CBI Student Sabbatical Proposal

13 September 2010

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Oxford University

10 weeks

March/April 2011

During my sabbatical, I will perform electrochemical studies on copper bound in a His_3 coordination environment in peptides of the TRI family. In particular, I will use protein film voltammetry to determine Cu(I)/Cu(II) redox potentials of the sites in the presence and absence of CO, NO, and NO_2^-. Design modifications will be made to the peptides and the corresponding effect on the redox potential will be observed and all values compared to those of similar natural systems as reported in the literature.

***De novo* design of $CuN_3(X)$ sites in metallopeptides**

Background:

De novo designed metallopeptides offer simplified constructs that retain sufficient complexity to resemble a natural protein environment. These can be utilized to study structure-function relationships in detail by systematically correlating changes in the three dimensional structure with chemical properties of the metallopeptide. While a number of structural motifs have been targeted, the most extensively studied are α-helical bundles or coiled coils.[1] Our research group has focused its attention on the **TRI** family [(Ac-$G(L_aK_bA_cL_dE_eE_fK_g)_4G$-$NH_2$)] of peptides, the design of which is based on the heptad repeat approach.[2,3] This sequence results in the formation of amphipathic α-helices, which aggregate in aqueous solution to form parallel, left-handed coiled coils with hydrophobic interiors. Metal-binding sites can be designed or engineered into the hydrophobic interior of these structures by substituting the leucine (Leu) residues with amino acids capable of metal-binding.[3]

The substitution of internal residues with cysteine (Cys) leads to coiled coils capable of selectively binding soft, heavy metals (Hg(II), Pb(II), Cd(II)), and our research group is able to, by proper engineering of the **TRI** sequence, control the geometric and spectroscopic properties and stability of the metalloproteins.[4] We have recently synthesized nitrogen sites, using histidine (His) residues, that can bind harder metals like Zn(II) and Cu(I)/Cu(II) which prefer a nitrogen-rich environment. The importance of having achieved the synthesis of His_3 metallopeptides resides in the fact that they represent outstanding models of enzymes like the zinc metalloenzyme carbonic anhydrase (CA), Cu nitrite reductase (CuNiR), and peptidylglycine α-hydroxylating monooxygenase (PHM).

CuNiR uses two copper sites to catalyze the one electron reduction of nitrite (NO_2^-) to nitric oxide (NO) during bacterial denitrification. The $Cu(His)_3$ site (type 2) binds NO_2^- and catalyzes its conversion to NO. A separate type 1 copper, ligated by two His residues, Cys, and methionine (Met), participates in electron transfer (ET) to the active type 2 center.[5] While many studies have been performed (crystallographic, spectroscopic, and theoretical) in attempts to understand the mechanism of NO_2^- reduction, a number of questions remain, especially regarding the proposed copper-nitrosyl intermediate.[6,7,8,9,10,11] Because the Cu(I)-NO binding mode was initially inferred from synthetic modeling studies to be end-on N-bound, it was very surprising when NO was found bound side-on in crystal structures of CuNIR. DFT calculations have demonstrated the importance of a nearby isoleucine (Ile) residue in stabilizing the side-on form[11], and this in turn demonstrates the need for a precise knowledge of the structural features at the $Cu(I)His_3$ site in the **TRI** metallopeptides for proper tuning of

the binding of NO and other diatomic molecules. Additionally, the mechanism of reduction of NO_2^- is not completely understood, as it is not clear whether NO_2^- binding to the type 2 Cu center is required for subsequent ET from type 1 Cu or if it is reduced prior to substrate binding.[5]

PHM, like CuNIR, also contains two copper sites. It is a eukaryotic enzyme which catalyzes the hydroxylation of the α-C atom of glycine-extended propeptides as a first step in the amidation of peptide hormones.[12] The active site consists of Cu bound to two His residues and Met, while in the ET site Cu is ligated by three His residues.[13,14] It is interesting that CuN_3 acts directly as the catalytic center in one enzyme yet in another is believed to participate only in electron storage and transfer. Modeling a CuN_3 site in our designed metallopeptide system will allow us to tune the sterics around the metal site and may give important information regarding binding of Cu(I)/Cu(II), its interaction with NO, NO_2^-, and CO, and its redox potential.

Preliminary Work

The Cu(I) **TRI**L23H metallopeptide can be prepared by reaction between the three-stranded coiled coil and Cu(I) acetonitrile. A pH titration of **TRI**L23H monitored by ^{1}H NMR demonstrates deprotonation of the His residues (imidazolium to imidazole) by pH 7.2. ^{1}H NMR at pH 8.0 displays evidence of Cu(I) and Cu(I)-CO binding. More excitingly, IR spectroscopy demonstrates CO binding with a stretching frequency of 2063 cm^{-1}. This value is very similar to those reported for both natural metalloenzymes and synthetic model complexes (a value of 2064 cm^{-1} has been reported for amine oxidase and 2050 cm^{-1} for CuNIR).[15] The complex can undergo Cu oxidation in the presence of dioxygen, so it must be handled under strictly anaerobic conditions. Visible absorption studies on the Cu(II)-peptide showed that the absorption maximum is at 655 nm, with an extinction coefficient of 131 M^{-1} cm^{-1}. These two values are in agreement with those for a metal-centered d-d transition on a Cu(II)-$(His)_3$ center in a distorted tetrahedral environment, where the fourth position is occupied by a solvent molecule. The binding of Cu(II) at the His site is also confirmed by broadening then disappearance of the imidazole CH signals in the ^{1}H NMR spectrum for addition of Cu(II) up to one equivalent. These results have proven that these Cu(I)/Cu(II)-$(His)_3$ metallopeptides potentially represent interesting models of the active site of CuNIR and in general of CuN_3-type sites in metalloproteins.

While definitive structural information has yet to be obtained on these Cu metallopeptides, crystallographic data on an analog peptide (**CoilSer**L9PenL23H where penicillamine is the methyl analog of cysteine) with Hg(II) bound to the sulfur site and Zn(II) bound to the His site indicates Zn(II) binding to all three His residues with water/hydroxide in the fourth position. Overlaying the ZnN_3O site with the active site in CuNIR gives an excellent structural match. Crystal growth conditions are currently being optimized for the Cu(II)-bound

peptide and XAS studies are also to be performed in order to gain more information on Cu(I)/Cu(II) coordination in our His site metallopeptides.

Proposed Studies

Copper Binding

Cu(I) is a spectroscopically silent metal, however, binding substrates such as CO, NO, and NO_2^- to the Cu(I) center may allow it to be probed using different spectroscopic techniques (such as IR or EPR for NO binding). One of the major aims of my Ph.D. project is to probe such NO and other small molecule binding in [Cu(I)](**TRI**L23H)$_3$. Studying the binding of NO to a Cu(I)N_3-type site is of special interest as relatively (to iron) little is known about Cu-NO chemistry and there are very few mononuclear Cu(I)N_3-NO model complexes in the literature. Also, understanding how NO_2^- binds to Cu and how binding affects the Cu(I)/Cu(II) redox potential in our metallopeptide models may contribute to the understanding of the mechanism of nitrite reduction in CuNIR. This is also particularly interesting because there is a debate as to whether the type 2 Cu center is first reduced, then binds nitrite or if the nitrite binds to an oxidized type 2 center, followed by electron transfer from the type 1 center.[16,17,18] Therefore, another major portion of my Ph.D. project will be to gain further understanding of the nature of the Cu sites through electrochemical measurements to determine Cu(I)/Cu(II) redox potentials. This is also of potential applicability in understanding the similarities and/or differences of the active site in CuNIR and/or the redox-only site in PHM.

I will begin my Cu studies by determining whether and how both NO_2^- and NO can bind to the His site in **TRI**L23H. As the binding of CO has proven to be similar to that found in natural metalloenzymes, we may expect NO and NO_2^- to also bind similarly. NO and NO_2^- binding may be probed using FTIR vibrational and Resonance Raman spectroscopy and the values obtained may be compared with those found in the literature for both CuNIR and for synthetic model complexes. The paramagnetic Cu(I)-NO adduct may also be characterized by electron paramagnetic resonance (EPR) spectroscopy and the data compared to reported *g* values and hyperfine couplings for both CuNIR and synthetic Cu(I)-NO complexes.

After I have shown that NO_2^- and/or NO can bind to Cu(I) in the His site of **TRI**L23H, my work will involve perturbing the Leu packing layers around the metal-binding His site in order to influence the binding modes of these small molecules and CO. I will prepare **TRI**L19AL23H, where the Leu residues in the 19 position are substituted for less sterically bulky Ala residues to create a hole above the His site (towards the N-terminus, where the solvent site is hypothesized to be, based on the crystal structure of Zn(II) bound to His_3). It might be expected that this would allow CO to bind in a more linear fashion, and can be assessed by observing if the

stretching frequency is lower (as a result of stronger metal-to-CO backbonding) than that of Cu(I)-CO in **TRI**L23H. Depending on what is seen for CO binding, this might alter the binding mode of NO, for example, from side-on to end-on based on steric crowding. An alternative amino acid to Ala is valine (**TRI**L19VL23H), which may retain the hole above the metal site (albeit smaller) yet incorporate some packing interactions in order to better stabilize the coiled coil structure. An opposite approach would be to substitute Ile (**TRI**L19IL23H), which should pack at least as well as Leu, yet will block any opening (hole) above the metal site. Here, we might observe a change from linear or bent to side-on Cu(I)-NO. Yet another option for controlling the steric effects, based on previous work in the group, is to substitute $_{L}$-Leu (or $_{L}$-Ile) in the 19 position with $_{D}$-Leu (or $_{D}$-Ile). The change in stereochemistry of this amino acid should cause an increase in the steric bulk around the metal site by pointing the Leu (or Ile) side chain down towards the site and may, therefore, enforce side-on NO binding (or possibly fully inhibit diatomic binding to the copper).

Copper electrochemistry

As an integral part of gaining an understanding of Cu and small molecule-Cu binding in these His site peptides, I will determine the Cu(I)/Cu(II) redox potentials of the sites described above. There are a couple of ways to approach measuring the redox potential of such a CuN_3-type site. The approach typically used for CuN_3-type sites is to perform redox titrations in solution in the presence of redox mediators, characterizing the optically-invisible CuN_3 features by EPR.[19,20] However, a more attractive technique I would like to utilize for measuring redox potentials is protein film voltammetry (PFV). There are several benefits to immobilizing the protein on an electrode, as compared to conventional voltammetry in which the molecules are free in solution, one of which is that direct control over the redox centers of all molecules of the protein can be achieved through the electrode potential, whereas conventional solution experiments must address freely diffusing molecules.[21,22] Additionally, PFV uses several orders of magnitude less sample than that required to measure an EPR spectrum (the electrode is coated with less than a picomole of protein whereas EPR experiments require about 10^3 to 10^4 times more material). PFV is recognized as a powerful method for probing redox-active sites in metalloproteins and will be of great utility in understanding the Cu(I)/Cu(II) redox potentials in our designed metallopeptide systems and, should we observe catalytic turnover in the presence of NO_2^-, can provide a means of probing this chemistry.

In particular, I will perform electrochemical studies to determine the redox potentials of Cu in the His site in the presence and absence of CO, NO, and NO_2^-, and compare my values to those available in the literature for similar CuN_3 sites (for example, the type 2 Cu in CuNIR, at pH 8.4, has a potential of 137 mV).[19,23] For the electrochemical studies, cyclic voltammetry will likely be utilized and the peptide immobilized on an electrode

(such as a rotating disk pyrolytic graphite edge working electrode), potentially with coadsorbates to stabilize the film if needed. In addition to all of the above-mentioned steric modifications that will be studied, another peptide design modification I will make is to substitute the glutamate residues around the metal site to glutamine. This will change the local charge around the metal site pocket and may, as a result, impact the Cu(I)/Cu(II) redox potential and possibly even the binding of CO, NO, or NO_2^- to the metal.

All of these studies combined, in addition to providing information regarding Cu(I)-NO binding, may be applicable to both clarifying the reaction mechanism of CuNIR and contribute to the general understanding of Cu-NO chemistry. If it is possible to demonstrate side-on Cu(I)-NO binding by controlling the sterics in our synthetic metallopeptides, this will support a CuNIR catalytic mechanism in which this complex can be an intermediate. Understanding how NO_2^- binding affects the Cu(I)/Cu(II) redox potential may provide insight into the mechanism of reduction in CuNIR. In addition, understanding how diatomic molecules can bind to and how to control the redox potential of the copper site in our designed metallopeptides may address the question of why a $Cu(I)N_3$-type site in one protein should act as a catalytic center yet in another as solely the electron storage/transfer cofactor.

References

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September 15, 2010

Professor Vincent L. Pecoraro
Director, Michigan Chemistry Biology Interface Training Program
University of Michigan
Ann Arbor, MI

Dear Me,

I am writing to you to provide my extremely strong support for the student sabbatical proposal of Ms. Melissa Zastrow. Melissa is at the start of her third year in the chemistry graduate program, presently working in my laboratory on the design of metallopeptides. Melissa selected my research group because she was interested in combining synthetic skills associated with de novo protein design, learning spectroscopic and enzymatic approaches for protein characterization and ultimately extrapolating what she has learned to relevant biological systems.

Melissa is interested specifically in designing metalloproteins. The idea is quite straightforward, preparing a single polypeptide chain that is capable of binding two different metals in structurally distinct sites in order to form a structural stabilizing site at one end of a three stranded coiled coil and a reactive site at the other end. In practice, achieving these goals is more difficult and requires a student of great aptitude. Melissa is now working with a construct, first designed by a postdoc and worked on by Melissa during her rotation project, that binds Hg(II) into a structural site containing sulfur ligands and Zn(II) with three histidines in an environment resembling the catalytic site of carbonic anhydrase. Melissa is finishing refinement of a mixed Hg(II)/Zn(II) protein at two different pH conditions. The structures demonstrate that the three histidine, water site has a remarkable similarity to the carbonic anhydrase active site. She has examined the reactivity of the Zn(II) site. Her studies using nitrophenylacetate as a substrate have been very encouraging showing a kcat/KM. that is within10,000 of the fastest carbonic anhydrase and which is 108 faster than anyprevious Zn model system. Therefore, her system is the best functional and structural mimic ever to be prepared for a zinc hydrolase. I suspect that we will soon write this paper for submission to Nature.

Melissa is also working on the Cu(I) analogue of this system as a model for nitrite reductase. A visiting professor in the group demonstrated that Cu(I) binds to the TRIL23H peptide, binds CO and, under the proper conditions can be oxidized to the Cu(II) form. More important, this Cu(I) protein reacts with nitrite to generate NO, just as does nitrite reductase. An important issue in characterizing this redox active system is determining the redox potentials under various conditions of pH, substrate concentration and protein modification. Unfortunately, direct redox potential measurements are prone to serious problems; however, an approach such as protein thin film voltammetry is ideal for extracting the desired information. The leading world expert on the application of this technique to metalloproteins is Fraser Armstrong at Oxford University. In addition to his outstanding expertise in this area, Fraser has
published the seminal papers on how electron transfer moderates the rate of nitrite reductases. For these reasons, Melissa has proposed to move to England for 10 weeks so that she can work with Fraser to explore the electrochemical behavior of our systems. I believe this is an excellent example of the student sabbatical as Melissa will go to one of the leading laboratories in the world to learn a technique that our group knows little about. The results will be an important part of her thesis. Hence, my strong support for this proposal.

Sincerely yours,

Vincent L. Pecoraro
John T. Groves Collegiate Professor of Chemistry

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September 13, 2010

Ms Melissa Zastrow
Graduate Student in Dr. Pecoraro's Group
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Re: Visit to Department of Chemistry, Oxford University.

Dear Ms Zastrow

This letter confirms my willingness and enthusiasm to have you join my research group for 8-10 weeks in Spring/Early Summer 2010, to carry out electrochemical experiments on the copper metallopeptide complexes that you have been studying as part of your PhD research. As you know, your supervisor Vince Pecoraro initiated the plan in discussions with me earlier this year, and it looks to be an excellent opportunity for collaborating on an important topic. Your proposal fits in well with the expertise that my group has in investigating complex redox chemistry of metalloenzymes including the Cu-containing nitrite reductase that is directly related to your research. I will arrange accommodation for you during the period of your stay, which will probably be at my own college, St Johns.

Kind regards,