Engineering a Purple Cu_A Site into the Blue Copper Protein Azurin: Construction, Spectroscopy and Metal Substitution Studies

Michael T. Hay Final Seminar June 5, 1998

The study of metal containing proteins, or metalloproteins, makes up a large portion of research in the field of bioinorganic chemistry.[1] Copper binding sites are some of the most common metal binding sites in biology.[2] Traditional copper binding sites, the blue copper (type 1), normal copper (type 2), and coupled binuclear copper (type 3) sites, are categorized based upon their geometry about the metal center and their unique spectroscopic properties.[3] Unlike the three established types of copper centers, a fourth type, the purple Cu_A site (Figure 1), displays an intense purple color with strong electronic absorption around 480 and 530 nm,[4] a characteristic resonance Raman Cu-S stretching frequency around 340 cm^{-1},[5] and a seven-line hyperfine splitting pattern in the EPR spectra.[6] The Cu_A binding site is found in cytochrome c oxidase (COX),[7] the terminal oxidase in the respiratory chain, and in nitrous oxide reductase (N_2OR),[8] an enzyme responsible for the reduction of N_2O in denitrifying bacteria.

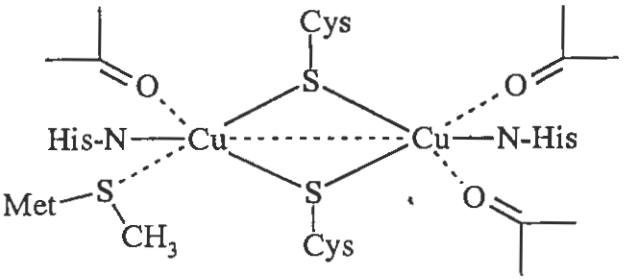

Figure 1. The general structure for a Cu_A center

A similarity in the tertiary structure between proteins containing a type 1 center and proteins containing a Cu_A center, the Greek key ß barrel fold, is noted.[9] Based upon this structural similarity, a Cu_A center was engineered into the framework of a blue copper protein, azurin from *Pseudomonas aeruginosa*, using loop directed mutagenesis (Figure 2).[10]

The electronic absorption (UV-vis), magnetic circular dichroism (MCD), multifrequency electron paramagnetic resonance (EPR), and resonance Raman spectra of holo-azurin-Cu_A are strikingly similar to other native or engineered Cu_A centers, indicating they all share similar geometric and electronic structures. Holo-azurin-Cu_A has the characteristic UV-vis absorption spectrum of a Cu_A center with absorption bands at 485 (ε = 3730), 530 (ε = 3370), 360 (ε = 550) and 770 nm (ε = 1640 $M^{-1} cm^{-1}$). The MCD spectrum of purple Cu_A azurin is dominated by a pair of intense, oppositely-signed features occurring at 480 nm ($\Delta\varepsilon$ = -118 $degM^{-1} cm^{-1} T^{-1}$) and 530 nm ($\Delta\varepsilon$ = 155 $degM^{-1} cm^{-1} T^{-1}$) and a negative feature occurring at 810 nm ($\Delta\varepsilon$ = -52 $degM^{-1} cm^{-1} T^{-1}$) (Figure 4). Multifrequency EPR spectra show a well-resolved seven-line hyperfine structure in the $g_{||}$ region, typical of a delocalized mixed-valence [Cu(1.5)···Cu(1.5)] binuclear center, and the Raman spectrum shows vibrational bands at 257- and 336-cm^{-1} consistent with the existence of a $Cu_2(\mu\text{-}S(Cys))_2$ core. Crystallographic characterization (1.5 Å resolution) of holo-azurin-Cu_A also confirms that the engineered metal

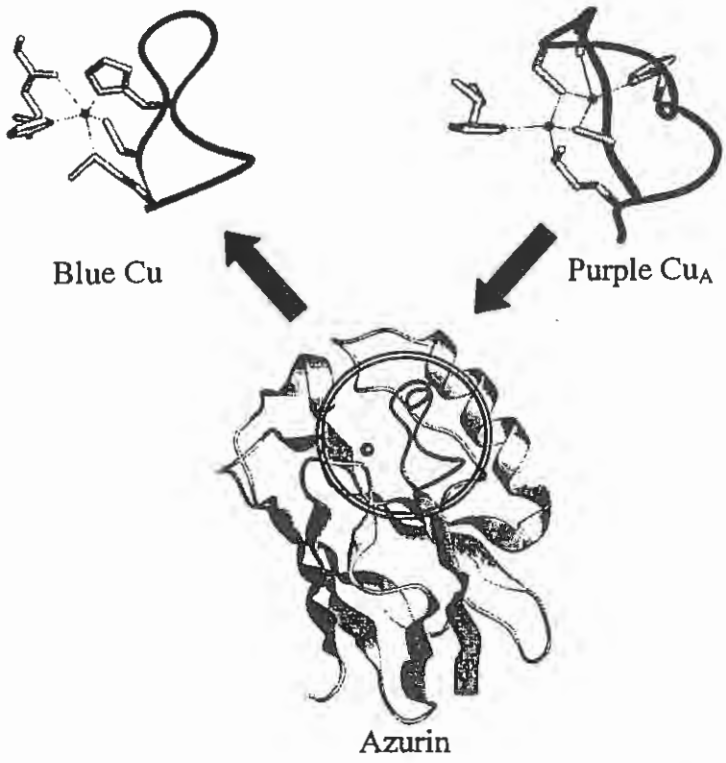

Figure 2. Structure of the blue copper azurin from *P. aeruginosa*. The loop containing the ligands to the blue copper center (highlighted in the circle) has been replaced by a ligand loop sequence similar to that of the purple Cu_A center in COX from *P. denitrificans*.

binding site in azurin is similar to that of the Cu_A site in the native system (Figure 3). Two independent structures are obtained in a single unit cell of the crystal structure. One contains a Cu-Cu distance of 2.41 Å and the other a distance of 2.32 Å. Compared with other delocalized mixed-valence Cu_A centers, azurin Cu_A has a relatively high energy near-IR Cu-Cu $\sigma \rightarrow \sigma^*$ absorption at 770 nm, the largest $A_{\|}$ at 55G, and the shortest Cu-Cu distance at 2.32 Å. These results may reflect a more sterically compressed Cu_A center in azurin.

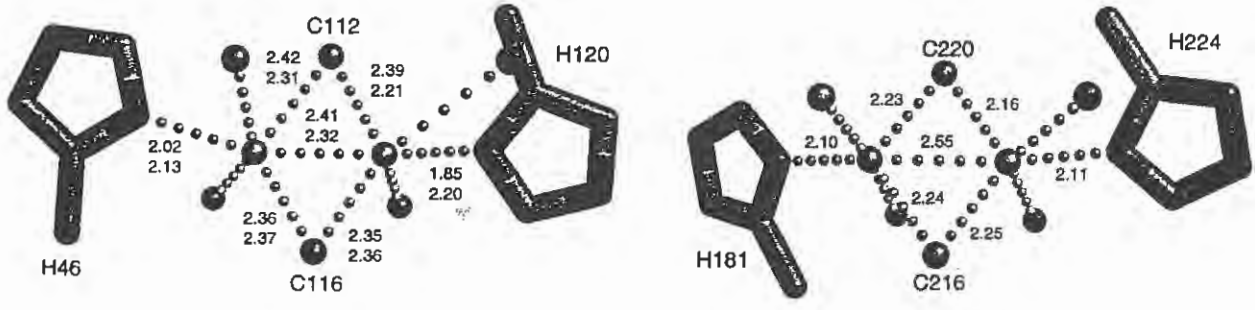

Figure 3. Schematic diagrams comparing the geometric parameters of the Cu_A site from azurin-Cu_A (left) and *P. denitrificans* COX (right).

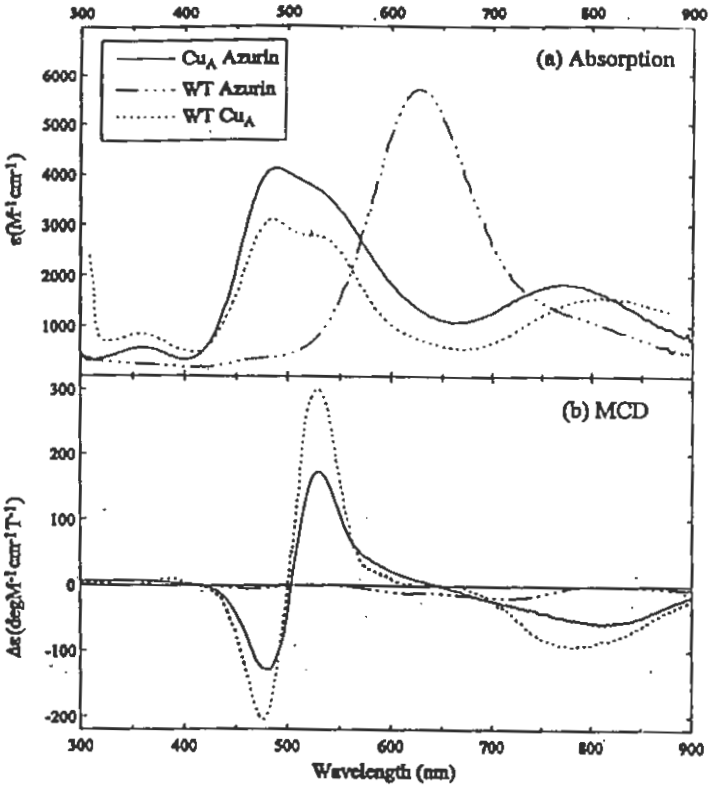

Figure 4. Room-temperature UV-vis absorption (a) and 4.2 K MCD (b) spectra

Metal substitution studies of azurin-Cu_A have been performed using various metal ions (i.e., Hg^{2+}, Ag^{+}, Cu^{+}, Cd^{+}, Rh^{2+}, Au^{+}, Co^{2+}, Ni^{2+}). Metal binding was determined by electronic absorption (UV-vis) and electrospray mass spectrometry (ES-MS). The results obtained from theses studies indicate that there are two factors that may influence binding in azurin-Cu_A: (1) the site tends to bind metal combinations which produce an overall +3 charge, and (2) the site binds soft thiophilic metals. Further characterization of the above derivatives will contribute to the understanding of the metal-binding affinity, rigidity, and other structural properties of purple copper centers.

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