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Identification of a Ferryl Intermediate of *Escherichia coli* Cytochrome *d* Terminal Oxidase by Resonance Raman Spectroscopy†

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ABSTRACT: The 680-nm-absorbing "peroxide state" of the *Escherichia coli* cytochrome *d* terminal oxidase complex, obtained by addition of excess hydrogen peroxide to the enzyme, is shown to be a ferryl intermediate in the catalytic cycle of the enzyme. This ferryl intermediate is also created by aerobic oxidation of the fully reduced enzyme. Resonance Raman spectra with 647.1-nm excitation show an Fe^{IV}=O stretching band at 815 cm^{-1}, a higher frequency than noted in any other ferryl-containing enzyme to date. The band shows an $^{16}O/^{18}O$ frequency shift of −46 cm^{-1}, larger than that observed for any porphyrin ferryl species. The Fe^{IV}=O formulation was unambiguously established by oxidations of the reduced enzyme with $^{16}O_2$, $^{18}O_2$, and $^{16}O^{18}O$. Only the use of a mixed-isotope gas permitted discrimination between a ferryl and a peroxo structure. A catalytic cycle for the cytochrome *d* terminal oxidase complex is proposed, and possible reasons for the high ν(Fe=O) frequency are discussed.

Cytochrome *d* terminal oxidase complex is one of two terminal electron acceptors of *Escherichia coli* catalyzing the four-electron reduction of dioxygen to water using ubiquinol as an electron donor (Anraku & Gennis, 1987; Gennis, 1987; Poole, 1983). The enzyme is fundamentally different from cytochrome *c* oxidase. It contains no copper, but instead, it contains two *b* hemes (one low spin, designated as b_{558},[1] and one high spin, designated as b_{595}) and a novel chlorin cofactor, chlorin *d* (Lorence et al., 1986). The chlorin *d* cofactor is postulated to be a dihydroxyprotochlorin (Timkovich et al., 1985; Vavra et al., 1986; Sotiriou & Chang, 1988; Andersson et al., 1987). The enzyme likely contains a single chlorin (Meinhardt et al., 1989; Rothery & Ingledew, 1989) but the possibility of a second chlorin cannot be ruled out (Bonner et al., 1991).

As purified, the enzyme has an intense absorption at 650 nm attributed to the oxygenated form of the reversibly dioxygen-binding chlorin *d* (Lorence & Gennis, 1989; Poole et al., 1983a). Addition of hydrogen peroxide to the enzyme above stoichiometric amounts leads to the partial loss of absorption at 650 nm and an increase in absorption at 680 nm (Lorence & Gennis, 1989; Poole & Williams, 1988). Intermediates with absorption maxima at 650 and 680 nm are seen in the low-temperature reaction of O_2 with reduced cytochrome *d* complex from *Acetobacter pasteurianus* (Williams & Poole, 1987) and *E. coli* (Poole et al., 1983b). In both cases, the 650-nm species is seen first, decreasing as the 680-nm intermediate appears.

We have used resonance Raman (RR) spectroscopy to show that this 680-nm-absorbing species (d_{680}) is a long-lived[2] ferryl

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[1] Abbreviations: amu, atomic mass unit, b_{558}, heme b_{558}; b_{595}, heme b_{595}; d_{650}, 650-nm-absorbing oxygenated intermediate of the cytochrome *d* complex; d_{680}, 680-nm-absorbing ferryl (Fe^{IV}=O) intermediate of the cytochrome *d* complex; FFT, fast Fourier transform; mW, milliwatt(s); OEP, octaethylporphyrin; RR, resonance Raman; TPP, tetraphenylporphyrin.

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(Fe^{IV}=O) intermediate in the catalytic cycle of the enzyme. This intermediate can be obtained by addition of excess H_2O_2 to the ferric enzyme or by O_2 oxidation of the fully reduced enzyme. It is identified by a very intense Fe^{IV}=O stretching band at 815 cm^{-1} in the Q-band excitation RR spectrum. This band moves to 769 cm^{-1} upon isotopic substitution of ^{18}O for ^{16}O. The observed Fe=O stretching frequency and magnitude of the isotope shift are larger than those observed for any other enzyme possessing a ferryl state.

Experimental Procedures

Protein samples were prepared in the oxygenated form at the University of Illinois according to standard procedures (Miller & Gennis, 1983). Initial enzyme concentrations ranged from 60 to 110 μM in a pH 7.5 buffer solution of 10 mM sodium phosphate with 5 mM EDTA, 0.5 mg/L leupeptin, and 0.025% *N*-lauroylsarcosine (sodium salt). Reduction of the enzyme was carried out under anaerobic conditions by addition of a minimum amount of freshly prepared 10–20 mM $Na_2S_2O_4$ solution (in 100 mM phosphate buffer). The amount of dithionite solution to give complete reduction was determined in a parallel experiment by monitoring the extent of reduction spectrophotometrically. The aerobically oxidized enzyme was prepared by introducing oxygen [bottled O_2 gas (or air), $^{18}O_2$, or isotopically mixed oxygen] to the reduced enzyme. Composition of the mixed-isotope dioxygen (1:1 $^{16}O^{18}O$, Cambridge Isotope Laboratory) was confirmed by Raman spectroscopy to be 1:2:1 $^{16}O_2/^{16}O^{18}O/^{18}O_2$. Oxidations with hydrogen peroxide were performed with the "as isolated", oxygenated enzyme by addition of ~8 mM H_2O_2 or ~60 μM $H_2{}^{18}O_2$. Similar results were obtained when anaerobically oxidized (with ferricyanide) enzyme was treated with H_2O_2.

Resonance Raman spectra were obtained with computer-controlled Dilor Z-24 and Jarrell-Ash 25-300 Raman spectrophotometers. Excitation was provided by a Spectra-Physics Kr^+ ion laser operating at 647.1 nm (≤50 mW). The Raman instruments were calibrated with indene or carbon tetrachloride and the Rayleigh line of the laser. Spectra were smoothed using a Wiener FFT spectral-filtering algorithm (Press et al., 1986). Spectra were collected from solution samples contained either in a melting-point capillary (1.5–1.8 mm o.d.) or in a 5-mm NMR tube. The NMR tubes were spun to decrease sample decomposition. Sample geometry was 90° for the capillaries and 180° backscattering for the NMR tubes. Sample temperature was held near 5 °C by contact with a cold finger immersed in ice/water or by blowing cold nitrogen over the sample.

Visible absorption spectra of the samples were obtained on a Perkin Elmer Lambda 9 spectrophotometer using a spectral bandwidth of 2 nm. Special cell holders made of black Delrin were used to mask the sample tubes (capillaries and NMR tubes). Water in the appropriate sample tube was used to set the background-blank correction. While this method is not useful for a quantitative measure of the sample concentration (owing to the curvature of the cells), it does give a good indication of any spectral changes. Absorption spectra of the samples were recorded before and after the Raman experiments to determine the extent of photoinduced reaction, which was judged to be less than 20% during the course of the experiment.[3]

[2] While we have not carried out quantitative studies on the stability of the ferryl species, we have noted that it persists for at least 12 h at room temperature and several months at −80 °C.

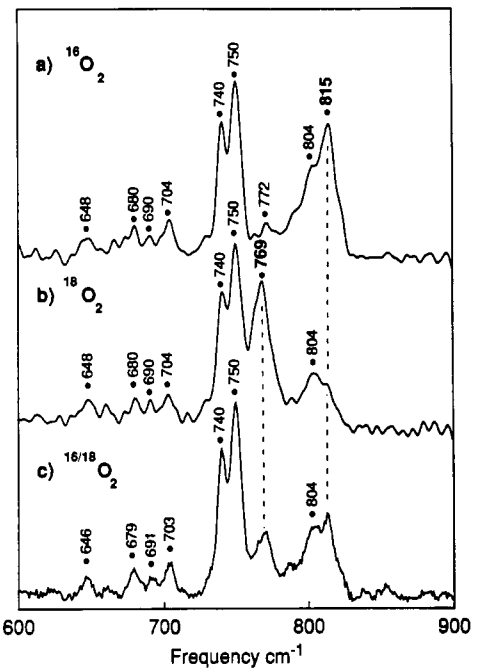

FIGURE 1: Resonance Raman spectra of the products of O_2 oxidation of dithionite-reduced cytochrome *d* complex. (a) Oxidation with dioxygen (natural-abundance isotopic composition), resulting in a strong band at 815 cm^{-1}. (b) Oxidation with $^{18}O_2$, showing the shift of the 815 cm^{-1} band to 769 cm^{-1}. (c) Oxidation with mixed-isotope $^{16/18}O$ dioxygen, showing the lack of a separate 792 cm^{-1} band. Conditions: 180° backscattering geometry; instrumental resolution 5.0 cm^{-1}; scan rate 1 cm^{-1}/s with repetitive scanning.

Results and Discussion

Reduced Enzyme plus O_2. Oxygen oxidation of the dithionite-reduced cytochrome *d* complex gives a species with a visible spectrum that appears to be primarily due to the d_{680} intermediate. Resonance Raman spectra with 647.1-nm excitation of dithionite-reduced enzyme oxidized with $^{16}O_2$, $^{18}O_2$, and mixed-isotope O_2 (50% ^{16}O, 50% ^{18}O) are shown in Figure 1. The 815-cm^{-1} band increases dramatically in intensity in the $^{16}O_2$-oxidized d_{680} species (Figure 1a) relative to that of the anaerobically (ferricyanide) oxidized enzyme (not shown). This band shifts to 769 cm^{-1} on oxidation with $^{18}O_2$ (Figure 1b). Two possibilities for this frequency are an O—O stretching mode of a coordinated peroxide or the Fe^{IV}=O stretching mode of a ferryl species. Our original inclination was to assign this feature as a peroxo O—O stretch. $[Fe^{III}(edta)(O_2^{2-})]^{3-}$ exhibits ν(O—O) at the identical RR frequency (Ahmad et al., 1988). The reaction product between Fe(OEP)Cl and KO_2 has an IR band at 806 cm^{-1} (759 cm^{-1} on substitution of ^{18}O) that has been interpreted as the O—O

[3] Since the optical spectrum of the sample exposed to the laser beam measures its bulk absorption, the possibility still exists that the scattering volume during the RR experiment may have a considerable concentration of a photodegradation product. However, repetitive RR scans give superimposable spectra even after several hours of data collection. The only loss in intensity occurs at the ferryl frequency, showing that some of this species is lost with time and exposure. The loss is minimized in samples that are spun or exposed to lower laser power. Under the stated conditions (647.1-nm excitation, 50-mW power at a spinning sample illuminated in a backscattering geometry), the reduction in the ferryl intensity was 30% over a period of 2.5 h relative to the intensity at the start of data collection. In addition, the very clear isotope effect observed for samples prepared from H_2O_2 or O_2 proves that the dominant RR scatterer is the ferryl species. Given that neither the optical spectrum nor the RR spectrum shows substantial changes outside of a loss of intensity at 680 nm and 815 cm^{-1} (769 cm^{-1} in the ^{18}O samples), respectively, we suggest that the decomposition product is still a ferric chlorin chromophore. Figure 3 suggests a possible state of the enzyme.

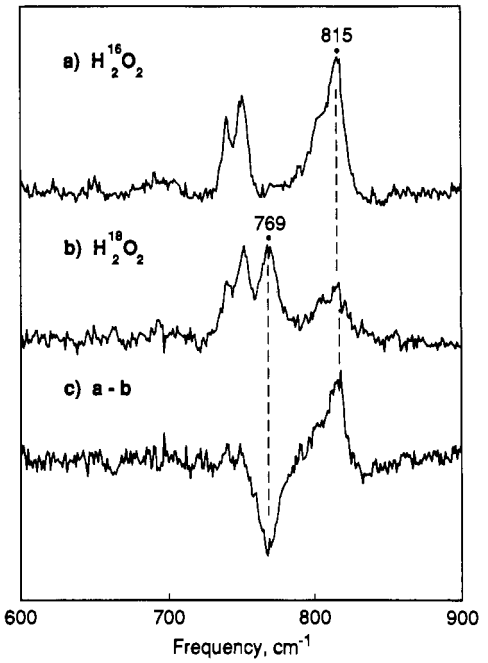

FIGURE 2: Resonance Raman spectra of the products of addition of hydrogen peroxide to oxidized cytochrome *d* complex. (a) Excess H_2O_2. (b) Excess $H_2{}^{18}O_2$. Conditions: 90° scattering geometry; resolution 5.5 cm^{-1}; scan rate 1 cm^{-1}/s with repetitive scanning. (c) Difference spectrum (a − b).

stretching mode of $[Fe^{III}(OEP)(O_2^{2-})]^-$ (McCandlish et al., 1980). We note, however, that this formulation has not been substantiated by a mixed-isotope dioxygen experiment. These results are very similar to those for the cytochrome *d* complex.

The assignment of a bound peroxo group, however, is ruled out by the experiment in which the reduced enzyme is oxidized with mixed-isotope oxygen (Figure 1c). If the d_{680} intermediate were a peroxo adduct, oxidation by the mixed-isotope oxygen would be expected to yield a Raman spectrum with three bands with intensities in a 1:2:1 ratio—$^{16}O_2$ (815 cm^{-1}), $^{16}O^{18}O$ (expected at 792 cm^{-1}), and $^{18}O_2$ (769 cm^{-1}). In the mixed-isotope experiment, bands are seen only at 815 and 769 cm^{-1}, with no band near 792 cm^{-1}. These results require O—O bond cleavage and lead us to assign the observed band as originating from an Fe^{IV}=O stretching mode. *We cannot overemphasize the importance of the mixed-isotope $^{16}O/^{18}O$ experiment to reach this conclusion.*

The fact that the stable ferryl form produced by O_2 addition to the dithionite-reduced enzyme is a three-electron-reduced species suggests that cytochrome *d* complex has a total of 3 reducing equiv: two *b* hemes and one chlorin *d*. This would argue against the proposal (Bonner et al., 1991) that the enzyme contains two *d* chlorins. Under natural conditions, it is likely that a fourth electron is available, since a stable ubisemiquinone is probably formed on the enzyme (W. J. Ingledew, T. M. Zuberi, S. W. Meinhardt, and R. B. Gennis, unpublished results).

Oxidized Enzyme plus H_2O_2. Similar results are obtained by direct formation of the d_{680} species by addition of hydrogen peroxide to the ferricyanide-oxidized enzyme. The RR spectrum (600–900 cm^{-1}) of the d_{680} intermediate formed by reaction with H_2O_2 is shown in Figure 2. On addition of excess H_2O_2 to either the oxidized enzyme or the oxygenated ("as isolated") enzyme, a large increase in the intensity of the 815-cm^{-1} feature is observed. If $H_2{}^{18}O_2$ is added to the oxygenated enzyme, this band shifts to 769 cm^{-1} (Figure 2b), the identical frequency as observed for the $^{18}O_2$-oxidized enzyme (Figure 1b). No evidence of ferryl oxygen exchange with water was observed under the conditions of either ^{18}O experiment.

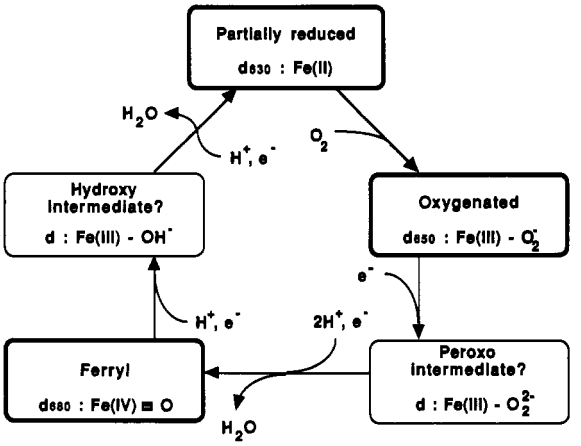

FIGURE 3: Proposed catalytic cycle of cytochrome *d* complex. Intermediates for which there is strong experimental evidence are enclosed in bold lines. Electron flow goes from ubiquinol through the two *b* hemes (not shown) to the chlorin *d*.

Reaction Scheme for the Native Enzyme. The identification of the d_{680} species as a ferryl intermediate of the catalytic cycle of cytochrome *d* complex allows us to propose a scheme for the enzyme reaction (Figure 3). This scheme is similar to that proposed by Hata-Tanaka and co-workers (Hata-Tanaka et al., 1987). Electron flow through the enzyme goes from b_{558} to b_{595} to *d* (Hata-Tanaka et al., 1987; Poole & Williams, 1987). Starting with the chlorin in the ferrous state, O_2 binds reversibly to form the oxygenated species characterized by an intense absorption at 650 nm (d_{650}). The reversible nature of this reaction has been suggested by optical spectroscopy, which shows that anaerobic removal of O_2 from the ferric–superoxide complex (d_{650}) results in a "partially reduced" enzyme (d_{630}) characterized by a ferrous chlorin *d* and ferric b_{558} and b_{595} (Lorence & Gennis, 1989).

Although the enzyme, as isolated, is mainly in the oxygenated state as evidenced by its 650-nm absorption, there is always a small shoulder at 680 nm. The latter is indicative of the presence of some of the ferryl intermediate (d_{680}) in the purified enzyme. It is accompanied by a 815-cm^{-1} feature in the RR spectrum, but with much lower intensity than that obtained from the addition of peroxide. The intensity of this Raman band in the purified enzyme is preparation-dependent. Moreover, addition of a small amount of O_2 during oxidation by ferricyanide leads to the appearance of both the 815-cm^{-1} feature in the RR spectrum and the 680-nm shoulder in the optical spectrum; the intensities of the two are directly correlated. Careful monitoring of the visible spectrum during reduction of the oxygenated enzyme by dithiothreitol shows that the kinetics of the disappearance of the 650- and 680-nm absorption bands proceed with dramatically different rates. Finally, the 815-cm^{-1} bands seen in the various forms of the enzyme all show the same behavior in the RR experiment: an ~2-fold decrease in intensity after 2 h of irradiation with 50 mW of 647.1-nm light. Thus, we conclude that the enzyme, as purified, consists of a mixture of d_{650} and d_{680} species.

Since ferryl is a three-electron-reduced iron–O_2 species (ferryl oxygen plus water) and the d_{650} intermediate is a one-electron-reduced dioxygen species ($O_2^{\bullet-}$), the catalytic reduction of d_{650} to d_{680} may occur through a two-electron-reduced intermediate (Figure 3). There is evidence for such an intermediate: (i) Williams and Poole (1987), in investigating the kinetics of O_2 reaction with reduced cytochrome *d* complex from *Acetobacter pasteurianus* spectrophotomet-

rically at low temperature, found that the disappearance of d_{650} and the appearance of d_{680} were inconsistent with the direct conversion of the 650-nm-absorbing to the 680-nm-absorbing species. This observation suggests the formation of a two-electron-reduced peroxo intermediate. (ii) Hata-Tanaka and co-workers (Hata-Tanaka et al., 1987) have observed a low-spin EPR signal at $g = 2.15$ while monitoring the reaction of reduced *E. coli* cytochrome *d* complex with O_2. They referred to this species as "oxygen binding intermediate II," that is formally equivalent to a ferric chlorin *d*–peroxo complex. (iii) Lorence and Gennis (1989) reported a "peroxy intermediate" state of the enzyme, formed by stoichiometric addition of H_2O_2 to the d_{650} (oxygenated) form of the enzyme. Upon addition of excess peroxide, this peroxy intermediate reacts to form the d_{680} species plus O_2.

Ferryl Intermediate. The d_{680} intermediate is analogous to the recently characterized ferryl of cytochrome *c* oxidase (Ogura et al., 1990; Varotsis & Babcock, 1990; Han et al., 1990), that can also be formed by addition of H_2O_2 (Larsen et al., 1990), as well as the Fe^{IV}=O states of several other heme enzymes. However, the observed frequencies and isotope shifts for ν(Fe=O) of the cytochrome *d* complex are higher than any previously reported for an enzyme. The ferryl stretching mode has been observed at 745 cm^{-1} in lactoperoxidase (Reczek et al., 1989), 753–767 cm^{-1} in cytochrome *c* peroxidase (Reczek et al., 1989; Hashimoto et al., 1986), 775 cm^{-1} in bovine liver catalase (Chuang et al., 1989), 782 cm^{-1} in myeloperoxidase (Oertling et al., 1988), 775 cm^{-1} (pH 7) or 787 cm^{-1} (pH 11) in horseradish peroxidase (Terner et al., 1985; Sitter et al., 1985a), 788 cm^{-1} in cytochrome *c* oxidase (Ogura et al., 1990; Varotsis & Babcock, 1990; Han et al., 1990), and 797 cm^{-1} in myoglobin (Sitter et al., 1985b). Cytochrome *d* complex has a ν(Fe=O) frequency that is $\geq$18 cm^{-1} higher than that seen in any of these other proteins. If one excepts myoglobin as a nonphysiological ferryl species, then ν(Fe=O) of chlorin *d* is $\geq$27 cm^{-1} higher in frequency.

Several factors may contribute to this high frequency. Model ferryl–porphyrin compounds with ν(Fe=O) frequencies ranging from 807 (Schappacher et al., 1986) to 861 cm^{-1} (Proniewicz et al., 1991) show a large trans ligand effect, with ν(Fe=O) being inversely dependent upon the electron-donating ability of the proximal ligand (Oertling et al., 1990). A trans effect has also been noted on ν(V=O) of six-coordinate vanadyl porphyrins (Su et al., 1988). Most of the enzymes investigated have histidine proximal ligands with the exception of catalase, that has a tyrosinate ligand (Fita et al., 1986). Both histidine and tyrosinate are strong electron donors. Preliminary ENDOR studies suggest that the proximal ligand of chlorin *d* is not a histidine (T. M. Zuberi, R. B. Gennis, F. Jiang, and R. L. Belford, unpublished results). Thus, one factor contributing to the high ferryl frequency of cytochrome *d* complex might be an axial ligand with lower electron donating ability than histidine or tyrosinate.

Hydrogen bonding of ferryl species causes a decrease in ν(Fe=O). For example, the ν(Fe=O) of certain peroxidases increases in frequency by 10–15 cm^{-1} at alkaline pH due to deprotonation of the distal histidine that serves as an H-bond donor at neutral pH (Reczek et al., 1989; Chuang et al., 1989; Sitter et al., 1985a). Thus, the high ferryl stretching frequency of cytochrome *d* complex could indicate a lack of hydrogen bonding of the ferryl moiety. Work is in progress to assess the effects of pH and D_2O on the Raman spectra of cytochrome *d* complex.

The third possible factor in the high ferryl stretching frequency is the dihydroxyprotochlorin IX macrocycle of the chlorin *d*. The disruption of the porphyrin conjugation by two strongly electronegative oxygen atoms might lead to a decrease in the electron-donating ability of the chlorin *d* versus protoporphyrin. [A similar argument has been evoked to account for the lowered cyanide affinity of iron chlorin *d* relative to iron protoporphyrin (Vavra et al., 1986).] Hence, substitution of porphyrins with electron-withdrawing groups has been shown to increase the ferryl stretching frequency. Proniewicz and co-workers (Proniewicz et al., 1991) have measured the Fe=O frequencies for a number of synthetic ferryl–prophyrin complexes in O_2 matrices. The ferryl complex of iron tetrakis(pentafluorophenyl)porphyrin shows the highest ν(Fe=O) frequency yet observed, 861 cm^{-1}, 8 cm^{-1} higher than that seen for O=Fe(TPP). This high frequency is explained by the electron-withdrawing effect of the pentafluorophenyl groups, making the porphyrin ring electron-deficient (Proniewicz et al., 1991). The withdrawal of electron density by the macrocycle increases the π-donor strength of the oxygen and strengthens the Fe^{IV}=O bond. It is possible that a similar loss of electron density for the chlorin *d* versus porphyrin could lead to an increased Fe^{IV}=O bond strength for ferryl cytochrome *d* complex relative to other enzymes.

The isotopic shift on ^{18}O substitution, -46 cm^{-1}, is higher than that for any previously studied Fe=O compound. The calculated oxygen isotope shift for ν(Fe=O) with an iron mass of 56 amu is -36 cm^{-1}. However, the expected isotope shift for an iron–chlorin core with a very tightly bound metal having an effective "infinite" mass gives $\Delta\nu = -47$ cm^{-1}. It is possible that the chlorin coordinates the iron atom more strongly than a porphyrin, thus contributing to a larger isotope shift.

Our major finding is that the 680-nm-absorbing species of cytochrome *d* complex formed by addition of peroxide or aerobic oxidation of the fully reduced enzyme is a very stable ferryl intermediate. Many additional questions remain. For example, what is the role of the b_{595} cofactor, and what are the identities of other catalytic intermediates? Do hydroporphyrin-containing systems exhibit discrete compounds I and II? The number of investigations of highly oxidized chlorins is still very limited (Hanson et al., 1981; Fujita & Fajer, 1983). Extension of the present work using RR spectroscopy to other possible intermediates and to site-directed mutants of cytochrome *d* complex (Fang et al., 1989) is in progress and should allow us to further characterize the reaction pathway of this enzyme.

Acknowledgments

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