Far-Red Resonance Raman Study of Copper A in Subunit II of Cytochrome *c* Oxidase

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Copper A (Cu_A) constitutes the proximate electron acceptor from cytochrome *c* in most cytochrome *c* oxidases (CcO). The structural and functional characteristics of Cu_A are key issues in respiratory bioenergetics. The Cu_A redox center resides in subunit II of CcO and previously was widely believed to have a simple single-copper structure similar to those of the chromophores in blue copper proteins.[1] However, more recent experimental results from a number of techniques[2−9] have suggested that Cu_A is actually a coupled two-copper center analogous to that inferred for N_2O reductase.[6,7,10] Results from EXAFS[9,11,12] and from X-ray crystallography[13−17] verify the two-copper nature of Cu_A. These structural studies all show a short Cu−Cu distance, approximately 2.5 Å, which is unprecedented in biological copper systems. Thus, the Cu_A site is composed of two copper atoms within possible bonding distance.[13,14] An outstanding question is whether there is an actual Cu−Cu bond, in addition to the two thiolate bridges between the Cu atoms.[13−15]

Previous EXAFS[9] experimental results are consistent with a Cu−Cu bond. The short separation between the two coppers is a reasonable distance for a bonding interaction, by comparison to $Mn_2(CO)_{10}$, 2.93 Å;[18] the KBr diatom, 2.821 Å;[19] and $[Co_2(CN)_{10}]6^-$, 2.798 Å.[20] Several inorganic dicopper model complexes contain copper−copper bonds with Cu−Cu distances in the 2.4−2.5 Å range.[21−23] Like Cu_A, these complexes exhibit intense electronic transitions near 800 nm. Unlike Cu_A, however, these cannot arise from charge transfer but are ascribed to transitions involving the Cu−Cu bond.

The present work employs far-red resonance Raman spectroscopy (RR) to investigate whether a Cu−Cu bonding interaction exists in Cu_A. The electronic transition of Cu_A near 830 nm[11,21,22,24] provides the opportunity for specific RR observation of the vibrations of this chromophore. We have employed RR with Cu isotopic substitution on genetically modified, solubilized forms of CcO subunit II[11,12] from *Bacillus subtilis* and *Paracoccus denitrificans*. We have also probed the Cu_A site of native beef heart CcO for comparison.

The RR spectra of *B. subtilis* and *P. denitrifcans*,[11,21,22,25,26] are shown in Figure 1A. The group of low-intensity peaks between 215 and 275 cm^{-1} and the relatively large peak at 361 cm^{-1} have previously been studied using blue excitation and were assigned to the metal−ligand stretches of Cu_A.[27,28] These earlier RR studies derived resonance enhancement from the Cu_A electronic absorption near 500 nm, assigned to Cu−S charge transfer.[8,25,26] The amplitudes of these peaks, relative to the ice peaks, were significantly larger for blue excitation than for the red excitation used in this study. In contrast, the peaks below 200 cm^{-1}, especially the peak at 125 cm^{-1} for *B. subtilis* and 139 cm^{-1} for *P. denitrifcans*, are much more strongly enhanced with red excitation than with blue excitation. These peaks are thus specifically coupled to the red electronic absorption. In Cu_A, this transition has commonly been assigned to a low-energy Cu−S charge transfer transition.[8,25,26] However, the model studies show[21,22] that low-energy transitions of a Cu−Cu bonded system are also expected near 800 nm. Thus, the far-red enhanced vibrational modes may be coupled either to Cu−S charge transfer or to transitions involving the Cu−Cu bond or to both simultaneously. In the latter two cases, selective far-red enhancement of a Cu−Cu stretch is expected.

Peak positions and isotope shifts[29] were determined from the zero crossings of the first derivatives of the spectra in Figure 1A. All of the peaks attributed to Cu_A vibrations exhibit isotopic shifts between the ^{63}Cu and ^{65}Cu isotopes as shown in Figure

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(29) We have experimentally determined the precision (σ) of the shift of the low-frequency peak to be ±0.3 cm^{-1}. This is determined by multiple independent measurements of the isotope shifts.

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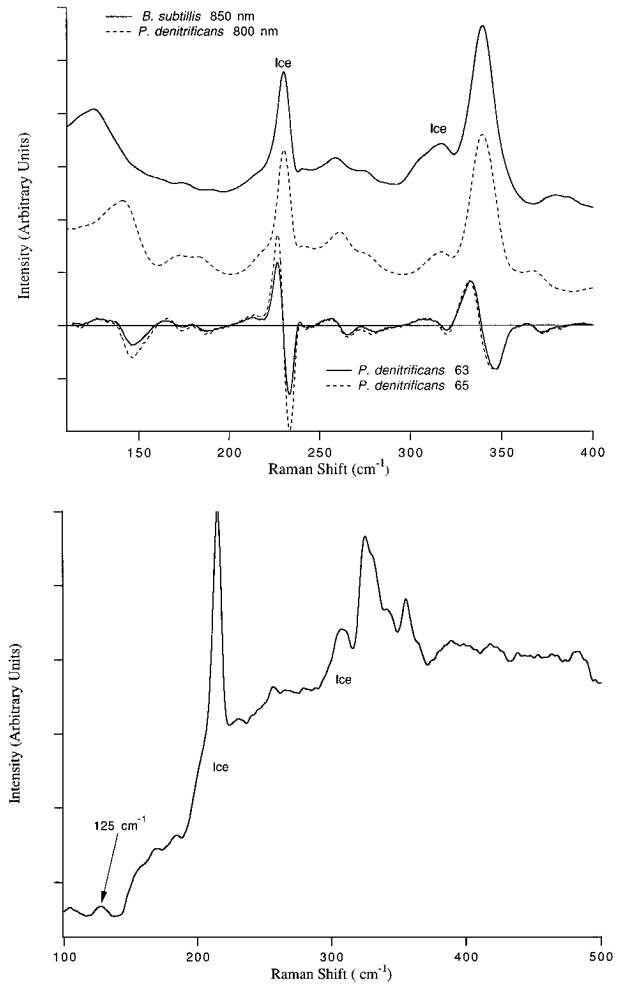

Figure 1. (A) Top two traces representing the resonance Raman spectra of the subunit II/Cu_A (^{63}Cu isotope) of *B. subtilis* and *P. denitrificans* using red excitation obtained at 30 K. The bottom overlapping traces are the derivative of the spectra of the two isotopes (^{63}Cu, ^{65}Cu) for the *P. denitrificans* species. (B) Resonance Raman spectra of beef heart cytochrome *c* oxidase using 850 nm excitation obtained at 30 K. No isotope data was obtained for the resting enzyme.

1A. Table 1 lists the observed positions and isotope shifts for the three dominant peaks of Figure 1A.

Given the structure of Cu_A, which is not rigorously centrosymmetric, the bonds (two Cu−S and two Cu−N) yield four predicted Cu−S and Cu−N stretching frequencies in this region. A fifth low-frequency mode representing the Cu−Cu stretch, or the symmetrically equivalent Cu−S−Cu angle bend,[30] is expected regardless of whether a Cu−Cu bond exists, and this mode will also shift with copper isotopic substitution. However, the frequency of this peak will strongly depend on the presence or absence of a Cu−Cu bond. A RR study of several of the Cu−Cu-bonded inorganic compounds has assigned the Cu−Cu stretching frequency from 160 to 185 cm^{-1}.[24] This peak was in maximum resonance with the Cu−Cu bond transition near 800 nm.[24] The Cu−Cu stretching vibration of a bonded structure of Cu_A is expected near this frequency range,[32,33] while a nonbonded vibration would occur at a substantially lower frequency.

In the blue RR study of Cu_A,[27,28] the peaks below 200 cm^{-1} were not significantly resonance enhanced and no attempt was made to assign them to specific modes. In particular, the peak occurring at 125 cm^{-1} for *B. subtilis* and at 139 cm^{-1} for *P. denitrificans* was very weak in the blue RR study. However, excitation into the band at ∼830 nm enhances this peak by more than a factor of 10.[31] As noted above, the 830 nm transition may have significant contributions from states involved in metal−metal bonding. Thus, the vibrations below 200 cm^{-1} which are selectively enhanced with far-red excitation may be associated with vibrations of a Cu−Cu bond.

Table 1. Cu_A Resonance Raman Isotope Shift Data

observed frequency[a]	observed $^{63/65}Cu$ isotope shift[a,b]	predicted[c] $^{63/65}Cu$ isotope shift[a]
	P. denitrificans	
139	1.9	2.0 (Cu−Cu)
		0.7 (Cu−S)
		1.1 (Cu−N)
261	1.1	4.2 (Cu−Cu)
		1.4 (Cu−S)
		2.1 (Cu−N)
339	1.3	5.4 (Cu−Cu)
		1.8 (Cu−S)
		2.8 (Cu−N)
	B. subtilis	
125	2.3	2.0 (Cu−Cu)
		0.7 (Cu−S)
		1.1 (Cu−N)
258	0.8	
340	0.8	

[a] All values reported in units of cm^{-1}. [b] Estimated error ±0.3. [c] Predicted isotope shifts calculated using the harmonic oscillator approximation for a diatomic molecule.

This peak also exhibits significant $^{63/65}Cu$ isotope sensitivity. The predicted shifts in Table 1 show that the observed shifts of 2.3 cm^{-1} for the peak at 125 cm^{-1} (*B. subtilis*) and 1.9 cm^{-1} for the 139 cm^{-1} peak (*P. denitrifcans*) are consistent with the assignment as the Cu−Cu stretching vibration. The RR excitation profiles are also consistent with this assignment. While this evidence is indicative of a Cu−Cu-bonded structure, neither of these observations constitute unique evidence as to whether the copper atoms are bonded or nonbonded.[30,32,33] However, the frequency location of this peak, compared to the Cu−Cu stretching frequency assigned in the model complexes, strongly suggests a direct Cu−Cu bond in addition to the thiolate bridges. Thus, the data reported in this study support the presence of a Cu−Cu bond for the Cu_A site of CcO.[5,6,9,11]

The RR spectrum of the resting beef heart cytochrome *c* oxidase is shown in Figure 1B for comparison. This spectrum is similar to those of the subunit II fragments (Figure 1A), thereby supporting Kitagawa's previous result[34] that the low-frequency Raman spectrum of the enzyme obtained with far-red excitation can be predominantly attributed to the Cu_A chromophore. The similarity of the CcO and Cu_A fragment (subunit II) spectra suggests that Cu_A in the isolated subunit II has essentially the same structure as that of Cu_A in intact CcO. Thus, the suggested Cu−Cu bonding in Cu_A, if correct, applies both to the isolated subunit II and native CcO. The functional reasons why nature may choose such a structure, or in fact why a two-copper center of any sort is constructed to perform a function which is commonly believed to be simple one-electron transfer, are unclear. Future studies will address these issues.

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