Engineered Cupredoxins and Bacterial Cytochrome *c* Oxidases Have Similar Cu_A Sites: Evidence from Resonance Raman Spectroscopy

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Cytochrome *c* oxidase (CCO) is the final enzyme of the respiratory chain in mitochondria and many aerobic bacteria.[2] The Cu_A center in this membrane protein accepts reducing equivalents from cytochrome *c* and passes them via intramolecular electron transfer to a low-spin heme Fe_a and finally to a dinuclear heme Fe_{a3}/Cu_B site, where the reduction of O_2 to H_2O takes place. Characterization of the purple Cu_A center has been greatly advanced by the engineering of soluble CCO fragments[3] and by the incorporation of the Cu_A ligands into the cytochrome *bo* quinol oxidase (purple CyoA),[4] as well as into the blue copper proteins amicyanin[5] and azurin.[6] Three X-ray crystal structures are now available for CuA domains from mammalian and bacterial (*Paracoccus denitrificans*) cytochrome *c* oxidases[7a,b] and from the purple Cyo construct.[7c] In each case, the metal site consists of two Cu ions 2.5 Å apart, each with a terminal histidine ligand and two cysteine thiolate bridges, thereby supporting the essential dinuclear Cu(1.5)···Cu(1.5) nature of Cu_A and N_2O reductase suggested by their seven-line EPR hyperfine splitting[8] and strong 2.5-Å EXAFS scattering.[9] Here we report the resonance Raman (RR) spectra of novel Cu_A constructs in *Pseudomonas aeruginosa* azurin and *Thiobacillus versutus* amicyanin and show that they closely resemble the Cu_A sites of the CCO fragments. The distinctive Cu_A RR fingerprint spectrum in both native and engineered sites provides strong evidence for a highly conserved dinuclear thiolate-bridged structure and for the existence of a flexible cupredoxin-like folding motif in the Cu_A domain. The RR spectra of these Cu_A-type sites are clearly distinguished from their blue Cu counterparts in having two intense Cu−S(Cys) stretching vibrations due to the presence of two bridging thiolate ligands.

Soluble Cu_A-containing fragments of CCO have been produced by gene cleavage in *Para. denitrificans*,[3a] *Bacillus subtilis*,[3b] and *Thermus thermophilus*.[10] Each of these proteins has the purple color, 480-, 530-, and 780-nm absorption bands, and seven-time EPR spectrum characteristic of a Cu_A chromophore. Amino acid sequence comparisons show that the Cu_A domain is evolutionarily related to the blue copper proteins, having a cupredoxin fold and a $Cys(His)_2Met$ ligand set but differing in the presence of a second Cys ligand.[4a] The mononuclear Cu site in the blue Cu protein azurin can accommodate a surprising range of Cu(II) coordination geometries upon mutagenesis of its Cu ligands, yielding an array of trigonal planar, tetrahedral, and tetragonal structures.[11] Addition of a second Cys ligand favors the conversion to a dinuclear Cu site. Thus, the *Escherichia coli* quinol oxidase has been engineered to form either a mononuclear (blue) or a dinuclear (purple) CyoA protein by the introduction of one or two Cys ligands, respectively.[4] Capitalizing on this flexibility in coordination, two blue copper proteins have now been converted into purple Cu_A-like proteins by the addition of a second Cys into the Cys-X_n-His-X_n-Met sequence of the Cu-binding loop. This has been accomplished by inserting the purple CyoA sequence into *T. versutus* amicyanin[5] and the *Para. denitrificans* Cu_A sequence into *P. aeruginosa* azurin.[6] All three of the engineered purple proteins have absorption and EPR properties similar to those of the Cu_A-containing CCO fragments.

RR spectroscopy selectively probes vibrations of the Cu−S(Cys) chromophore and is an invaluable method for characterizing copper site geometry because of its sensitivity to Cu−S bond distances and bond angles.[11] In our previous study of the *B. subtilis* Cu_A fragment, the same RR spectrum (Figure 1C) was obtained with excitation near 480, 530, and 780 nm, indicating that each of these absorption bands has significant (Cys)S→Cu CT character.[12] A remarkably similar pattern of RR frequencies has now been obtained for the Cu_A fragments of *Para. denitrificans* and *Ther. thermophilus* (Figure 1A,B) and for the Cu_A constructs in azurin and amicyanin (Figure 1D,E), providing strong evidence for the same Cu site geometry in all of these proteins. In each case, the two most intense vibrations occur near 260 and 340 cm^{-1}, with a number of weaker features between 115 and 400 cm^{-1}. Based on the analogy with blue Cu proteins, we first assigned the band at ~340 cm^{-1} as the predominant Cu−S(Cys) stretch on the basis of its high intensity, Cu isotope shift, and its being the generator of overtone and combination bands.[12] However, a more definitive experiment, based on the greater mass effect of S-isotope substitution, clearly shows that *both* the 260- and 339-cm^{-1} bands have large isotope shifts of −4.1 and −5.1 cm^{-1}, respectively (Figure 1A), indicating that both have substantial ν(Cu−S) character. The particularly high intensity of the 260-cm^{-1} peak in *Ther. thermophilus* Cu_A and the azurin construct (Figure 1B,D), as well as in N_2OR,[12] is consistent with the assignment of this mode to a second Cu−S(Cys) stretch.

A comparison of S isotope shifts in the RR spectra of Cu_A and blue Cu proteins highlights important distinctions arising from their different modes of cysteine coordination. In cupredoxins such as azurin[13] and plastocyanin,[14] multiple S-dependent bands are generated by kinematic coupling of a *single* Cu−S

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(2) Babcock, G. T.; Wikström, M. *Nature* **1992**, *356*, 301−9.

(3) (a) Lappalainen, P.; Aasa, R.; Malmström, B. G.; Saraste, M. *J. Biol. Chem.* **1993**, *268*, 26416−21. (b) von Wachenfeld, C.; de Vries, S.; van der Oost, J. *FEBS Lett.* **1994**, *340*, 109−13.

(4) (a) van der Oost, J.; Lappalainen, P.; Musacchio, A.; Warne, A.; Lemieux, L.; Rumbley, J.; Gennis, R. B.; Aasa, R.; Pascher, T.; Malmström, B. G.; Saraste, M. *EMBO J.* **1992**, *11*, 3209−17. (b) Kelly, M.; Lappalainen, P.; Talbo, G.; Haltia, T.; van der Oost, J.; Saraste, M. *J. Biol. Chem.* **1993**, *268*, 16781−7.

(5) Dennison, C.; Vijgenboom, E.; de Vries, S.; van der Oost, J.; Canters, G. W. *FEBS Lett.* **1995**, *365*, 92−4.

(6) Hay, M. T.; Richards, J. H.; Lu, Y. *Proc. Natl. Acad. Sci. U.S.A.*, in press.

(7) (a) Tsukihara, T.; Aoyama, H.; Yamashita, E.; Tomizaki, T.; Yamaguchi, H.; Shinzawa-Itoh, K.; Nakashima, R.; Yaono, R.; Yoshikawa, S. *Science* **1995**, *269*, 1069−1074. (b) Iwata, S.; Ostermeier, C.; Ludwig, B.; Michel, H. *Nature* **1995**, *373*, 660−669. (c) Wilmanns, M.; Lappalainen, P.; Kelly, M.; Sauer-Eriksson, E.; Saraste, M. *Proc. Natl. Acad. Sci. U.S.A.*, in press.

(8) Antholine, W. E.; Kastrau, D. H. W.; Steffens, G. C. M.; Buse, G.; Zumft, W. G.; Kroneck, P. M. H. *Eur. J. Biochem.* **1992**, *209*, 875−81.

(9) Blackburn, N. J.; Barr, M. E.; Woodruff, W. H.; van der Oost, J.; de Vries, S. *Biochemistry* **1994**, *33*, 10401−7.

(10) Fee, J. A.; Sanders, D.; Slutter, C. E.; Doan, P. E.; Aasa, R.; Karpefors, M.; Vänngård, T. *Biochem. Biophys. Res. Commun.* **1995**, *212*, 77−83.

(11) Andrew, C. R.; Yeom, H.; Valentine, J. S.; Karlsson, B. G.; Bonander, N.; van Pouderoyen, G.; Canters, G. W.; Loehr, T. M.; Sanders-Loehr, J. *J. Am. Chem. Soc.* **1994**, *116*, 11489−98.

(12) Andrew, C. R.; Han, J.; de Vries, S.; van der Oost, J.; Averill, B. A.; Loehr, T. M.; Sanders-Loehr, J. *J. Am. Chem. Soc.* **1994**, *116*, 10805.

(13) Dave, B. C.; Germanas, J. P.; Czernuszewicz, R. S. *J. Am. Chem. Soc.* **1993**, *115*, 12175−6.

(14) Qiu, D.; Dong, S.; Ybe, J.; Hecht, M.; Spiro, T. G. *J. Am. Chem. Soc.* **1995**, *117*, 6443−6.

0002-7863/95/1517-10759$09.00/0

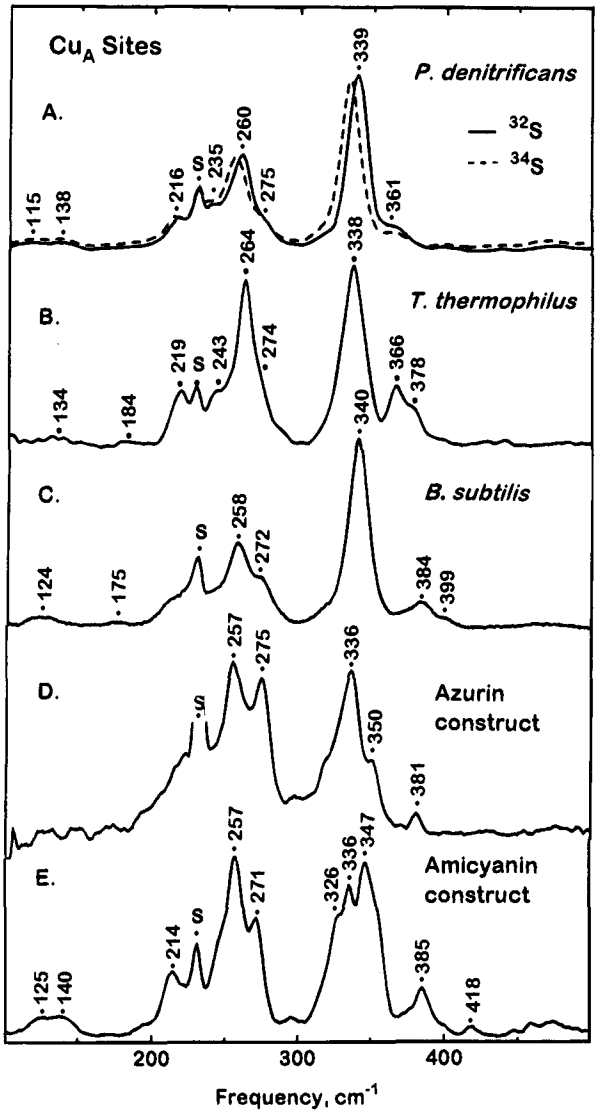

Figure 1. Resonance Raman spectra of Cu_A sites upon 488-nm (~150-mW) excitation at 15 K. (A) CCO fragment from *Para. denitrificans* (2.0 mM in Cu_A) in 20 mM Bis-Tris (pH 6.5) from bacteria grown in ^{32}S (—) or ^{34}S-substituted (- - -) Na_2SO_4, prepared as in ref 3a. (B) CCO fragment from *Ther. thermophilus* (1.8 mM in Cu_A) in 30 mM Tris-HCl (pH 8.0), prepared as in ref 10. (C) CCO fragment from *B. subtilis* (1.5 mM in Cu_A) in 20 mM Tris-HCl (pH 8.0), spectrum from ref 7. (D) Cu_A construct in *P. aeruginosa* azurin (~0.4 mM in Cu_A) in 50 mM NH_4OAc (pH 5.2), prepared as in ref 6. (E) Cu_A construct in *T. versutus* amicyanin (1.7 mM in Cu_A) in 50 mM HEPES buffer (pH 7.0), prepared as in ref 5. S, frozen solvent.

stretch with Cys ligand deformation modes of similar energy.[11] Thus, total S shifts of ~5 cm^{-1} are distributed in peaks within ~30 cm^{-1} of the ~400-cm^{-1} ν(Cu−S) mode, forming a cluster of intense bands with Cu−S stretching character (Figure 2B,C). However, in Cu_A, the increased total S isotope shift of ~10 cm^{-1}, together with the larger energy separation of ~80 cm^{-1} between the two predominant S-sensitive peaks (Figure 2A), implies that the 260- and 340-cm^{-1} bands originate from separate ν(Cu−S) modes due to the presence of *two* Cys ligands. Normal coordinate analyses[15] indicate that the large isotope shifts and large frequency separation between the two modes can be fit only by a model with bridging thiolates, in keeping with the X-ray structures[7] and ruling out the Cu−Cu bonded alternative.[9] Moreover, the RR spectrum can be well matched using only the vibrations of a six-atom $Cu_2N_2S_2$ cluster. This suggests that the Cu−S stretching modes of dinuclear Cu_A sites

(15) Andrew, C. R.; Sanders-Loehr, J.; Fraczkiewicz, R.; Czernuszewicz, R. Unpublished results.

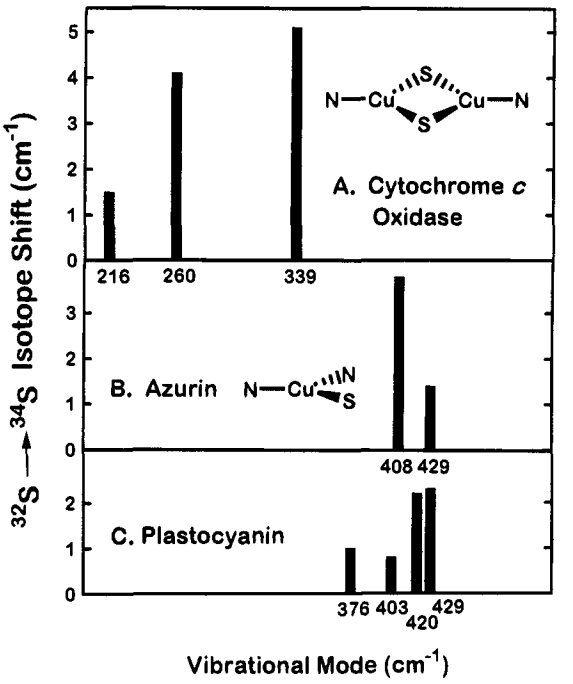

Figure 2. Sulfur isotope downshifts ($^{32}S \rightarrow {}^{34}S$) for different vibrational modes. Based on RR spectra for (A) Cu_A fragment from *Para. denitrificans* (from Figure 1A), (B) azurin from *P. aeruginosa* (from ref 13), and (C) plastocyanin from poplar (from ref 14).

are relatively pure, undergoing less kinematic coupling with internal Cys vibrations than in the mononuclear Cu sites.

In conclusion, the occurrence of two intense Cu−S stretching modes at ~260 and ~340 cm^{-1} in the RR spectra of Cu_A sites is consistent with a symmetric bridging arrangement for the two Cys ligands,[15] as observed in the X-ray structures.[7] In addition, the lower Cu−S frequencies in Cu_A compared to the predominant ν(Cu−S) near 400 cm^{-1} in blue Cu proteins (Figure 2) are consistent with a longer Cu−S distance of ~2.23 Å in Cu_A[16] compared to ~2.13 Å in the blue copper sites.[11] However, the vibrational frequencies in a bridged system are also strongly dependent on Cu−S−Cu bond angles and thus cannot be as readily correlated with Cu−S bond distances as in a mononuclear system.[17] Nevertheless, the striking similar ν(Cu−S) frequencies for the Cu_A constructs of amicyanin and azurin and the three different bacterial CCO fragments imply that their dinuclear sites have very similar Cu−S−Cu angles and Cu−S bond lengths. The ability to engineer a Cu_A site from a blue Cu site and vice versa confirms the presence of the same flexible Cu binding site in a cupredoxin fold, which can adopt different Cu coordination geometries upon substitution of a single amino acid ligand. The facile self-assembly of the dinuclear cluster in Cu_A is reminiscent of the $Fe_2S_2(Cys)_2$ clusters in ferredoxins. However, Cu_A domains differ in that their mixed valence state is fully delocalized over the two metal ions rather than having a trapped valence on each metal as in the ferredoxins.

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(16) The average Cu−S(Cys) distance in the *B. subtilis* CCO fragment has been measured at 2.24 Å by EXAFS (ref 9) and at 2.22 Å in the crystal structure of purple CyoA (ref 7).

(17) For example, in di-μ-oxo-bridged M−O−M systems, the M−O stretching frequencies are highly sensitive to the M−O−M angle, with a 10° change in angle causing shifts of 7% or more in vibrational frequencies [Wing, R. M.; Callahan, K. P. *Inorg. Chem.* **1969**, *8*, 871−4].