7.5. Thus, neither copper oxidation state fully reflects the present structural model for CuNiR.

An important parameter for any redox cofactor is its reduction potential. Using fluorescence quenching and UV–vis spectroscopy, we determined the binding affinities of both Cu(I) and Cu(II) to $(\mathbf{TRI}L23H)_3$ and then calculated standard reduction potentials. Values of ~400–500 mV were obtained, slightly higher than that reported for CuNiR (140–310 mV) suggesting that the trigonal planar Cu(I) state stabilizes the reduced $Cu(\mathbf{TRI}L23H)_3^+$.[58] This system was quantitatively

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reduced from Cu(II) to Cu(I) by sodium ascorbate and produced NO when Cu(I) is reacted with nitrite. Like CuNiR, the reaction is pH dependent with a maximal rate at pH 5.9 (5.2×10^{-4} s^{-1}). Although significantly less than CuNiR (~1500 s^{-1} at pH 5.8), Cu(**TRI**L23H)$_3^{n+}$ is the only stable and most highly active aqueous, homogeneous mimic for the native enzyme.[59]

The electrostatics surrounding the metal binding site can greatly affect the center's redox potential. Cu(**TRI**L23H)$_3^{n+}$ was redesigned to alter charged residues on the exterior of the 3SCC in hopes of optimizing both redox potential and catalytic efficiency. Inverting the K22 and E27 residues, (**TRI**K22EL23-HE27K), enhanced Cu(I) affinity (100-fold) and increased the rate 1.5-fold.[60] Additional peptides were designed, keeping the K22E mutation but altering other lysine or glutamate residues, changing the charge of the trimer from 0 to −3, −6, −9, and −12; however, all of these charge-modified peptides resulted in more positive reduction potentials (490−500 mV). These data demonstrated that surface mutations influence the reduction potential over a 100 mV range and the reaction rate by a factor of 4. Unpublished studies now suggest that modifications in the interior of the 3SCC can lead to rate enhancements of almost 1000-fold.

We can conclude from these catalytic studies that major advances in preparing water-soluble and stable catalysts can be realized using metalloprotein design. Furthermore, metal coordination geometries can be recapitulated in dramatically different protein folds. Nonetheless, there are major challenges for further synthetic development. We must learn how to place acid−base catalysts in defined orientations within the helical assembly. In some cases, substrate, water, and proton channels are important for catalysis, yet we still do not have a method to mimic such structures in these scaffolds. In order to broaden the range of catalytic sites, one must generate nonsymmetric scaffolds that allow for mixed first and second coordination spheres. These and other issues will be at the forefront of protein design research over the next decade.

AN ENTIRELY DIFFERENT APPROACH: METALLO-THREE-HELIX BUNDLES (3HB)

The previous systems focused on highly symmetric, self-assembling 3SCC. An alternative strategy to incorporate asymmetry is to employ a single polypeptide chain that can be site specifically mutated to generate channels or acid−base catalysts. An appealing system is α_3D, a 3HB from the DeGrado laboratory.[61] α_3D (Table 1) was optimized to have a helix−turn−helix−turn−helix motif. Computational modeling suggested four sites where three cysteine residues could replace hydrophobes. The fourth model, α_3D**IV** (Table 1), was chosen as the test peptide to assess heavy metal binding. Comparison of the NMR structures of α_3D and α_3D**IV** reveal a preorganized metal binding site (Figure 1) upon replacement of three leucines with cysteines with little perturbation of the scaffold.[62,63] Metal complexation was strong and led to higher protein stability with Hg(II) bound as a three coordinate complex, probably T-shaped, while Pb(II) adopted a trigonal pyramidal geometry. Cd(II) adopted two structures CdS_3O or CdS_3N, with either coordinated water or His72, respectively.[63]

We next incorporated histidines, yielding α_3DH_3 (Table 1), to form a CA analogue. While Zn(II) bound with higher affinity to α_3DH_3 than to Hg(II)$_S$(**TRI**L9CL23H)$_3^-$, the catalytic efficiency for *p*NPA hydrolysis was diminished. The CO_2 hydration efficiency was excellent and allowed for a detailed pH profile that again demonstrated an elevated Zn−OH_2 pK_a (9.4).[64] Nonetheless, these results illustrate that a more native-like fold with an antiparallel helical orientation can lead to both high affinity binding of metals and catalytic activity.

CONCLUSIONS

Based upon these promising results, and those that have been achieved in other laboratories, one can envision a promising future for *de novo* designed proteins. The greatest challenges in chemistry are underpinned by complexity, and protein design provides a vehicle to tackle these obstacles. At its basis, biology is the ultimate achievement of chemical complexity in which concepts of molecular recognition, spatial and temporal control of reactivity, and intricate molecular architecture define life. Our early steps to build, from scratch, systems adopting precise protein folds with controlled metal environments have been successful. The future will provide advances that allow for numerous more difficult challenges. First, how does one develop asymmetric environments that control both the properties of the metal (e.g., pK_a), orientation and activation of the substrate, and regio, stereo, and functional group discrimination? The α_3D analogs provide us with the foundation to interrogate biological systems as a single change in sequence controlling second coordination environments can easily be achieved; however, preparation of self-assembling peptides that confer similar control is in its infancy. How does one prepare and control the reactivity of multiple metal sites within and between artificial constructs? Recent studies building rubredoxin and cupredoxin centers suggest that mononuclear redox centers can be embedded in the α_3D helical fold. Based on these promising results, it appears that the next generation of designed peptides, those with redox centers capable of shuttling electrons to a catalytic center in the same peptide, may soon be upon us. However, more challenging will be developing systems that allow protein−protein interactions of sufficient selectivity and affinity to begin gating redox catalysis. Much of biology's magic occurs by separation and confinement of chemical reactions to discrete spatial locations. Ultimately developing systems that are capable of exploiting compartmentalization of designed proteins in small "factories" to carry out multistep catalysis or information processing has become the ultimate challenge.

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