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Axial Methionine Has Much Less Influence on Reduction Potentials in a Cu$_A$ Center than in a Blue Copper Center

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Cupredoxins are an important family of copper-containing redox-active proteins with electron transfer (ET) function.[1] They include mononuclear type 1 blue copper proteins and structurally related dinuclear purple Cu$_A$ proteins (Figure 1). Common to both proteins is the presence of a highly conserved methionine at the axial position. Spectroscopic studies and molecular orbital calculations have shown that axial ligand interaction is important for modulation of the electronic structure and control of ET reduction potentials and reorganization energy of both centers. The role of the axial Met in blue copper centers has been the subject of numerous studies.[2] In particular, much attention has been focused on how Met and its variants affect the reduction potential of the metal center.[3] In contrast, the role of the axial Met in purple Cu$_A$ centers is only beginning to be addressed.[4] No systematic study of its effect on the reduction potential of the Cu$_A$ center has been reported, and neither has a comparison between the two cupredoxin systems. Here we report electrochemical studies of variants of an engineered Cu$_A$ center in azurin (Cu$_A$ azurin) in which the axial Met has been changed to Glu, Asp, Leu, and Gln. From comparison with the same variants in blue copper azurin, we found the reduction potentials of the Cu$_A$ center were much less sensitive to the axial ligand substitution than those of the type 1 blue copper center. The significance of this finding in relationship with the functional roles of cupredoxins is discussed.

All Cu$_A$ azurin variants, prepared as previously described,[5] show characteristic purple color and display UV−vis spectra similar to that of Cu$_A$ azurin (Figure 2A). The main difference is variation of the energy of the near-IR band among the variants, with the largest red-shift resulting from the Met123Glu mutation (from 760 to 855 nm). A similar red shift was observed in the Met to Glu variant of the *Thermus thermophilus* Cu$_A$ center, and it was interpreted as a result of an elongated Cu−Cu distance due to a strong interaction between the Glu and the Cu.[4b] It is, however, interesting that a similar red shift of the near-IR band was observed in the Met123Leu variant which has a noncoordinating axial ligand. A significant increase in the intensity of a peak around 350 nm was observed in Met123Glu and Met123Asp variants. This band was previously assigned as the equatorial His to Cu charge transfer (CT) transition.[6] A comparison of crystal structures from several Cu$_A$ centers revealed a correlation between angular position of the two imidazole rings relative to the Cu$_2$S$_2$(Cys) plane and Cu-axial ligand distance.[4d,7] The increased intensity observed here is probably associated with modulation of Cu−His interaction upon axial ligand replacement.

Additional information on the geometric and electronic states of the mutants was obtained from EPR spectra (Figure 2B). A seven-line hyperfine, indicative of a delocalized mixed valence dinuclear species, was observed for Met123Asp and Met123Glu variants, with different hyperfine coupling in the $g_{||}$ region.[8] Due to the protonation of the carboxylate side chain of Met123Glu (pK_a ≈ 5.0) and Met123Asp (pK_a ≈ 5.5), EPR spectra of these variants
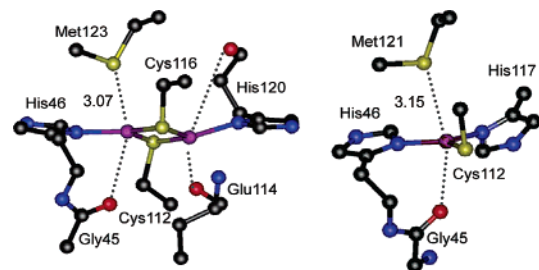
Figure 1. Active sites of an engineered purple CuA azurin (left, PDB ID: 1CC3)[7] and blue copper azurin from *P. aeruginosa* (right, PDB ID: 4AZU).[17]
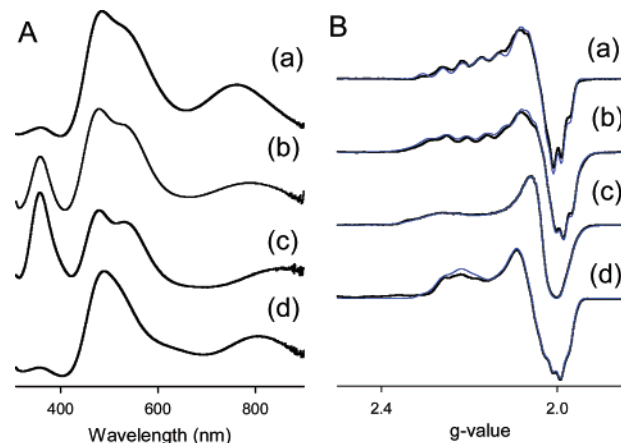

Figure 2. (A) UV−vis spectra and (B) X-band EPR spectra of Cu$_A$ azurin and its Met123X variants. (a) Cu$_A$ azurin, (b) Met123Asp, (c) Met123Glu, (d) Met123Leu. (Black line: exptl, blue line: simulated, pH 5.5, 50 mM ammonium acetate. The X-band (9.05 GHz) spectra were recorded at 15 K with 50% glycerol).

were simulated with a mixture of two valence-delocalized species.[9] Met123Leu has narrowly spaced four-line hyperfine structure in the $g_{||}$ region. Detailed simulation of the EPR spectrum revealed two species that contribute to the observed EPR spectrum: the major species having an unpaired electron localized primarily on only one copper and valence-delocalized dinuclear copper species as a minor species. Thus it can be concluded that replacement of axial Met with Leu resulted in a valence-trapped species. The shift of the near-IR peak to lower energy observed in the optical spectrum of Met123Leu can be interpreted as an intervalence charge-transfer transition.[10]

The reduction potentials of the Met variants were measured as described previously.[11] The reduction potential of Cu$_A$ azurin obtained at pH 5.5 (239 mV versus NHE) matches closely the value reported for other Cu$_A$ centers using different methods.[12] Replacing Met123 with negatively charged Glu and Asp and more hydrophobic Leu resulted in small changes of reduction potentials (Figure 3). This is in contrast with the potential changes of the same mutations in blue copper azurin, where the Met variants modulate
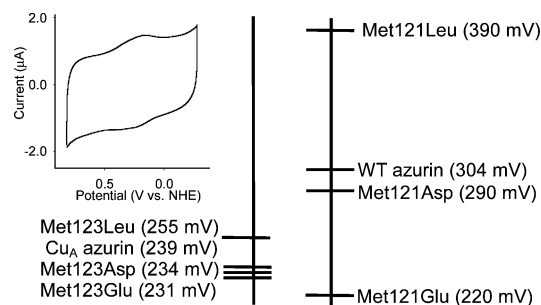
Figure 3. Comparison of the reduction potentials of Cu$_A$ azurin and its Met123 variants (left) with wt azurin and its Met121 variants (right).[3b] Inset: a representative cyclic voltammogram.

the reduction potential to a greater extent (Figure 3).[3b] For example, Met to Glu mutation in Cu$_A$ azurin resulted in only an 8 mV decrease (from 239 to 231 mV), while the same mutation in blue copper azurin caused an 84 mV decrease in reduction potential (from 304 to 220 mV). Similarly, replacing Met with Leu increased the reduction potential of Cu$_A$ azurin only by 16 mV, while the same substitution in azurin increased the potential by 86 mV.[13] Previous studies on a soluble domain of the Cu$_A$ center from *T. thermophilus* cytochrome *ba$_3$* subunit II showed that replacing axial Met with selenomethionine resulted in no change in the reduction potential,[4a] in contrast to a larger (25 mV) increase by the same substitution observed in the azurin from *Pseudomonas aeruginosa*.[2e] On the basis of these results, we can conclude that variations in the axial Met exert much less influence on reduction potentials in the Cu$_A$ center than in blue copper center.

Given that the Cu—S(Met) distance in Cu$_A$ azurin (3.07 Å) is slightly shorter than that in blue copper azurin (3.15 Å) (Figure 1), the lessened sensitivity of the reduction potential of Cu$_A$ center on Met perturbation is remarkable.[14] The small changes in the reduction potential must have little to do with the distance and interaction between the Met and the copper center, but more to do with the "diamond core" nature of the Cu$_A$ center; the bonding interactions between the Cu and sulfur ligands and between the Cu ions in the plane must be strong enough to resist perturbations in the axial positions. This difference may be one of the keys to understanding different functional roles played by otherwise structurally related Cu$_A$ and blue copper proteins. Since blue copper proteins are used in a wide range of ET functions from photosynthesis to denitrification, their potentials must be tuned in a wide (>1000 mV) range, mostly through the axial ligand interactions.[1] On the other hand, the Cu$_A$ center is part of cytochrome *c* oxidase that is at the end of a respiration chain with very small potential differences between the reduction partners (e.g., the driving force for the ET is only ~20 mV between the Cu$_A$ and cytochrome *c*,[12,15] and 90 and 10 mV between Cu$_A$ and heme *a* for oxidized and half-reduced enzyme, respectively[16]). In this case, a large variation of reduction potentials is not desirable and could result in a loss of electron flow in the right direction. The Cu$_2$S$_2$(Cys) diamond core structure of the Cu$_A$ is one way to minimize changes in reduction potential by axial ligand variations.

Supporting Information Available: Tables of EPR simulation parameters and reduction potential, EPR spectra of Met123Asp and Met123Glu at pH 5 and 6, CV of Cu$_A$ azurin and Met123 variants, and UV–vis and EPR spectrum of Met123Gln. This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) EPR spectra were simulated with the automated fitting program, SIM-POW6, a program developed at the University of Illinois based on POW (Nilges, M. J. Ph.D. Thesis, University of Illinois, Urbana-Champaign, 1979) and MPOW (Chang, H.-R.; Diril, H.; Nilges, M. J.; Zhang, X.; Potenza, J. A.; Schugar, H. J.; Hendrickson, D. N.; Isied, S. S. *J. Am. Chem. Soc.* **1988**, *110*, 625–627). Hyperfines for the two Cu atoms were found to be slightly inequivalent for Cu$_A$ azurin and Met123Asp. For detailed parameters, see the Supporting Information.

(9) EPR spectra of Met123Asp and Met123Glu obtained at pH 5 and 6 are included in the Supporting Information.

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(13) Met123Leu Cu$_A$ azurin is, however, a mixture of valence-delocalized and localized species. The presented results cannot determine if the two species are in rapid equilibrium or which species was measured. However, since previous results showed that valence-localized species of Cu$_A$ azurin has a reduction potential ~70 mV higher than that of the valence-delocalized species of the same protein,[5c] the small 18 mV reduction potential increase of Met123Leu derivative suggests that the valence-delocalized species was measured. Regardless of which species was measured, the conclusion of this communication (i.e., the axial Met exert much less influence on reduction potentials in the Cu$_A$ center than in blue copper center) is still valid

(14) Exposure of the Cu$_A$ centers to solvent could contribute to their modest difference in reduction potentials. However, a crystal structure shows that the metal center in Cu$_A$ azurin is shielded from solvent. If its mutant proteins have solvent-exposed metal-binding sites, their reduction potentials should be dramatically different from WT Cu$_A$ azurin. The small difference in potentials is indicative of little influence from solvation. Furthermore, the reduction potentials of blue copper and Cu$_A$ azurins are very different in their native forms (320 and 240 mV, respectively), but very similar in their denatured forms (420 and 410 mV, respectively), (ref 10b and Malmström, B. G.; Wittung-Stafshede, P. *Coord. Chem. Rev.* **1999**, *185–186*, 127–140), suggesting that solvation contributes much more significantly to the denatured states of both proteins than to the native states.

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