Published in final edited form as:

Acc Chem Res. 2019 May 21; 52(5): 1160–1167. doi:10.1021/acs.accounts.9b00032.

Noncoded Amino Acids in *de Novo* Metalloprotein Design: Controlling Coordination Number and Catalysis

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CONSPECTUS:

The relationship between structure and function has long been one of the major points of investigation in Biophysics. Understanding how much, or how little, of a protein's often complicated structure is necessary for its function can lead to directed therapeutic strategies and would allow one to design proteins for specific desired functions. Studying protein function by *de novo* design builds the functionality from the ground up in a completely unrelated and noncoded protein scaffold. Our lab has used this strategy to study heavy and transition metal binding within the TRI family of three stranded coiled coil (3SCC) constructs to understand coordination geometry and metalloenzyme catalytic control within a protein environment. These peptides contain hydrophobic layers within the interior of the 3SCC, which one can mutate to metal binding residues to create a minimal metal binding site, while solid phase synthesis allows our lab to easily incorporate a number of noncoded amino acids including D enantiomers of binding or secondary coordination sphere amino acids, penicillamine, or methylated versions of histidine. Our studies of Cd(II) binding to Cys₃ environments have determined, largely through the use of ¹¹³Cd NMR and ^{111m}Cd PAC, that the coordination environment around a heavy metal can be controlled by incorporating noncoded amino acids in either the primary or secondary coordination spheres. We found mutating the metal binding amino acids to L-Pen can enforce trigonal Cd(II)S₃ geometry exclusively compared to the mixed coordination determined for L-Cys coordination. The same result can be achieved with secondary sphere mutations as well by incorporating D-Leu above a Cys₃. We hypothesize this latter effect is due to the increased steric packing above the metal binding site that occurs when the L-Leu oriented toward the N-terminus of the scaffold is mutated to D-Leu and oriented toward the C-terminus. Mutating the layer below Cys₃ to D-Leu instead formed a mixed 4- and 5-coordinate Cd(II)S₃(H₂O) and Cd(II)S₃(H₂O)₂ construct as steric bulk was decreased below the metal binding site. We have also applied noncoded amino acids to metalloenzyme systems by incorporating His residues that are methylated at the δ - or ϵ -nitrogen to enforce Cu(I) ligation to the opposite open nitrogen of His and found a 2 orders of magnitude increased catalytic efficiency for nitrite reductase activity with ϵ -nitrogen coordination compared to δ -nitrogen. These results exemplify the ability to tune coordination environment and catalytic efficiency within a *de novo* scaffold as well as the utility of noncoded amino acids to increase the chemist's toolbox. By furthering our understanding of metalloprotein design one could envision, through our use of amino acids not normally available to nature, that protein design laboratories

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Notes

The authors declare no competing financial interest.

will soon be capable of outperforming the native systems previously used as their benchmark of successful design. The ability to design proteins at this level would have far reaching and exciting benefits within various fields including medical and industrial applications.

Graphical Abstract

INTRODUCTION

Understanding the relationship between protein structure and function is among the most important goals within Biophysics. Using *de novo* protein design, researchers can probe this relationship from a bottom-up approach by transplanting the function of one native protein into the tertiary structure of another previously unknown scaffold (unrelated to any natural sequence).^{1–3} It is estimated that 30–40% of proteins require a metal to function.^{4,5} *De novo* metalloprotein design allows one to determine the requirements to achieve a target metal coordination environment in a simplified protein scaffold. Our lab has focused on using three stranded coiled coils (3SCC) as scaffolds through the TRI system of peptides (Table 1).⁶ These scaffolds use a heptad repeat strategy with 3, 4, or 5 heptads of the sequence LKALEEK making up a single coil. Hydrophobic Leu residues in the a and d positions enforce the creation of a 3SCC. Mutating one or more of the Leu layers into the metal binding residues Cys or His allows us to create simplified binding sites for heavy or transition metals, respectively.

One of Nature's most impressive accomplishments is the breadth of functions that have been obtained utilizing a restrictive set of 20 commonly available amino acids and a pool of cofactors. Proteins designed in the lab, however, are under no such restriction, and several laboratories have shown the utility of noncoded amino acids to tune metal binding properties.^{7,8} Our lab benefits from the simplicity with which one can insert a noncoded amino acid through solid phase peptide synthesis. Thus, we have explored their use in several applications centered around either heavy metal coordination control^{9–13} or transition metal catalysis tuning.¹⁴ This Account will describe the tactics we have implemented using noncoded amino acids to understand coordination number and geometry

control of Cd binding to sulfur rich proteins or modifications of the first and second coordination of Cu nitrite reductase (NiR) catalysts.

HEAVY METAL COORDINATION CONTROL

One of our lab's larger goals is to probe heavy metal coordination to sulfur rich environments that are relevant to understanding metalloregulators and metallochaperones.^{15–17} When we began these studies in the early 1990s, we hypothesized that an atomistic understanding of how these toxic metals bind to designed proteins would provide structural information that was difficult to obtain using the native systems. **TRIL16C**, which presents a Cys₃ binding site between two Leu hydrophobic layers, was one of the earliest successes of this strategy binding Hg as HgS₃.⁶ Hg(II) prefers to bind as a linear 2-coordinate complex with sulfurs, but evidence at the time had suggested that the metalloregulatory protein MerR utilized a Cys₃ motif.¹⁸ Through spectroscopic and crystallographic studies, it was shown that Hg(II) could form such trigonal planar structures far in advance of the ultimate X-ray structure of mercurated MerR.^{6,19} Other metalloids such as trigonal pyramidal Pb(II) and As(III) could be interrogated in a similar vein.^{20,21} Utilizing similar control on Cd(II) proved more difficult since this ion has a less restrictive coordination number in the presence of thiolate ligands. **TRIL16C** successfully created a CdS₃ binding site, but ^{111m}Cd perturbed angular correlation (PAC) experiments of this system determined that Cd(II) bound in two geometries with 40% being trigonal planar CdS₃ and 60% being tetrahedral CdS₃O.²²

Our first application of noncoded amino acids sought to exploit changes in steric bulk around the **TRIL16C** binding site to control Cd(II) coordination. We compared Cd(II) binding in **TRIL16C**, **TRIL16Pen** (Pen = penicillamine), **TRIL12AL16C**, and **TRIL12HfLL16C** (HfL = hexafluoroleucine) (Table 1). Decreasing steric bulk above the Cys₃ binding site in **TRIL12AL16C** successfully created a single species of CdS₃(H₂O) as determined by ^{111m}Cd PAC and allowed us, using our previous results with Cd(**TRIL16C**)₃[–], to predict the ¹¹³Cd NMR chemical shift for a pure CdS₃ system as ~680–700 ppm (Figure 1). We have since determined through examination of the Hg(**TRIL12AL16C**)₃[–] crystal structure that opening this space allows four waters to insert within the layer above the bound metal, one of which binds to the metal creating the CdS₃(H₂O) site.²³

Increasing steric bulk in the 12th position using hexafluoroleucine produced unexpected results as it shifted the ratio of 4-/3-coordinate Cd(II) toward more 4-coordinate based on ¹¹³Cd NMR. We now realize that the incorporation of 18 fluorine atoms to the layer at the N-terminal side of the cysteine sulfur layer likely caused the Cd(II) to shift to the C-terminal side of the helix, which has more space to accommodate water allowing an increased coordination number (Figure 1). The second strategy utilizing non-native amino acids was to increase the steric bulk within the plane of the first coordination sphere by mutating the Cys₃ binding residues to penicillamine. This replaces the β -methylene hydrogen atoms of Cys with methyl groups (Figure 2). The ¹¹³Cd NMR of Cd(II)(**TRIL6Pen**)₃[–] contained a single peak at 684 ppm, a value within the calculated range, leaving us confident that we had achieved a CdS₃ binding geometry. This was confirmed by ^{111m}Cd PAC that identified a single 3-coordinate trigonal planar species. Hence, via small structural changes to either the

second or first coordination spheres, we altered the energy landscape to obtain the desired geometries. These lessons can be applied to both heavy metal binding and metalloenzyme catalytic sites.

SECONDARY COORDINATION SPHERE NONCODED AMINO ACIDS

Penicillamine yielded the desired CdS_3 center by modifying the sulfur atom plane. We next sought to create a construct that could enforce the same coordination on a Cys_3 binding site by modifying steric bulk above the sulfur layer. Because the bulky hexafluoroleucine mutation above the Cys_3 layer was unable to enforce a CdS_3 structure, we used an alternate strategy exploiting the chirality of amino acids. We altered the original L-Leu diastereomer to its D-Leu variant to create **TRIL12D-LL16C** (Table 2). Given that the β carbon atoms of L-amino acids point toward the N-terminus of a 3SCC (while the D-diastereomer orients the side chain toward the C terminus), one should be able to remove the small solvent cavity above the metal without increasing the side chain steric bulk (Figure 3). ^{113}Cd NMR of $\text{Cd(II)(TRIL12D-LL16C)}_3^-$ exhibited a peak at 697 ppm, similar to that of **TRIL16Pen**. Also, ^{111}mCd PAC was fit with a trigonal planar binding site,¹⁰ indicating that this design bound Cd(II) solely as CdS_3 .

Analysis of the X-ray structures of $\text{Hg(II)(TRIL6Pen)}_3^-$ and $\text{Hg(II)(TRIL12D-LL16C)}_3^-$ revealed that the exclusion of solvent was achieved in two different ways. With penicillamine, the β carbon atoms of cysteine must accommodate the more bulky methyl substitution, causing a change in the preferred side chain rotamers. The consequence is that the cysteine sulfur plane is translated toward the N-terminus decreasing the space for solvent. The D-Leu retains the original positioning for the sulfur plane, moving the Leu side chains toward the C-terminus of the scaffold. Effectively, we have built a small house within the 3SCC that can allow a small amount of water in when Cd(II) is present. By using penicillamine, we raise the floor of the house (sulfur layer forming the metal binding site) to displace the water, while D-Leu substitution effectively lowers the ceiling (hydrophobic layer above the metal binding site). Extending this analogy, placing an alanine above the metal binding site moves the ceiling up one level, generating pure 4 coordination.

The power of this strategy is exemplified by the creation of **GRANDL12D-LL16CL26AL30C** (dichromeric 3SCC), a peptide of increased length designed to incorporate at different sites exclusive 3-coordinate and 4-coordinate Cd(II) structures simultaneously (Table 2). This design separated the two metal binding sites by 20 Å and 2 layers of hydrophobic residues so one would expect the two sites to act independently of each other. Analyzing Cd(II) titrations of this dichromeric 3SCC by UV-vis gave the expected stoichiometry of 2:1 Cd/trimer and $[\text{Cd(II)}]_2(\text{GRANDL12D-LL16CL26AL30C})_3^{2-}$. ^{113}Cd NMR experiments showed two resonances with the first peak at 589 ppm and the second at 690 ppm. Thus, a 5% change in primary sequence created a scaffold that bound Cd(II) selectively in two different binding modes. The $\text{Cd(II)S}_3\text{O}$ site bound Cd(II) at least 10 times more tightly than the Cd(II)S_3 site and fully formed at much lower pH. Thus, we determined that secondary sphere changes can influence the primary coordination sphere of a heavy metal, and such coordination sphere changes can have a notable effect on important thermodynamic properties such as binding affinity.

We hypothesized that incorporating D-Leu below the Cys₃ site would lower the floor while keeping the ceiling at the same level. This change could allow for the first 5-coordinate Cd(II) (i.e., Cd(II)S₃O₂) in this scaffold. **TRIL16CL19D-L** with D-L below the Cys₃ layer addresses this question (Table 2). This comparison utilized both TRI and CoilSer (CS) families of peptides.²⁴ CS derivatives of TRI peptides have proven to have comparable properties while CS more easily forms diffraction quality crystals.^{25,26}

Cd NMR experiments of Cd(II)(**TRIL16CL19D-L**)₃[−] exhibited a peak at 600 ppm, which one would assign as exclusively Cd(II)S₃O using previously established models.¹¹ ¹¹¹mCd PAC, however, required two unique sites to fit the data; one with parameters matching that expected for Cd(II)S₃O ($\omega_0 = 0.316 \text{ rad ns}^{-1}$) and a second with $\omega_0 = 0.159 \text{ rad ns}^{-1}$, which we had not previously observed for the TRI system, in a roughly 1:1 mixture. Lower angular frequency is indicative of more symmetric charge distribution with perfectly tetrahedral or octahedral geometries exhibiting ω_0 of 0 rad ns^{-1} ; herefore, we proposed two different geometric possibilities²⁷ (Figure 4). The first was a standard pseudotetrahedral Cd(II)S₃O ($\omega_0 = 0.316 \text{ rad ns}^{-1}$) as previously observed. The second structure ($\omega_0 = 0.159 \text{ rad ns}^{-1}$) was a Cd(II)S₃O₂ coordination in which another exogenous water is bound to the C-terminal side of the bound Cd(II) as well as the N-terminal side. This latter hypothesis is strongly supported by the X-ray structure of the Hg(II)(**TRIL16CL19D-L**)₃[−], which exhibits water molecules above and below the mercury ion.

PRIMARY COORDINATION SPHERE NONCODED AMINO ACIDS

Changing the stereochemistry of the second coordination sphere can have a large effect on the structure of the metal center, but what about perturbing the chirality of an inner sphere ligand? Clearly, substitution of methyl groups on cysteine dramatically altered Cd(II) binding with an L-Pen derivative. What other changes might be observed using D-Pen? By crystallizing apo versions of both L- and D-containing peptides, we sought to understand how the S₃ layer in (**TRIL16Pen**)₃ was reoriented by inverting a stereocenter. This is an important design issue as we have shown that the cysteine sulfur layer is predisposed (sulfurs in a layer, but significant rotamer reorganization required) to bind Hg(II), Cd(II), and Zn(II) or preorganized (sulfurs form a layer that does not require rotamer reorganization) to bind Pb(II) and As(III).²¹ Thus, thermodynamic preferences will be influenced based on whether reorganization energy is necessary to incorporate the ion.

Crystal structures of (CSL16Pen)₃ and (CSL16D-Pen)₃ were solved to 1.86 and 1.71 Å resolution, respectively. Clear differences in the S₃ layer were noted, with the L-Cys diastereopeptide forming a cavity that was predisposed to metal binding, with sulfur atoms oriented toward the N terminus. In contrast the structure of the D-Cys system placed the thiols down toward the C-terminus with the sulfur atoms pointed at the helical interface (Figure 5). Spectroscopic evidence indicated that the binding of Cd(II) differed greatly between these two peptides. ¹¹³Cd NMR results for CSL16Pen matched those observed for **TRIL16Pen** with a peak at 683 ppm while Cd(II)(CSL16D-Pen)₃ exhibited a peak at 557 ppm, an even more upfield shifted resonance than seen for CdS₃O structures (range of δ 570–600 ppm).¹¹ Thus, this Cd(II) is likely either CdS₃O₂ or possibly CdS₂O₂.²⁸ Thus,

changing the stereoisomer of a side chain in the primary coordination sphere can change the coordination environment and the physical positioning of the bound metal.

Given the difference in chemistry between L-Cys and L-Pen with Cd(II), we felt it prudent to evaluate how Cd(II) D-Cys peptides would behave. Previously, we had determined that the side chains of (CSL16C)₃ were predisposed but not preorganized for Cd(II) binding in the hydrophobic interior.²¹ Upon metal binding, Cys side chains reorient, increasing the interlayer distance between sulfur atoms and the hydrophobic layer in the 12th position. This increase in interhelical distance was likely the cause of Cd(II)TRIL16C's mixed coordination state of Cd(II)S₃ and Cd(II)S₃O. Our hypothesis was that using D-Cys, which points toward the C-terminus, would further increase the distance between the 12th and 16th layers allowing for a greater proportion of CdS₃O.

Analysis of the Cd(TRIL16D-Cys)₃⁻ spectral properties led to conflicting conclusions. ¹¹³Cd NMR experiments gave a peak at 646 ppm, which predicts a 60% Cd(II)S₃ and 40% Cd(II)S₃O distribution. In contrast, ^{111m}Cd PAC spectra were fit with a single species of Cd(II)S₃O. We hypothesized that this discrepancy was linked to the sensitivity of ¹¹³Cd NMR peaks to ligand to metal charge transfer (LMCT) changes in the Cys–Cd bond.^{29,30} This bond is affected by the Cys p-orbital and thus one would expect differences between L-Cys and D-Cys, while ^{111m}Cd PAC is unaffected by such differences in LMCT but is affected by Cd–Cys distance. This illustrates that the ^{111m}Cd PAC and ¹¹³Cd NMR structural correlation may have limitations when not strictly applied to L-Cys in an “a” site peptide environment. The final proposed model for Cd(II)(TRIL16D-Cys)₃⁻ was Cd(II)S₃O with an elongated Cd–O bond and shorter Cd–S distances.

Diffraction quality crystals for apo-(CSL16D-C)₃ as well as Zn(II)Cl(CSL16D-C)₃⁻ were compared to apo-(CSL16C)₃ and Zn(II)(GRAND-CSL12AL16C)₃⁻ to evaluate the differences between L-Cys and D-Cys using Zn(II) as a mimic for tetrahedral Cd(II) (Figure 6). Comparison of the apo structures found no significant deviation from α -helicity, but an increased distance between the 12th and 16th layer from 4.92 Å (L-Cys) to 6.10 Å (D-Cys), which we hypothesized allowed the Cd(II)S₃O coordination seen in Cd(II)(CSL16D-C)₃⁻. Comparing apo-(CSL16D-C)₃ to Zn(II)(CSL16D-C)₃⁻, we found little reorganization upon metalation. Zn(II) binds D-Cys with a distorted tetrahedral Zn(II)S₃Cl coordination environment, which we hypothesized would be a reasonable model for the structure of Cd(II)S₃O. That the Zn(II) bound Cl⁻ had 100% occupancy within the crystal structure left no doubt that a smaller exogenous water ligand could occupy the same space. A similar analysis of Zn(II) binding to L-Cys using apo-(CSL16C)₃ and Zn(II)(GRAND-CSL12AL16C)₃ determined that the Cys ligands rotated ~80° to bind Zn(II) while D-Cys ligands rotated ~10–30°. Thus, we had added primary sphere D-Cys mutation to the growing list of methods one could use to control Cd(II) coordination and determined that it could create a site that was preorganized for a tetrahedral metal rather than predisposed as were the L-Cys sites.

APPLYING NONCODED AMINO ACIDS TO *DE NOVO* CATALYTIC SYSTEMS

A second major thrust of our work is to apply lessons of *de novo* metalloprotein design we have learned from heavy metal systems to metalloenzymes utilizing His₃ binding to transition metals. It should be noted that converting the metal binding ligand from cysteine to histidine has significant consequences. These include moving from a soft to an intermediate Lewis base, a large increase in size going from a sulfur atom to an imidazole ring, a decrease in negative charge, and a significant change in orientational bonding between a spherical thiolate ligand and two different azole nitrogens. Nonetheless, we were able to show that incorporating His₃ sites into TRI systems and binding either Zn(II) or Cu(I) allowed for the recapitulation of carbonic anhydrase or Cu nitrite reductase (CuNiR) activities.^{19,31} X-ray structures of the Zn(II) containing peptides demonstrated that the TRI scaffolds were able to accommodate a tetrahedral metal by increasing the interhelical separations of the 3SCCs.¹⁹ Moreover, X-ray absorption spectroscopy of the copper peptides revealed that metal complexes ranging from 2- to 5-coordinate with as many as 3 bound histidines could be isolated. Reviews of our work on *de novo* carbonic anhydrases and, recently, nitrite reductases have been published, so this Account will focus largely on our application of noncoded amino acids with CuNiR.^{32,33}

Our first CuNiR mimic within a 3SCC system was created by substituting Zn(II) with Cu(I/II) within the **TRIW-H** peptide used to recapitulate carbonic anhydrase activity (Figure 7).³¹ X-ray absorption spectroscopy (XAS) indicated that the Cu(I) coordination environment was likely His₃ based on the Cu(I)–N/O distance of 1.93 Å, the presence of long distance scatterers expected with His ligation, and the intensity of the 1s → 4p pre-edge transition, which inversely correlates with Cu(I) complex coordination number.³⁴ Electronic absorption and EPR spectroscopies were initially used to estimate the Cu(II) coordination geometry as His₃(H₂O)₂, but more recently, we have revisited this question using XAS, and the average calculated Cu(II)–N/O distance of 1.94 Å as well as analysis of the multiscattering intensity leaves His₃(H₂O) as the most likely model.

Cu(I) and Cu(II) *K_D* values at pH 5.9 were determined to be 3.1 pM and 40 nM, respectively. The reduction potential of the Cu peptide at pH 5.9 was calculated using the Nernst equation to be +400 mV, a value more reminiscent of type 1, rather than type 2, copper proteins. An activity assay monitored the oxidation of ascorbate as NO₂ was converted to NO. A pH dependent activity, with a maximum value for the first order rate constant of $4.6 \times 10^{-4} \text{ s}^{-1}$, was found. While this value is 7 orders of magnitude below native CuNiR, this model was capable of 1000s of turnovers without degradation and provided a starting point for investigating CuNiR activity within a *de novo* scaffold.

We hypothesized that one could apply secondary coordination sphere mutations around the Cu(I)His₃ site of **TRW-H** to affect substrate access and coordination geometry similar to our above work with Cd(II)(Cys₃) sites.¹⁴ The resting state of this catalyst is Cu(I)(**TRIW-H**)₃⁺ due to an excess of the reducing agent ascorbate, which was confirmed as Cu(I)His₃ by X-ray absorption spectroscopy (XAS). Native CuNiR systems, however, have a CuHis₃(H₂O) coordination environment in both the cuprous and cupric forms.^{35,36} We created a series of **TRIW-H** variants with differing steric bulk above or below the active site to investigate

whether steric controls seen with heavy metal binding constructs could be used to enforce a Cu(I)His₃(H₂O) coordination state and what effects this would have on catalysis. We will focus on two constructs, **TRIW-HL19A** and **TRIW-HL19D-L**, which are direct analogues of changes made to control the metal coordination number of Cd(II) in the cysteine containing peptides (Table 3).

We determined by XAS that opening space above the His₃ site with **TRIW-HL19A** decreased Cu(I)'s coordination number to Cu(I)His₂ based on the calculated Cu–N/O distance of 1.86 Å and the relative intensity of the 1s → 4p absorption band³⁴ (Figure 8). Packing of Leu above the Cu(I)His₃ precluded the His from rotating to create a linear 2-coordinate center. Upon decrease of that steric hindrance, Cu(I) adopted its preferred linear geometry. Cu(I)(**TRIW-HL19D-L**)₃⁺ was, instead, practically indistinguishable from Cu(I)(**TRIW-H**)₃⁺ in both Cu–N/O distance at 1.92 Å and 1s → 4p absorption band intensity and was assigned as a Cu(I)His₃ geometry. Thus, we found that secondary sphere changes allowed for tuning the coordination and reactivity of a transition metal His₃ site.

Surprisingly, while Cu(I)(**TRIW-HL19A**)₃⁺ had moved further away from our goal of mimicking the coordination environment of CuNiR, its first-order rate constant was 75 times that of Cu(I)(**TRIW-H**)₃⁺ or Cu(I)(**TRIW-HL19D-L**)₃⁺. Other secondary sphere mutations, either above or below the His₃ layer, revealed that decreasing the steric bulk above the CuHis₃ decreased the coordination number to 2, while the same modification below the CuHis₃ site left the coordination number unchanged. Surprisingly, both mutation types increased rates equivalently. This increase in first-order kinetics was correlated with a 1 eV shift to higher energy of the 1s → 4p absorption bands (8982.8 ± 0.2 eV seen in Cu(I)(**TRIW-H**)₃⁺ vs 8983.6 ± 0.2 eV for Cu(I)(**TRIW-HL19A**)₃⁺). This energy shift could be related to the net charge on the bound Cu(I) that could then affect the energy of the Cu(I)His₃NO₂[−] transition state. This could explain how systems with both 2- and 3-coordinate Cu(I) have the same increase in first order rate constant. Alternatively, the rate limitation could occur from the Cu(II) state.

We next applied noncoded amino acids to the primary coordination sphere of our CuNiR constructs, employing methylated histidine residues to enforce His binding by either the δ- or ε-nitrogen (using N_ε or N_δ methylated histidine, respectively) (Figure 9). Previous metastudies of crystal structures deposited into the PDB had postulated that δ- or ε-nitrogen bound copper correlated with differing functions in nature, with δ-N being found in more electron transfer centers and ε-N in metalloenzymes.^{37,38} This rationale was further supported by the later discovery of the CuH center of peptidylglycine α-hydroxylating monooxygenase (PHM), an electron transfer center with the same His₃ metal binding site as CuNiR but binding Cu with the δ-nitrogens rather than ε-nitrogens.³⁹ Based on these factors, we hypothesized the CuNiR efficiency would be higher using N_ε coordination than N_δ within our *de novo* constructs. We designed **TRIW-_{δm}H** and **TRIW-_{εm}H** and compared these to the original **TRIW-H** to investigate this effect (Table 3).

EXAFS, XANES, and EPR analysis indicated that Cu(II) coordination to (**TRIW-H**)₃ was similar to Cu(II) coordination to (**TRIW-_{δm}H**)₃ with EXAFS best fit to 4-coordinate Cu(II)His₃(H₂O). Cu(II)(**TRIW-_{εm}H**)₃ on the other hand was more tetrahedral in its

geometry based on intensity of the $1s \rightarrow 3d$ transition and compressed hyperfine coupling constant, while EXAFS was best fit to 4-coordinate Cu(II)-His₂(H₂O)₂. The reasoning for this difference, based on modeling the site within the crystal structure of Hg(II)₅[Zn-(II)_N(H₂O)](CSL9CL23H)₃⁺ (PDB 3PBJ), is likely that ligation to N_δ within a 3SCC does not enable all three His to ligate simultaneously and a Cu(II)His₂(H₂O)₂ coordination was instead preferred similar to Cd(II) binding to (CSL16D-Pen)₃. Investigations of Cu(I) coordination gave similar results with EXAFS and XANES analyses of Cu(I)(**TRIW- δ mH**)₃ matching Cu(I)(**TRIW-H**)₃ with a 3-coordinate Cu(I)His₃ geometry while Cu(I)(**TRIW- ϵ mH**)₃ was bound as a 2-coordinate Cu(I)His₂.

Dissociation constants were determined for Cu(I) and Cu(II) for (**TRIW- δ mH**)₃ and (**TRIW- ϵ mH**)₃ and compared to literature values for (**TRIW-H**)₃. While Cu(I) K_D 's were largely unaffected by methylation of δ - or ϵ -nitrogen (all three constructs ~2–6 pM), the Cu(II) K_D of (**TRIW- δ mH**)₃ was 2 orders of magnitude looser binding (3.3 μ M) than either (**TRIW- ϵ mH**)₃ or (**TRIW-H**)₃ (0.04 μ M). This difference led to a 130 mV increase in reduction potential for Cu-(**TRIW- δ mH**)₃²⁺ (530 mV) versus Cu(**TRIW- ϵ mH**)₃²⁺ or Cu(**TRIW-H**)₃²⁺ (400 mV). We hypothesize that this shift in reduction potential is caused by the inductive effect of the methyl group in comparison to hydrogen. Interestingly, we have shown that changing charge of residues at the helical interface of Cu(**TRIW-H**)₃²⁺ also caused a 100 mV shift in potential; however, this change was primarily due to changes in Cu(I) K_D . This reduction potential shift came with a minimal perturbation to CuNiR activity. We hypothesized that these reduction potential changes were due to a hydrogen bond between the outer sphere amino acids and the His₃ site. The comparison of methylated histidine containing constructs cannot be influenced by such a hydrogen bond, so other factors must be at play.

Looking at CuNiR activity, Cu(I)(**TRIW- δ mH**)₃⁺ had a 2 orders of magnitude higher first-order rate constant than Cu(I)(**TRIW- ϵ mH**)₃⁺. This is a significant difference between N_ε or N_δ coordination (Table 4) and may be due to the inductive effect of the methyl group. A similar effect was hypothesized for catalytic differences between models of CuNiR using pyrazole versus imidazole.^{40,41} However, we caution that the differing Cu(I) and Cu(II) coordination environments between these two constructs and the shift in reduction potential complicates such analysis. Interestingly, the shift of 100 mV in potential that occurs due to modification of interhelical residues involved in H-bonding with the imidazole that influences Cu(I) affinity has only a 4-fold change in rates, whereas alkylation of the histidine causing the same 100 mV shift, but this time lessening Cu(II) affinity, results in a 100-fold increase in activity. This result may point to the sensitivity of the kinetics on Cu(II) rather than Cu(I). Cu(I)-(**TRIW- δ mH**)₃⁺ also has 300 times higher CuNiR activity based on first order rate constant than the original Cu(I)-(**TRIW-H**)₃⁺, with a new record for a homogeneous aqueous system at 0.12 s⁻¹. We argue that this is the first quantitative evidence for the catalytic differences between N_ε or N_δ Cu coordination within a protein environment. It is true that the differing coordination spheres of these constructs can complicate such a direct comparison, but given our previous results showing several 2- and 3-coordinate Cu(I) constructs with indistinguishable activity, we do not believe the change in

coordination between Cu(**TRIW- δ_{NH} H**)₃ and Cu(**TRIW- ϵ_{NH} H**)₃ is the primary origin of differences in NiR activity between these two constructs.¹⁴

In conclusion, we have shown that noncoded amino acids can be used to influence dramatically coordination number and the physical properties of designed metalloproteins. These modifications may be achieved through installation of residues with altered chirality, modified steric bulk, or changes in the electronic nature of a coordinating ligand. With Cd(II), coordination number could be controlled across the series CdS₃, CdS₃(H₂O), and CdS₃(H₂O)₂. With copper, coordination numbers from 2 to 5 could be observed. More important, through the use of noncoded amino acids, we not only created a new benchmark for homogeneous model CuNiR systems in aqueous solution but provided the first quantitative evidence for the catalytic differences between N_e or N₈ Cu coordination within a protein environment. Thus, we conclude that exploitation of a broader toolset of noncoded amino acids promises even greater structural and reactivity control in metalloprotein design.

Biographies

Karl J. Koebke obtained his Ph.D. from the University of Wisconsin—Milwaukee working with Prof. Andy Pacheco exploring the kinetics of nitric oxide dioxygenation in heme proteins. He then spent three years working as a postdoctoral researcher in the lab of Vincent Pecoraro where he designed a plethora of *de novo* constructs including nitrite reductase and cupredoxin mimics. He is currently working at Wayne State University with Prof. Timothy Stemmler on mitochondrial FeS cluster biogenesis.

Vincent L. Pecoraro is the John T. Groves Collegiate Professor of Chemistry at the University of Michigan, Ann Arbor. He has studied bioinorganic chemistry for his entire career with emphasis on manganese and vanadium systems. In addition to discovering metallocrowns, he has been a pioneer in metalloprotein design. He served as an Associate Editor of *Inorganic Chemistry* for over 20 years and is a Fellow of the ACS.

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Figure 1. PyMOL models of (left) $\text{Cd(II)(TRIL16Pen)}_3^-$, (middle) $\text{Cd(II)(H}_2\text{O)(TRIL12AL16C)}_3^-$, and (right) $\text{Cd(II)(H}_2\text{O)(TRIL12HfLL16C)}_3^-$ using the crystal structures of apo- $(\text{CSL16Pen})_3$ [pdb 3h5f] and $\text{Zn(II)(H}_2\text{O)(GRANDCSL12AL16C)}_3^-$ [pdb 5kb2] based on chemical knowledge. Cd(II) ions and water are represented as white and red spheres, respectively. Peptides are depicted with the N-terminus on top and C-terminus on bottom.

Figure 2.
Structure of (left) penicillamine and (right) hexafluoroleucine.

Figure 3. Schematic representation of the L- to D-Leu mutation at position 12. Reorientation of the amino acid side chain serves to increase the steric bulk in the plane above the Cys ligands. Reproduced with permission from ref 10. Copyright 2008 National Academy of Sciences, U.S.A.

Figure 4. PyMOL models of hypothesized Cd(II) coordination environments: (left) $\text{CdS}_3(\text{H}_2\text{O})$ in a tetrahedral geometry and (right) $\text{CdS}_3(\text{H}_2\text{O})_2$ in a trigonal bipyramidal geometry using the crystal structure of $\text{Hg}(\text{II})(\text{TRIL16CL19D-L})_3^-$ based on chemical knowledge. Models created using $\text{Zn}(\text{II})(\text{H}_2\text{O})_6^{2+}$ - (**GRANDCSL12AL16C**) $_3^-$ [pdb 5kb2]. Cd(II) ions are represented as white spheres, while water is represented as red spheres. Peptides are depicted with the N-terminus on top and C-terminus on bottom.

Figure 5. PyMOL representations of (left) (CSL16Pen)₃ [pdb 3h5f] and (right) (CSL16D-Pen)₃ [pdb 3h5g]. Top-down view is done looking down from the N-terminus.

Figure 6.

Preorganization of D-Cys versus the predisposition of L-Cys upon binding to Zn(II) in the 3SCCs. (top panels) Top down view from the N termini representing the 16 L-Cys side chain orientations in (a) [apo-(CSL16L-C)₃] [PDB ID 5K92] and (b) Zn(II)(H₂O)-(GRANDCSL12AL16L-C)₃⁻ [PDB ID 5KB2] and (c) an overlay between structures from panels a and b. (bottom panels) (d) Apo-(CSL16D-C)₃, (e) Zn(II)Cl(CSL16D-C)₃⁻, and (f) an overlay between structures from panels d and e. Metal binding ligands are represented as sticks (sulfur atoms in yellow). Zn(II) ions in Zn(II)Cl(CSL16D-C)₃⁻ and Zn(II)(H₂O)(GRANDCSL12AL16L-C)₃⁻ are given in gray and green, respectively. The coordinated water molecule is red, and the chloride ligand is orange. Adapted with permission from ref 12. Copyright 2017 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Figure 7.

(A) Representation of the model for Cu(TRIH)₃ based on the structure of Hg(II)_S[Zn(II)_N(H₂O)](CSL9CL23H)₃⁺ [PDB 3PBJ]. (B) View of the Zn(II)(H₂O)His₃²⁺ site along the pseudo-3-fold axis of Hg(II)_S[Zn(II)_N(H₂O)](CSL9CL23H)₃⁺ (Cyan), superimposed on the type 2 Cu(II)H₂O(His)₃ site in *R. sphaeroides* NiR (PDB 2DY2, backbone, green; side chain, pink). Coordinated water molecules are represented as spheres. (C) Side view of the same superposition in panel B. Peptides are depicted as either a side view with the N-terminus on top and C-terminus on bottom or top-down view looking down from the N-terminus. Adapted with permission from ref 31. Copyright 2012 National Academy of Sciences, U.S.A.

Figure 8.
XANES of Cu(I) bound constructs of **TRIW-H** and secondary coordination sphere variants.

Figure 9.
Structure of (left) N ϵ methylated His and (right) N δ methylated His.

Table 1.

TRI Family Peptide Sequences Discussed in this Section^a

peptide	1	2	9	16	23	30	37
TRI	Ac-G	LKALEEK	LKALEEK	LKALEEK	LKALEEK	G-NH ₂	
TRIL16C	Ac-G	LKALEEK	LKALEEK	CKALEEK	LKALEEK	G-NH ₂	
TRIL12AL16C	Ac-G	LKALEEK	LKAAEEK	CKALEEK	LKALEEK	G-NH ₂	
TRIL16Pen	Ac-G	LKALEEK	LKALEEK	PenKAAEEK	LKALEEK	G-NH ₂	
TRIL12HHLL16C	Ac-G	LKALEEK	LKAHLEEK	CKALEEK	LKALEEK	G-NH ₂	

^aThe first residue within each heptad is labeled in the column heading with its overall position within the peptide.

Table 2.

TRI Family Peptide Sequences Used to Study Secondary Coordination Sphere Effects of Noncoded Amino Acids Discussed in this Section^a

peptide	1	2	9	16	23	30	37
TRI	Ac-G	LKALEEK	LKALEEK	LKALEEK	LKALEEK	G-NH ₂	
TRIL16C	Ac-G	LKALEEK	LKALEEK	CKALEEK	LKALEEK	G-NH ₂	
TRIL12D-LL16C	Ac-G	LKALEEK	LKADLEEK	CKALEEK	LKALEEK	G-NH ₂	
GRANDL12D-LL16CL26AL30C	Ac-G	LKALEEK	LKADLEEK	CKALEEK	LKAAEEK	CKALEEK	G-NH ₂
TRIL16CL19D-L	Ac-G	LKALEEK	LKALEEK	CKAD-LEEK	LKALEEK	G-NH ₂	
CoilSer (CS)	Ac-E	WEALEKK	LAALESK	LQALEKK	LEALEHG	-NH ₂	
GRAND	Ac-G	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	G-NH ₂

^aThe first residue within each heptad is labeled in the column head with its overall position within the peptide. Nomenclature for **CS** and **GRAND** follow the same numbering pattern as for **TRI**.

Table 3.

TRI-H Family Peptide Sequences for CuNiR Constructs^a

peptide	1	2	9	16	23	30
TRIW-H	Ac-G	WKALEEK	LKALEEK	LKALEEK	HKALEEK	G-NH ₂
TRIW-HL19D-L	Ac-G	WKALEEK	LKALEEK	LKAD-LEEK	HKALEEK	G-NH ₂
TRIW-HL19A	Ac-G	WKALEEK	LKALEEK	LKAAEEK	HKALEEK	G-NH ₂
TRIW-Δ₉H	Ac-G	WKALEEK	LKALEEK	LKALEEK	Δ ₉ HKALEEK	G-NH ₂
TRIW-Δ₉H	Ac-G	WKALEEK	LKALEEK	LKALEEK	Δ ₉ HKALEEK	G-NH ₂

^aThe first residue within each heptad is labeled in the column head with its overall position within the peptide.

Table 4.

Comparison of Cu(I) Coordination and Nitrite Reductase Rate for TRIW-H CuNiR Models

peptide	Cu(I) coordination	rate (s ⁻¹)
TRIW-H	Cu(I)(His) ₃	4.6×10^{-4}
TRIW-HL19A	Cu(I)(His) ₂	3.5×10^{-2}
TRIW-HL19D-L	Cu(I)(His) ₃	9.7×10^{-4}
TRIW-_{an}H	Cu(I)(His) ₂	1.2×10^{-3}
TRIW-_{an}H	Cu(I)(His) ₃	0.12