METALLOPROTEIN DESIGN

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Design and construction of metal-binding sites in proteins is an attractive approach toward the elucidation of the structure and function relationships in metalloenzymes. Metal centers in proteins are important for both stability and enzymatic activity, however, the principles required to engineer and control the properties of metal-binding sites are not yet fully understood. The design or re-design of metal-binding sites in proteins is in some respects similar to *de novo* design of proteins. The goal of both approaches is to engineer a protein with new or enhanced properties. Whereas *de novo* design involves creation of both the protein and the binding site for the metal co-factor, our approach focuses on just the metal site by building on a pre-existing protein scaffold. This is in fact similar to the approach used in nature in which a limited number of thermodynamically stable protein folds are used but diversity in function is still obtained by altering the protein active site.

The protein scaffolds we have chosen to build upon are yeast cytochrome *c* peroxidase (C*c*P) and and sperm whale myoblobin (swMb).[1,2] C*c*P is a 34kDa heme protein that utilizes oxidizing equivalents derived from hydrogen peroxide to oxidize two molecules of ferrous cytochrome *c*.

$$\text{C}c\text{P-Fe(III)} + H_2O_2 \rightarrow \text{C}c\text{P-Fe(IV)=O, Trp191}^{+\cdot} + H_2O$$
$$\text{C}c\text{P-Fe(IV)=O, Trp191}^{+\cdot} + 2\text{Cyt}c\text{-Fe(II)} \rightarrow \text{C}c\text{P-Fe(III)} + 2\text{Cyt}c\text{-Fe(III)}$$

Mb is a small, 17 kDa molecular weight, protein that reversibly binds dioxygen. These proteins are ideal templates for protein model studies because both have recombinant expression systems that have been optimized and have been extensively characterized by various spectroscopic techniques.[2,3] Furthermore, X-ray crystal structures have been solved for both the native and several mutant forms of the proteins. C*c*P and swMb have been used as templates for the purpose of engineering metal binding sites characteristic of cytochrome P450 (cyt P450) and cytochrome *c* oxidase (C*c*O), respectively.

The cyt P450 class of enzymes are known to perform a variety of reactions including hydroxylations, epoxidations, dealkylations, and sulfoxidations.[4] Thiolate ligation from an axial cysteine residue has been shown to be essential for the interesting spectroscopic properties and activity of this enzyme.[5] For instance, replacement of the axial histidine residue in myoglobin with cysteine resulted in an enzyme with similar spectroscopic properties to high-spin ferric cyt P450.[6] In previous work, a proximal His175Cys mutant in C*c*P was constructed and demonstrated that the sulfur of cysteine did not ligate to the heme.[7] The authors concluded that the cysteine was too far from the heme to bind and X-ray crystal structure refinement of this mutant showed that the cysteine was instead oxidized to cysteic acid. Clearly, other structural elements may be important in facilitating ligation of a proximal cysteine in this protein. Crystallographic studies of cyt P450 show that a conserved non-polar phenylalanine residue and the protein backbone form an enclosure around the proximal cysteine ligand.[8] In C*c*P the amino acid at the analogous position to this non-polar amino acid is Asp235 (see figure 1). Therefore, the mutation of Asp235 to Leu, which is similar in shape

to Asp yet non-polar, has been made to stabilize cysteine ligation in the C*c*P(H175C) variant. UV-vis,

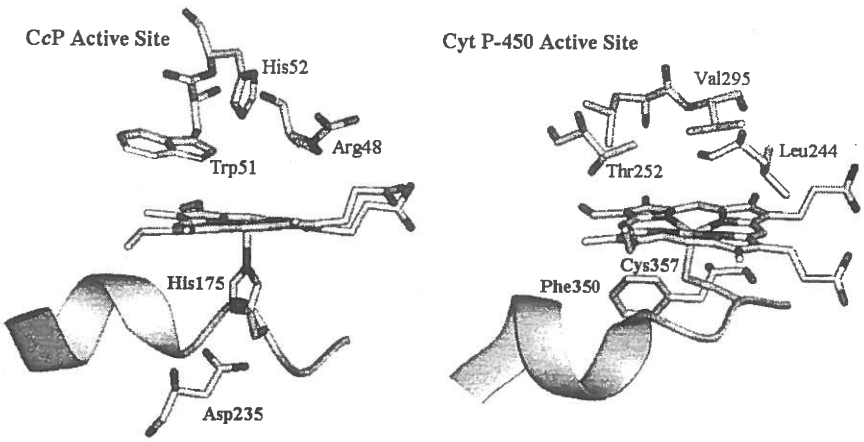

Figure 1

MCD, and EPR characterization of this mutant will be presented and provide evidence for similar heme environment to cyt P450 in both high-spin and low-spin imidazole and cyanide derivatives and confirms that cysteine is the axial ligand in this mutant of C*c*P. This work will demonstrate the utility in using protein models to study secondary coordination effects at the metal-binding sites in protein.

Finally, in an effort to make a protein model of Cu_B-heme center of respiratory oxidase, a Cu(II)-binding site has been designed above the heme center in swMb. Cu_B-heme centers are at the heart of the superfamily of terminal respiratory oxidases that include both cytochrome *c* oxidases (C*c*O) and quinol oxidases.[9-12] They catalyze the reduction of O_2 to H_2O, which is coupled to the generation of a proton gradient through the mitochondrial or cytoplasmic membrane. X-ray crystal structures of the bovine and *Paracoccus denitrificans* cytochrome *c* oxidases revealed the structure of the Cu_B-heme center as shown in Figure 2.

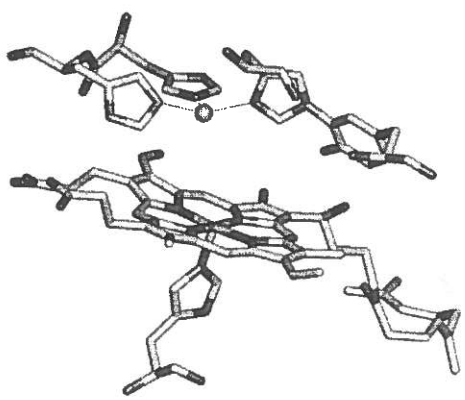

Figure 2

The center contains a heme with a proximal histidine ligand and a single copper atom coordinated by three histidines about 5 angstroms above the heme iron. A copper-binding site has been designed in sperm whale myoglobin (swMb) based on structural comparison and molecular modeling of Mb and C*c*O. UV-vis studies of the resting state of the designed protein swMb((L29H, F43H) (called Cu_BMb) show that a single copper-binding site is created in swMb. UV-vis, elemental analysis, and EPR studies of the cyanide-bound Cu_BMb indicate that a spin-coupled, CN^--bridged Cu_B-heme center is formed in the designed model protein, as in the native HCOs. Parallel spectroscopic studies with Zn(II) in the place of Cu(II) further support the conclusion. The study also reveals that the presence of Cu(II) and Ag(I) (as a Cu(I) mimic) increased the affinity of heme for diatomic ligands such as CN^- and O_2. New oxygen activity of the protein will also be demonstrated that is dependent upon the designed metal-binding site. This study shows that it is possible to design and engineer metal-binding sites in proteins with little sequence and structural homology.

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