The Role of Histidine Ligands in the Structure of Purple Cu_A Azurin

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The structure of purple Cu_A centers,[1] found in cytochrome *c* oxidase and nitrous oxide reductase, has now been firmly established as a binuclear, mixed-valence Cu_2S_2(Cys) complex through extensive biochemical,[2−7] biophysical,[8−11] and X-ray crystallographic studies.[12−15] The two copper ions, each coordinated to a histidine, are bridged by the thiolate sulfurs of two cysteine ligands (Figure 1). Weak axial ligands, such as the thioether sulfur of methionine and backbone carbonyl oxygens, are also present. These structural elements are known to be responsible both for the unique spectroscopic features, such as strong charge-transfer absorption bands around 485 and 530 nm and a high-energy mixed-valance $\sigma \rightarrow \sigma^*$ transition around 800 nm, and for its functional properties, such as high reduction potential, low reorganization energy, and efficient electron-transfer rate. Comprehensive spectroscopic characterization[16−24] and molecular orbital calculations[24] have identified three structural elements, a weak axial ligand interaction with the copper ions, Cu−Cu core compression, and the equatorial position of the histidine nitrogen, to be critical for modulating the structure and function of the Cu_A center. A comparison of different Cu_A X-ray structures allowed us to identify that the angular position of the His rings with respect to the Cu_2S_2(Cys) core can influence the axial ligand interaction with the copper ions.[15] Since both the axial ligands and the angular position of the His ring are important for modulating the Cu−Cu distance, the three structural elements critical in modulating the structure and function of the Cu_A center can be traced to the angular position of His rings. Therefore, site-specific changes of histidine ligands and study of the consequences of these changes on structure and function of the Cu_A center are of great interest.

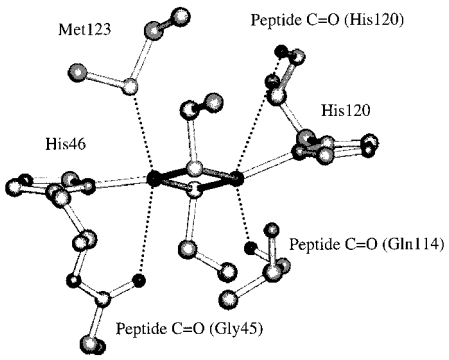

Figure 1. The active site structure of Cu_A azurin.[15]

The His ligands in Cu_A have been changed to Asn in the Cu_A-binding domain isolated from *P. denitrificans* cytochrome *c* oxidase[11] and the restored Cu_A domain of quinol oxidase.[17,25] In both cases, spectroscopic characterization of the mutant proteins strongly suggests that the His-to-Asn mutation transformed the mixed-valence [Cu(1.5)···Cu(1.5)] Cu_A center into a valence-trapped [Cu(II)Cu(I)] center. In this paper we report the first examples of a mixed-valence [Cu(1.5)···Cu(1.5)] center where one of the histidine ligands in an engineered Cu_A azurin has been replaced by Asn, Asp, or Ala. Electronic absorption (UV−vis) and electron paramagnetic resonance (EPR) studies demonstrate that one of the histidine ligands can be substituted with all three amino acids with little destructive effects on the binuclear core of the protein. The results presented here suggest that in the engineered azurin construct His120 is not critical for maintaining the binuclear core. However, His120 is important in modulating the Cu−Cu distance as all three mutants display low-energy $\sigma \rightarrow \sigma^*$ transitions.

The purple Cu_A azurin mutants were constructed and purified as described previously.[7,23] The genes coding the mutant proteins were confirmed by DNA sequencing. The identity of the mutant proteins was verified by electrospray mass spectrometry. Changing His46, a ligand buried deeply in the structural core of the protein, to Asn resulted in a protein that remained colorless when Cu(II) was added, indicating the Cu_A center in the parent purple azurin is disrupted by the H46N mutation. However, when His120, a ligand in the ligand loop, is changed to Asn, Asp, or Ala, the intense purple color appears after Cu(II) addition. The electronic absorption spectra of the three mutants (Figure 2A) display strong absorption bands around 485 and 530 nm, and weaker absorption bands around 360 and 810 nm. This spectral pattern is quite

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Table 1. UV−Vis and EPR Properties of H120N, H120D, and H120A Cu_A Azurins and Other Cu_A Centers

protein	source	abs (nm)	g_z	A_z	g_y	g_x	ref
Cu_A in N_2OR[a]	*P. stutzeri*	350, 480, 540, 780	2.18	31	2.03	2.03	17
Cu_A from *caa*$_3$ oxidase[b]	*B. subtilis*	365, 480, 530, 790	2.18	38.2	2.03−1.99	2.03−1.99	4
Cu_A from *ba*$_3$ oxidase[b]	*T. thermophilus*	360, 480, 530, 790	2.19	30	2.00	2.00	5
Cu_A from *aa*$_3$ oxidase[b]	*P. denitrificans*	354, 485, 530, 808	2.19	31	2.03	2.03	17
Cu_A in azurin[c]	*P. aeruginosa*	360, 485, 530, 770	2.17	55	2.04	2.01	23
H120N Cu_A azurin[c]	*P. aeruginosa*	360, 485, 530, 810	2.18	45	2.01	2.00	this work
H120D Cu_A azurin[c]	*P. aeruginosa*	360, 485, 530, 810	2.18	45	2.03	2.01	this work
H120A Cu_A azurin[c]	*P. aeruginosa*	360, 485, 530, 810	2.18	45	2.01	2.00	this work
Cu_A in amicyanin[c]	*T. versutus*	360, 483, 532, 790	2.18	32.4	2.02−1.99	2.02−1.99	6
Cu_A in CyoA[c]	*E. coli*	358, 475, 536, 765	2.20	53, 68	2.02	2.00	17

[a] Native Cu_A protein. N_2OR: nitrous oxide reductase. [b] Soluble Cu_A domain. [c] Engineered Cu_A protein.

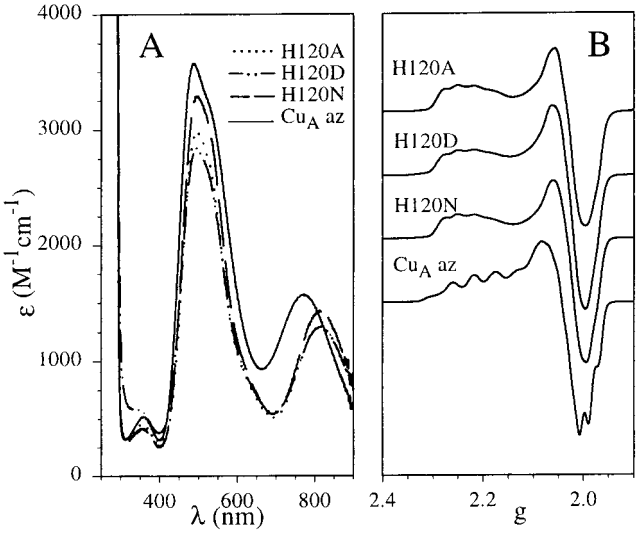

Figure 2. UV−vis (A) and X-Band EPR (B) spectra of the engineered H120N, H120D, and H120A mutant Cu_A azurin. The spectra of the parent purple Cu_A azurin[7,23] are also shown for comparison. The proteins are in 50 mM ammonium acetate buffer, pH 5.1. The UV−vis spectra were taken at ambient temperature, while EPR spectra were collected at 10 K with 2 mW microwave power and 12.5 G modulation amplitude.

similar to that of the parent purple Cu_A azurin,[7,23] and those of other native[3−5] and engineered Cu_A centers[2,6] studied to date (Table 1). The same conclusion can be drawn from the X-band EPR spectra of the engineered H120N, H120D, and H120A mutants shown in Figure 2B and summarized in Table 1.[26] The major difference between the mutant proteins and the parent protein is that the mutant proteins display ∼10 G smaller A_z. These results indicate that removal of His120 in the engineered purple Cu_A azurin has no disruptive effect on the binuclear construction of the system.[27]

The similarity of the UV−vis and EPR spectra between the parent and H120 mutant purple Cu_A azurins and those of native Cu_A sites suggests similar geometric and electronic structures, as well as similar bonding characteristics. The differences, primarily in the specific energies and intensities of the observed transitions, therefore, provide a useful link between geometric and electronic perturbations within the valence-delocalized Cu_2S_2(Cys) unit. In particular, the electronic transition responsible for the near-IR absorption maximum at 810 nm in the three mutant purple Cu_A azurins can be assigned, on the basis of previous spectroscopic studies of Cu_A centers, as involving a one-electron promotion between symmetric and antisymmetric dimer orbitals having significant Cu−Cu $\sigma \rightarrow \sigma^*$ character.[24,28] The high-energy nature of this absorption in the parent purple Cu_A azurin has been shown to correlate to the contraction of the $Cu_2(SR)_2$ core.[23,24] Figure 2A shows that this transition shifts to lower energy by ∼40 nm in the three H120 mutants, indicating a change in Cu−Cu distance. Therefore, we conclude that the Cu−Cu distance can be modulated by changing the His ligand.

The most interesting result of this study is that H120A also results in a purple Cu_A center that is essentially identical with that of H120N and H120D. To investigate whether the H120A mutation created an open binding site for exogenous ligands, as in blue copper His-to-Gly mutant azurins,[29−31] up to 1000 equiv of external ligands, such as chloride, azide, and imidazole, were added to 0.1 mM of H120A purple Cu_A azurin. No spectral shifts were observed (data not shown), suggesting that the site is not accessible to these ligands either through steric blocking or through coordination to copper ion by an internal amino acid.[32] However, after addition of more than 500 equiv of the above-mentioned exogenous ligands, the spectrum of H120A disappears at a rate slightly faster than the parent Cu_A azurin, suggesting that the mutant protein is not as stable.

In conclusion, our results strongly suggest that His120 is not critical for maintaining the binuclear structural core in the engineered Cu_A azurin and that it is possible to modulate the Cu−Cu bond distances in mixed-valence binuclear copper proteins by changing the equatorial histidine ligand on the ligand loop.

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Supporting Information Available: One figure showing the potential residues (Glu114 and Asn119) that might ligate to Cu in the three mutant Cu_A azurins (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(26) EPR simulation suggests that a blue copper signal also contributes to the EPR spectra of all three mutant proteins.

(27) The observed molecular weights of the holo forms of the H120N, H120D, and H120A mutant proteins (based on the electrospray mass spectrometry) correspond to the molecular weights of respective apo-protein plus two copper ions, indicating that each mutant protein binds two copper ions, just like the parent purple azurin.

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(32) Other explanations of this observation include that (a) the purple Cu_A spectrum is not perturbed by binding of these exchangeable ligands and (b) the binding affinity of these ligands to the proteins is weak so that an excess amount of ligands destablizes the protein before binding to the site. However, the nearly identical UV−vis and EPR spectral patterns of all three mutants argue strongly for the same internal protein group replacing His120. The likely candidates are Glu114 and Asn119 that are in close proximity to His120 (see Supporting Information). Further investigations are under way to probe the role of these residues in the formation of the Cu_A centers in the mutant proteins described in this communication.