The Oxygenated Complex of Cytochrome *d* Terminal Oxidase: Direct Evidence for Fe–O_2 Coordination in a Chlorin-Containing Enzyme by Resonance Raman Spectroscopy

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The cytochrome *d* terminal oxidase complex (CDO) is a terminal electron acceptor for many gram-negative bacteria.[1] It catalyzes the four-electron reduction of dioxygen to water using ubiquinol as an electron donor.[2] Resting CDO contains a total of three cofactors: two *b* hemes (low-spin b_{558} and high-spin b_{595}) and a novel chlorin cofactor, chlorin *d*.[3] The cyanide-resistant enzyme differs from cytochrome *c* oxidase or cytochrome *o* oxidase in that it contains no additional copper cofactors. Although CDO catalyzes transmembrane separation of protons and electrons, it does not function as a proton pump.[4]

The heme b_{558} is associated with the ubiquinol oxidase site of the enzyme,[5] whereas the heme b_{595} and chlorin *d* appear to be closely associated at the dioxygen reductase site. This chlorin *d* is postulated to be a dihydroxyprotochlorin[6] and very similar to the prosthetic groups of *Escherichia coli* catalase HPII[7] and *Neurospora crassa* chlorin catalase.[8] Given these properties, CDO provides an interesting alternative to the cytochrome *c* oxidases for the study of terminal oxidase properties and reaction mechanisms. Recent work has shown that CDO is also essential for aerobic nitrogenase activity in *Azotobacter*[9] and is present in *Klebsiella pneumoniae* during nitrogen fixation.[10] Its major role in nitrogen-fixing organisms is presumably that of an oxygen scavenger.

In a recent study of CDO, we identified a long-lived ferryl (Fe^{IV}=O) intermediate by resonance Raman (RR) spectroscopy and proposed a catalytic cycle for the reduction of O_2 by the enzyme complex.[11] The cycle, modeled after that first proposed by Hata-Tanaka and co-workers,[12] is shown in Figure 1. Dioxygen binds reversibly to ferrous *d* (d_{630}) to give a one-electron-reduced[13] Fe–O_2 adduct designated as d_{650} owing to its prominent absorbance at ~645 nm. The bound O_2 is then reduced through several intermediate states: a poorly characterized 2-electron-reduced peroxo intermediate,[12,14] an isolable ferryl intermediate with notable absorbance at 680 nm (d_{680}),[11] and a stable ferric *d* state in which both atoms of the originally bound O_2 have been reduced to water.[15] Another one-electron equivalent returns the enzyme to the ferrous *d* state. Anaerobic addition of a strong reductant (such as dithionite) will reduce all of the porphyrinic cofactors of the enzyme, yielding a totally reduced species.

In the present study, we have obtained RR spectroscopic evidence for the oxygenated enzyme. By using red excitation within the absorption of the d_{650} chromophore, we have observed an Fe–O_2 stretching vibration at 568 cm^{-1}; the frequency shifts to 542 cm^{-1} in Fe–$^{18}O_2$, as shown in Figure 2. This is the first report of the vibrational frequency of ν(Fe–O_2) for a biological, oxygenated hydroporphyrin complex. Remarkably, these values are essentially identical to those of oxyhemoglobin.[16]

As purified, the enzyme exists in an ~70:30 mixture of two forms: the oxygenated (Fe^{II}–O_2 ↔ Fe^{III}–O_2^-) and ferryl (Fe^{IV}=O) species, respectively. The optical absorption spectrum of the enzyme shows two major features in the 600–800-nm region: a prominent absorption at 645 nm and a shoulder at 680 nm (Figure 3, inset). The 645-nm band is indicative of O_2 binding to the chlorin *d*,[17] and the 680-nm shoulder has recently been assigned to the ferryl intermediate of the enzyme.[11] In the oxygenated and ferryl states of CDO, the two *b* hemes are oxidized and lose their prominent Q-band features characteristic of the fully reduced hemes. A mutant of CDO, His186Leu, lacks the low-spin heme b_{558}[18] and is isolated solely in the oxygenated form. Preliminary studies show that this H186L mutant, as isolated, lacks the 680-nm feature seen in the visible spectrum of the wild-type enzyme and that it has a more prominent 645-nm band (data not shown). It is conceivable that the elimination of the b_{558} cofactor prevents the enzyme from redox cycling, effectively trapping it in the oxygenated state.

Whereas Soret excitation gives rapid photoreduction of the enzyme, excellent RR spectra are attainable with excitation into its 645-nm absorption band (Figure 3). Resonance Raman spectra of both wild-type (Figure 3) and H186L enzymes are nearly identical, showing that the low-spin heme b_{558} makes no significant contribution to the RR spectrum recorded with 630–650-nm excitation. Similarly, the high-spin heme b_{595} is not expected to exhibit such strongly enhanced modes at these excitation wavelengths.

The appearance of an intense peak at 1247 cm^{-1} (Figure 3) and the polarized nature of the Raman bands are both indicative of a chlorin chromophore. The 1247-cm^{-1} feature correlates with the porphyrin ν_{42a} (E_u) mode that is Raman-forbidden for a

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(13) "*i*-electron reduced" (*i* = 0,1,2,3,4) refers to the state of reduction of the dioxygen molecule. When *i* = 1, O_2 is formally superoxide (O_2^-); *i* = 2 is peroxide; *i* = 3 implies that O—O bond cleavage has occurred to yield a ferryl species (Fe^{IV}=O) and H_2O; and when *i* = 4, O_2 is fully reduced to $2H_2O$.

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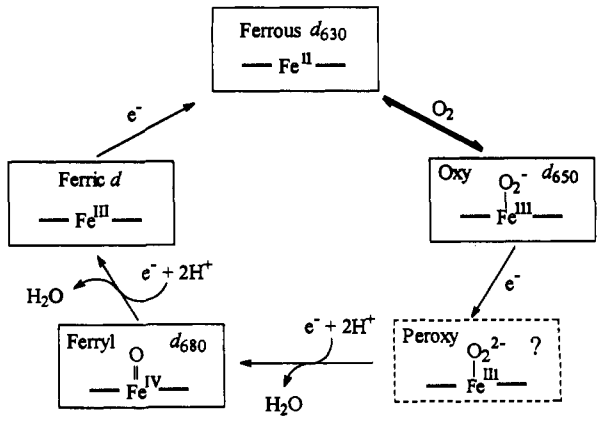

Figure 1. Proposed catalytic cycle of cytochrome *d* oxidase showing the chemical changes in the chlorin/O_2 species. The two *b* hemes, which participate in electron transfer during this cycle, are not indicated.

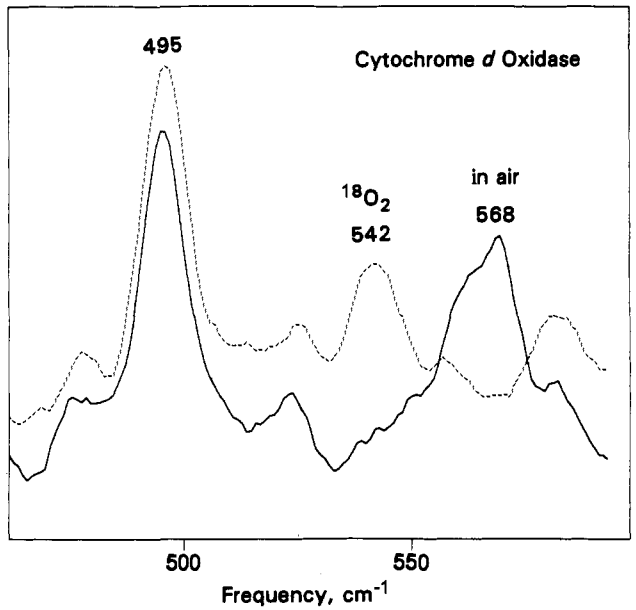

Figure 2. Resonance Raman spectra showing the isotope-sensitive Fe–O_2 mode. The samples were prepared by first deoxygenating the resting enzyme by addition of protocatechuate-3,4-dioxygenase and a minimum amount of the substrate protocatechuic acid, followed by exposure to air ($^{16}O_2$) or $^{18}O_2$. Conditions: Dilor Z-24 with cooled Hamamatsu R943-02 PMT, 630-nm excitation (R6G dye laser), <50 mW, multiple scans, 1 cm^{-1}/pt, 3 s/pt, and an instrumental resolution of ~5 cm^{-1}. Similar results were obtained with 647.1-nm excitation.

porphyrin but strongly enhanced in metallochlorins with Q-band excitation.[19,20] All features of the high-frequency Raman spectra of CDO (except for the band at 1576 cm^{-1}) are polarized, as is expected for a chlorin.[19] The 1576-cm^{-1} band, which is depolarized, most likely correlates with the porphyrin ν_{19} (A_{2g}) anomalously polarized mode.[19,21] These observations, taken together, indicate that the RR spectra observed with red excitation are probably due to the chlorin chromophore. The ν(Fe–O_2) isotope effect was observed in RR spectra with excitation wavelengths from 630 to 647.1 nm throughout the chlorin *d* absorption. While red excitation is not normally used for enhancement of axial ligand modes for a porphyrin, ν(Fe–O_2) has been seen with Q-band excitation for hemoglobin.[22]

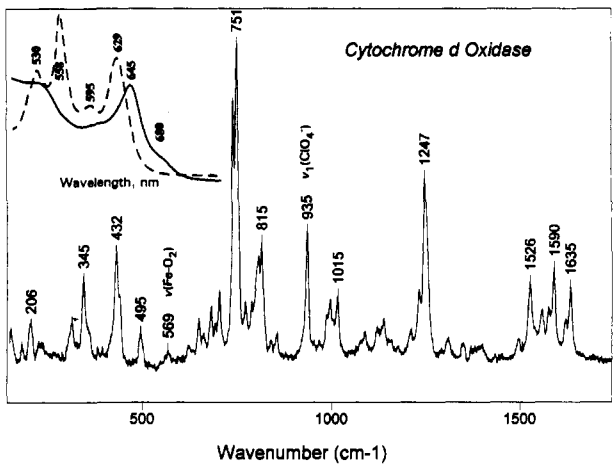

Figure 3. Resonance Raman spectrum of cytochrome *d* oxidase, as purified. Conditions: as in Figure 2, 647.1-nm excitation. The inset shows the Q-band absorption spectrum of the enzyme: solid line, as purified; dashed line, dithionite reduced.

Resonance Raman spectroscopy of CDO with 647.1-nm excitation had been attempted by Poole et al.[23] These authors reported a band at 1088 cm^{-1} which they assigned to an O—O stretch, but this finding was not confirmed by isotopic substitution. Although we also see a weak band at ~1085 cm^{-1} (Figure 3), this band does not shift in the $^{18}O_2$ sample, and, hence, we cannot confirm this assignment.

The ν(Fe–O_2) frequencies for CDO are very similar to those noted for other oxygen-binding proteins, such as hemoglobin,[16,22] myoglobin,[24] and cytochrome *c* oxidase.[25] This suggests that the nature of the binding of O_2 to the chlorin *d* iron is essentially identical to that seen in heme enzymes, despite the high oxygen affinity of CDO and the altered cofactor structure. This is consistent with the findings of Ozaki and co-workers, who found that the Fe–axial ligand vibrations of Fe–octaethylchlorin derivatives are very close to those of the corresponding Fe–octaethylporphyrins.[26] Others have shown that, for porphyrins, the electron-donating capabilities of the ring substituents exert a significant effect on the porphyrin–O_2 binding constant, yet the Fe–O_2 bond strength and stretching frequency remain unchanged.[27] The lack of correlation of Fe–O_2 stretching frequencies with macrocycle substituents apparently extends to chlorin *d*.

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