Vibrational Characteristics of Mutant and Wild-Type Carbon Monoxy Cytochrome *c* Oxidase: Evidence for a Linear Arrangement of Heme *a*, a_3, and Cu_B

Jonathan P. Hosler,[†] Younkyoo Kim,[‡,∥] J. Shapleigh,[§,▽] R. Gennis,[§] J. Alben,[⊥] S. Ferguson-Miller,[†] and G. Babcock*[,‡]

Departments of Biochemistry and Chemistry and LASER Laboratory, Michigan State University East Lansing, Michigan 48824 Department of Biochemistry University of Illinois, Urbana, Illinois 61801 Department of Medical Biochemistry Ohio State University, Columbus, Ohio 43210

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The combined application of genetic, biochemical, and spectroscopic techniques to bacterial versions of the key oxygen-metabolizing enzyme of aerobic organisms, cytochrome oxidase, has led to topological models of the heme and copper cofactors within the protein framework.[1-5] The current view places the oxygen-activating center, heme a_3/Cu_B, and the low-spin heme, heme *a*, within subunit I of the enzyme. In the most recent model (Figure 1), both hemes are bound to transmembrane helix X, with His102 and His421 functioning as the ligands for heme *a* and His419-ligating heme a_3 (see refs 1 and 2). Three remaining conserved histidines, His284, -333, and -334, are assigned as Cu_B ligands. A polypeptide loop located on the outer side of the membrane connects helices IX and X and contains a number of conserved residues, including Asp412. According to the model, Asp412 is on the proximal side of heme a_3, while His333 is on the distal side, as a ligand of Cu_B. Although there is substantial evidence to support these metal ligand assignments,[1-5] there have been some conflicting data regarding the designation of His419 or His284 as the ligand of heme a_3.[6] This assignment dictates whether Cu_B is located between or distal to the two hemes, and thus is critical for understanding its function.[7,8]

Here we have tested the model of Figure 1 by analyzing mutants at the 333 and 412 positions for their vibrational properties in both the unliganded fully reduced and carbon monoxy forms. Mutants in cytochrome aa_3 of *Rhodobacter sphaeroides* in which His333 or Asp412 of subunit I was substituted with asparagine (His333Asn and Asp412Asn, respectively) were constructed as

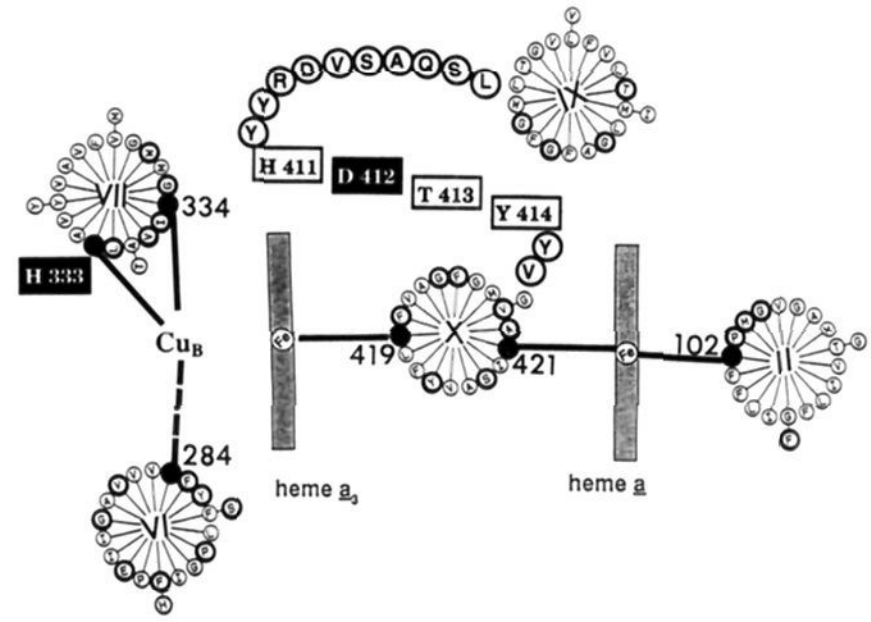

Figure 1. Helical wheel model of five transmembrane helices of subunit I of *Rhodobacter sphaeroides* cytochrome *c* oxidase, showing the proposed location of the three metal centers and an extramembrane loop that caps the heme *a*, heme a_3, and Cu_B metal centers. The histidine ligands of the metals are numbered according to the *Rb. sphaeroides* sequence and shown as solid black circles; other conserved residues in the transmembrane helices are shown in bold circles. The two residues that are the focus of this study are in black boxes.

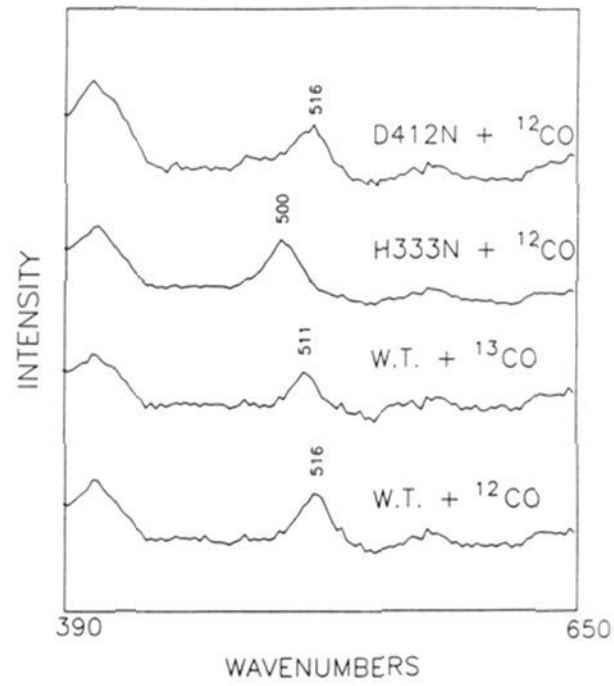

Figure 2. Resonance Raman spectra of CO-bound forms of wild-type *Rb. sphaeroides* cytochrome *c* oxidase and mutant forms Asp412Asn (D412N) and His333Asn (H333N), as indicated. The spectra were obtained at room temperature with 5-cm^{-1} resolution by using a Spex 1877B triple spectrograph and an EG&G Model 1421 diode array detector. The oxidase samples (30–50 μM) were placed in a spinning cell and excited at 424 nm with a Coherent 590 dye laser connected with a Coherent Innova 200 argon laser. Laser power at the sample was less than 2 mW, and spectra were collected within 5 min of initiating laser irradiation. ^{12}CO was added to the reduced enzyme by repeated evacuation and flushing with CO. ^{13}CO (Cambridge Isotope Laboratories) was exchanged for ^{12}CO by the same method.

previously described;[1,3] the mutant enzymes were purified as in Hosler et al.[9]

Figure 2 shows resonance Raman spectra in the low-frequency region for the carbon monoxy complexes of the mutant and wild-type oxidases. The stretching vibration of the iron–carbon bond of heme a_3, ν(Fe–CO), is identified at 516 cm^{-1} for the wild-type enzyme. This is confirmed by the 5-cm^{-1} downshift in the ^{13}CO isotope-labeling experiment, in agreement with the earlier work by Rousseau and co-workers.[10] In His333Asn, ν(Fe–CO) is

† Department of Biochemistry, Michigan State University.
‡ Department of Chemistry and the LASER Laboratory, Michigan State University.
§ Department of Biochemistry, University of Illinois.
⊥ Department of Medical Biochemistry, Ohio State University.
∥ Present address: Department of Chemistry, Hankuk University of Foreign Studies, Yongin-gun, Kyungki-do, 449-791, Korea.
▽ Present address: Department of Microbiology, Cornell University, Ithaca, New York.

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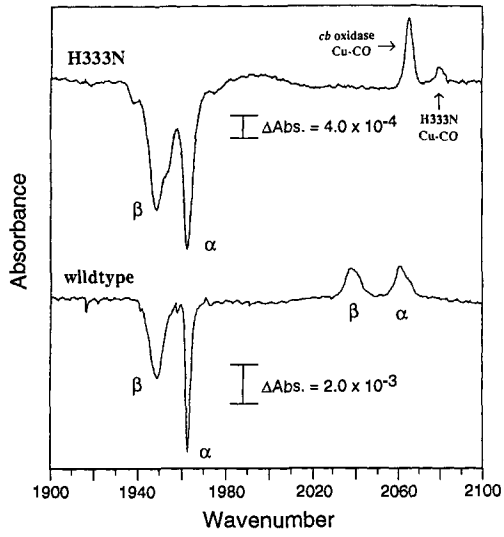

Figure 3. Low-temperature (10 K) FTIR difference spectra (light *minus* dark) of membrane bound, CO-ligated wild-type *Rb. sphaeroides* cytochrome *c* oxidase and His333Asn (H333N). Conditions are as described in Shapleigh et al.[14] The α and β forms[14] of $\nu(C{\equiv}O)_{a_3}$ and $\nu(C{\equiv}O)_{Cu_B}$ are labeled where they can be identified. The peak at 2065 cm^{-1} in the spectrum of His333Asn membranes arises from the C≡O (Cu) stretch of a *cb*-type cytochrome *c* oxidase that is overexpressed in mutants containing an inactive aa_3-type oxidase.[1,14] The C≡O (Fe) stretching mode of this enzyme underlies the β form of $\nu(C{\equiv}O)_{a_3}$ of the aa_3-type oxidase.

shifted down by 16 cm^{-1} to 500 cm^{-1}, while in Asp412Asn, ν(Fe–CO) is the same as that for the wild-type.[11]

These two mutants were also studied by low-temperature FTIR, providing the stretching frequencies for CO bound to heme a_3, $\nu(C{\equiv}O)_{a_3}$, as well as to Cu_B, $\nu(C{\equiv}O)_{Cu_B}$. As previously described for Asp412Asn,[1] $\nu(C{\equiv}O)_{Cu_B}$ is the same as that for wild-type, while $\nu(C{\equiv}O)_{a_3}$ is slightly shifted to 1666 cm^{-1}. The FTIR difference spectrum for CO-bound His333Asn is compared to wild-type in Figure 3. In the wild-type spectrum, the negative peak at 1964 cm^{-1} and the positive peak at 2060 cm^{-1} represent the stretching frequencies for the α forms of CO bound to heme a_3 and Cu_B, respectively. (β forms are also observed; see ref 14.) In His333Asn, $\nu(C{\equiv}O)_{a_3}$ is unchanged, although the signal is slightly broadened. In contrast, $\nu(C{\equiv}O)_{Cu_B}$ is dramatically shifted to 2080 cm^{-1}, and the signal is attenuated. Thus, in His333Asn, Cu_B shows less CO binding with a stronger carbon–oxygen bond, indicating an altered environment that may weaken the copper–carbon bond. This is consistent with the assignment of His333 as a ligand of Cu_B.[2,3,5]

Additional information comes from previous resonance Raman analyses of the fully reduced, unliganded enzymes.[1-3] In His333Asn, the iron–histidine bond of heme a_3 is unperturbed, while in Asp412Asn, $\nu(Fe{-}N_{his})$ is shifted from 214 to 218 cm^{-1}, indicating strengthening of this bond (Table 1).

The vibrational data from these resonance Raman and FTIR analyses are summarized in Table 1. Two important conclusions can be drawn. First, Li and Spiro[12] have correlated ν(Fe—CO) and $\nu(C{\equiv}O)$ vibrational data for a variety of heme proteins and model compounds, showing that an inverse linear relationship

Table 1: Vibrational Data for Mutant and Wild-type Cytochrome *c* Oxidases[a]

enzyme	$\nu(Fe{-}N_{his})$ (cm^{-1})	ν(Fe–CO) (cm^{-1})	$\nu(C{\equiv}O)a_3$ (cm^{-1})	$\nu(C{\equiv}O)Cu_B$ (cm^{-1})
wild-type	214	516	1964	2060
His333Asn	214	500	1964	2080
Asp412Asn	218	516	1966	2060

[a] The $\nu(Fe{-}N_{his})$ vibrational results are taken from refs 9 (wild-type), 3 (His333Asn), and 1 (Asp412Asn). The $\nu(C{\equiv}O)$ data for wild-type and Asp412Asn are taken from refs 14 and 1, respectively.

exists between the frequencies of these two modes. The underlying physical phenomenon for this correlation was identified as Fe to CO back-bonding which simultaneously strengthens the Fe—C bond and weakens the C≡O bond. The carbon monoxide complexes of heme/Cu terminal oxidases, however, deviate significantly from the linear correlation.[12,13] Mutant oxidase Asp412Asn, with its wild-type Fe–CO stretching frequency, continues to be an outlyer, but the altered ν(Fe–CO) mode of His333Asn brings this species into good agreement with the Li/Spiro correlation. Since the $Fe{-}N_{his}$ bond of His333Asn in its unliganded form is unperturbed relative to that of the wild-type (Table 1), the proposed explanations for the deviation of the wild-type enzyme as reflecting weak ligation by the proximal histidine[12] or a tyrosine[13] appear unlikely. Rather it seems that distal effects in the cytochrome oxidase heme a_3–Cu_B pocket may act to strengthen the Fe–CO bond and produce the upshifted Fe–C vibrational frequency in the wild-type enzyme and that alteration of His333 to asparagine lessens this effect. For example, distal forces introduced by the proximity of Cu_B to the heme in the wild-type enzyme may cause a tilt of the CO bound to the iron; movement of Cu_B away from heme a_3 in His333Asn could lessen the tilt. By the arguments of Li and Spiro,[12] a less tilted Fe—C≡O configuration would decrease the π back-bonding between CO and the heme iron and produce the observed weakening of the Fe–CO bond in His333Asn. These results argue that distal forces, due to the proximity of Cu_B to the heme in heme/Cu oxidases, are likely to account for the deviation of these proteins from the ν(Fe—CO)/$\nu(C{\equiv}O)$ correlation.

Second, perturbation of the Cu_B ligand, His333, modifies the vibrational properties of the distal CO ligand but leaves the proximal $Fe{-}N_{his}$ bond unaffected. Conversely, mutation of Asp412 produces a shift in the iron–histidine stretching vibration but does not significantly alter the properties of the CO ligand in the distal pocket. These results provide strong support for the model of Figure 1 and are inconsistent with previously published models in which His284 of helix VI was proposed to ligate heme a_3. In these earlier proposed structures,[3,5] it is difficult to model Asp412 proximal to heme a_3 while taking into account the spectral evidence for the proximity of Tyr414 to heme *a*.[1] Thus, these data buttress the developing consensus that the redox centers in subunit I of cytochrome *c* oxidase are situated toward the outer side of the membrane and arranged in the linear order of Cu_B/heme a_3/heme *a*, with helix X providing ligands to both hemes.

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(11) The ν(Fe–CO) mode in the mutant enzymes was assigned by ^{13}CO isotopic labeling, and in each case a 4–5-cm^{-1} downshift was observed. The δ(Fe—C≡O) bending mode was also detected in the mutant and wild-type enzymes at frequencies characteristic of heme a_3–CO complexes[10] (see also ref 12).

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