Engineered Cupredoxins and Bacterial Cytochrome c Oxidases Have Similar Cu₆ Sites: Evidence from Resonance Raman Spectroscopy

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Received July 24, 1995

Cytochrome c oxidase (CCO) is the final enzyme of the respiratory chain in mitochondria and many aerobic bacteria. a The Cu₆ center in this membrane protein accepts reducing equivalents from cytochrome c and passes them via intramembrane electron transfer to a low-spin heme Fe₃. f,g Finally, it delivers the electrons to a Cu₆ center consisting of two Cu ions in a dinuclear Cu₆ site, where the reduction of O₂ to H₂O takes place. Characterization of the purple Cu₆ center has been greatly advanced by the engineering of soluble CCO fragments a,b and by the incorporation of the CUA ligands into mammalian and bacterial (Paracoccus denitrificans) cytochrome c oxidases a,b and from the purple Cyo construct. a,b,c,d,e,f 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 Cu₅.₆-Cu₇.₈ nature of Cu₆, and N₂O reductase suggested by their seven-line EPR hyperfine splitting a,b and strong 2.5-Å EXAFS scattering. a,b,c,d,e,f

Here we report the resonance Raman (RR) spectra of novel Cuo constructs in Pseudomonas aeruginosa azurin and Thiobacillus versutus amicyanin and show that they closely resemble the Cuo sites of the CCO fragments. The distinctive Cuo 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 Cu₆ domain. The RR spectra of these Cu₆-type sites are clearly distinguished from their blue Cuo counterparts in having two intense Cu-S(Cys) stretching vibrations due to the presence of two bridging thiolate ligands.

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Soluble Cu₆-containing fragments of CCO have been produced by gene cleavage in Para. denitrificans,a Bacillus subtilis,b 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 Cu₆ chromophore. Amino acid sequence comparisons show that the Cu₆ domain is evolutionarily related to the blue copper proteins, having a cupredoxin fold and a Cys(His)₇Met ligand set but differing in the presence of a second Cys ligand.4 The mononuclear Cu₆ site in the blue Cu₆ protein azurin can accommodate a surprising range of Cu(I) coordination geometries upon mutation of its Cu₆ ligands, yielding an array of trigonal planar, tetrahedral, and tetragonal structures.11 Addition of a second Cys ligand favors the conversion to a dinuclear Cu₆ 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 Cu₆-like proteins by the addition of a second Cys into the Cys-X-His-X-Met sequence of the Cu₆-binding loop. This has been accomplished by inserting the purple CyoA sequence into T. versutus amicyanin12 and the Para. denitrificans Cu₆ sequence into P. aeruginosa azurin.6 All three of the engineered purple proteins have absorption and EPR properties similar to those of the Cu₆-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 Cu₆ 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.11 A remarkably similar pattern of RR frequencies has now been obtained for the Cu₆ fragments of Para. denitrificans and Ther. thermophillus (Figure 1A,B) and for the Cu₆ 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 Cu₆ proteins, we first assigned the band at ~340 cm⁻¹ as the predominant Cu₆–S(Cys) stretch on the basis of its high intensity, Cu₆ isotope shift, and its being the generator of a second Cys ligand favors the conversion to a dinuclear Cu₆ site. The particular high intensity of the 260–340 cm⁻¹ peak in Ther. thermophillus Cu₆ and the azurin construct (Figure 1B,D), as well as in N₂OR,12 is consistent with the assignment of this mode to a second Cu₆–S(Cys) stretch.

A comparison of S-isotope shifts in the RR spectra of Cuo and blue Cu₆ proteins highlights important distinctions arising from their different modes of cysteine coordination. In cupredoxins such as azurin13 and plastocyanin,14 multiple S-dependent bands are generated by isotope coupling of a single Cu-S


implies that the 260- and 340-cm\(^{-1}\) bands originate from between the two predominant S-sensitive peaks (Figure 2A), using only the vibrations of a six-atom Cu\(_2\)N\(_2\)S\(_2\) cluster. This is consistent with a symmetric bridging arrangement for the two Cys ligands,\(^{13}\) 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 \(\sim 2.23\) Å in Cu\(_A\)\(^{16}\) compared to \(\sim 2.13\) Å in the blue copper sites.\(^{1}\) 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 Fe\(_2\)S\(_2\)(Cys)\(_2\) clusters in ferredoxins. However, Cu\(_A\) domains differ in that their mixed valence state is commensurate with each metal as in the ferredoxins. 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].

**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).

stretch with Cys ligand deformation modes of similar energy.\(^{11}\) Thus, total S shifts of \(\sim 5\) cm\(^{-1}\) are distributed in peaks within \(\sim 30\) cm\(^{-1}\) of the \(\sim 400\)-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 \(\sim 10\) cm\(^{-1}\), together with the larger energy separation of \(\sim 80\) 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 thioclates, 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\(_2\)N\(_2\)S\(_2\) cluster. This suggests that the Cu–S stretching modes of dinuclear Cu\(_A\) sites 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 \(\sim 260\) and \(\sim 340\) cm\(^{-1}\) in the RR spectra of Cu\(_A\) sites is consistent with a symmetric bridging arrangement for the two Cys ligands,\(^{13}\) 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 \(\sim 2.23\) Å in Cu\(_A\)\(^{16}\) compared to \(\sim 2.13\) Å in the blue copper sites.\(^1\) 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\(_2\)S\(_2\)(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.

**Acknowledgment.** This work was supported by grants from the National Institutes of Health (GM18865 to J.S.-L. and GM35342 to J.A.F.), the EC (SC1-CT91-0698 to M.S.), the Academy of Finland (P.L.), National Science Foundation (Career Award CHE 95-02421 to P.L.), National Institutes of Health (GM18865 to J.S.-L. and GM35342 to J.A.F.), the EC (SC1-CT91-0698 to M.S.), the Academy of Finland (P.L.), National Science Foundation (Career Award CHE 95-02421 to P.L.), and NATO (CRG 930170 to G.W.C. and J.S.-L.).