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Comparison of Cysteine and Penicillamine Ligands in a Co(II) Maquette

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L-Penicillamine (Pen) has been investigated as a ligand for metalloprotein design by examining the binding of Co(II) to the sequence NH_2–KL(Pen)EGG•(Pen)**IG**(Pen)G**A**(Pen)•GGW–$CONH_2$. For comparison, we have studied Co(II) binding to the analogous sequence with Cys ligands, the ferredoxin maquette ligand **IGA** that was originally designed to bind a [4Fe-4S] cluster. The Co(II) affinity and UV–vis spectroscopic properties of **IGA** indicate formation of a pseudotetrahedral tetrathiolate ligated Co(II). In contrast, **IGA-Pen** showed formation of a pseudotetrahedral complex with Co(II) bound by three Pen ligands and an exogenous H_2O. EXAFS data on both Co(II) complexes confirms not only the proposed primary coordination spheres but also shows six Co(II)-C_β methyl group distances in Co(II)-**IGA-Pen**. These results demonstrate that ligand sterics in simple peptides can be designed to provide asymmetric coordination spheres such as those commonly observed in natural metalloproteins.

Metalloprotein design is an active field of bioinorganic chemistry aimed at delineating the structure−function relationships of natural metalloproteins.[1] De novo metalloprotein design implemented using either rational[2] or combinatorial[3] methodologies provides a constructive approach to the design of novel metalloproteins. These studies have provided detailed insight into the role of transition metal ions in protein folding,[4] oligomerization[5] and stability[6] as well as revealing the engineering specifications of their design[7] and the factors that modulate metalloprotein chemical properties and reactivities.[8]

We are utilizing peptide-based coordination complexes, protein maquettes,[9] as aqueous soluble and stable synthetic analogues of natural metalloproteins to explore the chemical consequences of using noncoded amino acid ligands.[10] By studying natural to non-natural ligand amino acid substitutions in otherwise invariant peptide sequences, we are expanding the repertoire of ligands available for metalloprotein design. Our approach is to use solid-phase peptide synthesis methods coupled with de novo design to evaluate potential ligands prior to their incorporation into natural metalloprotein scaffolds via expressed protein ligation[11] or sophisticated molecular biological methods.[12]

In this contribution, we introduce the cysteine analogue L-penicillamine (Pen, Figure 1) as a noncoded amino acid ligand for metalloprotein design. L-Penicillamine, whose enantiomer is a systemic treatment for copper overload in Wilson's disease,[13] was chosen for its similar basicity (pK_a of 7.9 vs 8.3 for Cys)[14] and yet greater steric bulk at the C_β in comparison to cysteine. Pen was incorporated at the cysteine positions of the **IGA** ferredoxin maquette sequence to evaluate the effect on Co(II) metal ion affinity and spectroscopy.[15] The designed primary structure of each ligand of the penicillamine containing peptide ligand (**IGA-Pen**),

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(15) The peptide ligands were synthesized using standard Fmoc/^{t}Bu solid-phase peptide synthesis methodologies. For **IGA-Pen**, double coupling was employed for the Pen amino acids followed by resin capping.

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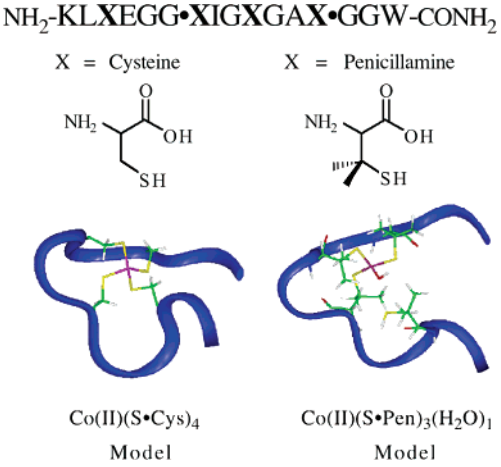

Figure 1. Primary structure of the maquette, chemical structures of cysteine (Cys) and penicillamine (Pen) and molecular models of their corresponding Co(II) maquette complexes.

NH$_2$−KL**(Pen)**EGG·**(Pen)**IG**(Pen)**GA**(Pen)**·GGW−*CONH*$_2$, is that of the **IGA** ferredoxin maquette which binds a $[4Fe\text{-}4S]^{2+}$ cluster.[16] The data demonstrate that **IGA-Pen** binds Co(II) with high affinity using only three of the four potential Pen ligands whereas the **IGA** maquette binds Co(II) avidly via four cysteine thiolates. The Co(II) affinities of **IGA** and **IGA-Pen** are compared with **IAA**, recently shown to bind Co(II) with a K_d of 5 μM at pH 6.5,[17] to determine the effect of the Ala9Gly sequence modification and the use of Pen ligands on Co(II) affinity, respectively. Furthermore, the spectroscopic effects of Pen ligation are evaluated in comparison to the Cys analogue to assess the role of sterics in modulating the metal coordination environment.

Figure 2A shows that the **IGA** ligand containing four Cys residues binds 1 equiv of Co(II) as evidenced by UV−vis spectroscopy. Titration of Co(II) into **IGA** (75 μM) at pH 7.5 (20 mM HEPES, 100 mM KCl) results in increasing absorbance at 304 nm [$\epsilon = 3800$ M^{-1} cm^{-1}] with a shoulder at 340 nm [$\epsilon = 3200$ M^{-1} cm^{-1}] consistent with S → Co(II) charge-transfer bands.[18] The extinction coefficient of the highest energy CT transition suggests four Cys thiolate ligands (ϵ = 950 M^{-1} cm^{-1} per Co−S bond).[19] The appearance of ligand field bands at 630 nm [$\epsilon = 400$ M^{-1} cm^{-1}], 686 nm [$\epsilon = 570$ M^{-1} cm^{-1}], and 728 nm [$\epsilon = 540$ M^{-1} cm^{-1}] consistent with $^4A_2 \rightarrow {}^4T_1(P)$ transitions demonstrates that the Co(II) is in a tetrahedral coordination environment.[19] Indeed, the UV−vis spectrum of Co(II)-**IGA** is comparable to Co(II)-substituted rubredoxin[20] and other designed tetrathiolate proteins.[21−23] Figure 2B shows a sharp break in the titration curve due to the tight formation of a 1:1 metal/ligand complex between the **IGA** and Co(II) at pH 7.5 whose dissociation constant, $K_d^{Co(II)}$ value, is tighter than 500 nM.

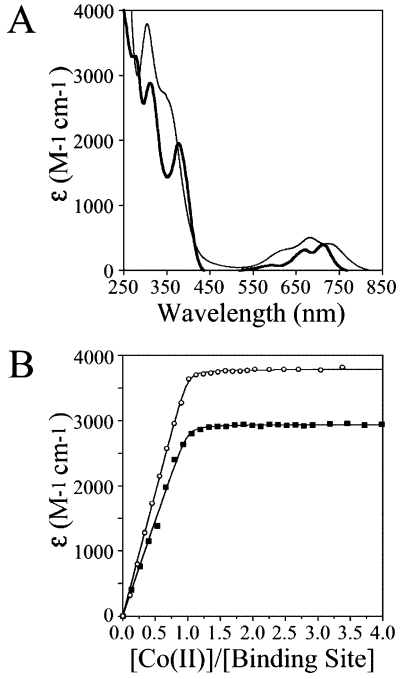

Figure 2. (A) UV−vis spectra of the Co(II) complexes of **IGA** and **IGA-Pen** (bold) (B). Evaluation of the metal−ligand stoichiometry of the Co(II)-**IGA** (○) and Co(II)-**IGA-Pen** (■) complexes. All experiments were performed at 75 μM protein concentration in 20 mM HEPES, 100 mM KCl at pH 7.5. A binding site is defined in **IGA** as four S-donors and three in **IGA-Pen**.

Figure 2A also shows the UV−vis spectrum of Co(II)-complex of **IGA-Pen** (λ_{max} [ϵ] values at 311 nm [2970 $M^{-1}cm^{-1}$], 377 nm [1950 $M^{-1}cm^{-1}$], 588 nm [90 $M^{-1}cm^{-1}$], 668 nm [330 $M^{-1}cm^{-1}$], 713 nm [420 $M^{-1}cm^{-1}$]) which is markedly different from that of Co(II)-**IGA**. In particular, the high energy absorption bands at 311 and 377 nm are red-shifted and less intense in comparison to those of the Co(II)-**IGA** complex. The 2970 M^{-1} cm^{-1} molar extinction coefficient value of the 311 nm band suggests three (990 M^{-1} cm^{-1} per Co−S bond) rather than four (742 M^{-1} cm^{-1} per Co−S bond) sulfur ligands in the primary coordination sphere.[19] These data are further supported by the titration curve shown in Figure 2B, which demonstrates formation of a tight 1:1 complex in Co(II)-**IGA-Pen** in which a Co(II) binding site is defined at three Pen ligands. Despite the change in primary coordination sphere, sedimentation equilibrium analytical ultracentrifugation demonstrates that Co(II)-**IGA-Pen** is monomeric in solution (Figure S2). The strength and blue-shift of the ligand field bands indicate a stronger tetrahedral ligand field which is consistent with substitution of a thiolate ligand for a neutral H_2O ligand.[21] Despite the loss of one sulfur donor, the $K_d^{Co(II)}$ value for Co(II)-**IGA-Pen** at pH 7.5 is tighter than 500 nM, a value too tight to accurately measure.

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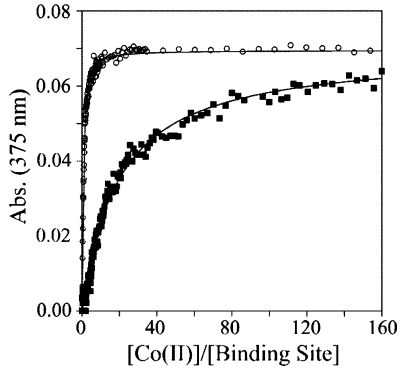

Figure 3. Determination of the equilibrium dissociation constants of the Co(II)-**IGA** (○) and Co(II)-**IGA-Pen** (■) complexes at pH 6.6. The absorbance change at 375 nm was fit to a 1:1 metal to binding site model. Binding sites are defined as in Figure 2. Both experiments were conducted at 5 μM peptide in 20 mM HEPES, 100 mM KCl at pH 6.6.

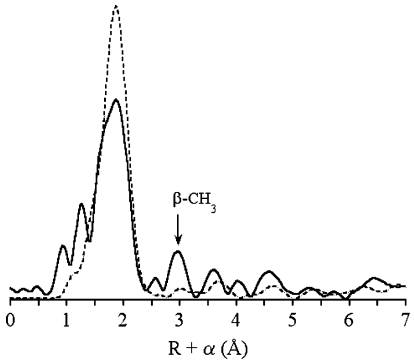

Figure 4. Fourier transformed EXAFS of Co(II)-**IGA** (- - -) and Co(II)-**IGA-Pen** (—).

Accurate Co(II) dissociation constants were obtained for Co(II)-**IGA** and Co(II)-**IGA-Pen** at pH 6.6 where proton competition for metal ion binding is more pronounced. Figure 3 shows the anaerobic titration of Co(II) into 5 μM peptide ligand at pH 6.6 as followed by UV−vis spectroscopy in a 10 cm path length quartz cuvette. The Co(II)-**IGA** titration curve (○) was fit to a 1:1 metal/peptide binding model with a $K_d^{Co(II)}$ value of 2 μM at pH 6.6. This value is slightly tighter than the 5 μM value measured at pH 6.5 for **IAA** suggesting that the Ala9Gly sequence change results in a minimal change in Co(II) affinity. In contrast, the binding curve of Co(II)-**IGA-Pen** (■) shows significantly weaker Co(II) affinity than observed for Co(II)-**IGA**, consistent with loss of one S ligand. Fitting to a 1:1 binding model in which each binding site is defined as three penicillamine ligands resulted in a $K_d^{Co(II)}$ value of 98 μM at pH 6.6, some 49-fold weaker (2.2 kcal/mol) than Co(II)-**IGA**. The expected $[H^+]^3$ dependence of the Co(II)-**IGA-Pen** $K_d^{Co(II)}$ value suggests this value is 78 nM at pH 7.5.

Further evidence for the proposed primary coordination spheres of Co(II)-**IGA** and Co(II)-**IGA-Pen** is provided by their EXAFS spectra at the Co K-edge as shown in Figure 4. The FT for Co(II)-**IGA** (dashed line) shows a single peak at $R + \alpha$ of 1.9 Å, corresponding to 4 S donors at 2.31 Å (Table S1, Figure S4). In contrast, the FT for Co(II)-**IGA-Pen** shows a first shell scattering peak that is substantially lower in magnitude with its center of gravity shifted, owing to the appearance of a shoulder to low *R*. These data are best modeled with 3 S donors at 2.27 Å and one N/O donor at 2.13 Å. Interestingly, the substitution in the Pen side-chains is also apparent in the EXAFS. A new peak, absent in the FT for Co(II)-**IGA**, appears in the FT for Co(II)-**IGA-Pen** (indicated by the arrow). Single scattering fits are consistent with a shell of 6 C at 3.45 Å. This distance is too short to be the β-carbons of Pen, and smaller coordination numbers led to unreasonably small Debye−Waller factors, supporting its assignment as scattering from the β-CH_3 carbons consistent with the model in Figure 1.

These simple Co(II)-peptide complexes serve as water soluble synthetic analogues of Co(II)-substituted Zn(II) proteins. The pseudotetrahedral $(S{\cdot}Cys)_4$ coordination motif observed in Co(II)-**IGA** is a model for both structural $(S{\cdot}Cys)_4$ coordinated Zn(II) sites, e.g., DNA polymerase III[24a] and alcohol dehydrogenase,[24b] as well as for the reactive Zn(II) site in the DNA repair enzyme Ada.[24c] The $(S{\cdot}Cys)_3(H_2O)_1$ site in Co(II)-**IGA-Pen** mimics the structural site in TRAIL[25a] and the catalytic Zn(II) site observed in 5-aminolevulinate dehydratase.[25b]

In conclusion, we have shown the feasibility of using the sterics of a non-natural amino acid ligand to produce an asymmetric primary coordination sphere at a mononuclear metal ion. Our efforts are now focused on detailed spectroscopic and electronic structure studies of these Co(II) complexes, evaluating the reactivity of the corresponding Zn(II) maquettes and introducing further asymmetry into the metal primary coordination sphere.

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Supporting Information Available: Mass spectrum of **IGA-Pen**, derivation of K_d fitting equation, sedimentation equilibrium analytical ultracentrifugation data, and fits to Fourier transformed EXAFS data for Co(II)-**IGA** and Co(II)-**IGA-Pen**. This material is available free of charge via the Internet at http://pubs.acs.org.

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