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Redox transitions between oxygen intermediates in cytochrome-*c* oxidase

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ABSTRACT **Some intermediates in the reduction of O_2 to water by cytochrome-*c* oxidase have been characterized by optical, Raman, and magnetic circular dichroism spectroscopy. The so-called "peroxy" (P) and "ferryl" (F) forms of the enzyme, which have been considered to be intermediates of the oxygen reaction, can be generated when the oxidized enzyme reacts with H_2O_2, or when the two-electron reduced ("CO mixed-valence") enzyme reacts with O_2. The structures as well as the overall redox states of P and F have recently been controversial. We show here, using tris(2,2′-bipyridyl)ruthenium(II) as a photoinducible reductant, that one-electron reduction of P yields F, and that one-electron reduction of F yields the oxidized enzyme. This confirms that the overall redox states of P and F differ from the oxidized enzyme by two and one electron equivalents, respectively. The structures of the P and F states are discussed.**

The heme–copper oxidases catalyze the reduction of O_2 to water in cell respiration. This redox reaction is uniquely coupled to proton translocation across the mitochondrial or bacterial membrane, creating an electrochemical proton gradient, which is subsequently used to drive the synthesis of ATP in oxidative phosphorylation. The reduction of oxygen to water in these enzymes takes place at a binuclear heme iron/copper (Fe_{a3}, Cu_B) site. These enzymes are classified as cytochrome-*c* oxidases or quinol oxidases according to the electron donor. In the case of cytochrome-*c* oxidases, the initial electron acceptor is a copper center, Cu_A (1). In all heme–copper oxidases, there is a low-spin heme (Fe_a) which serves as the immediate electron donor to the oxygen reduction site. Two different groups have recently published three-dimensional crystal structures of cytochrome-*c* oxidase (2–4).

On the basis of a large amount of time-resolved spectroscopic work (see ref. 5 for a review), the catalytic cycle of these enzymes may be described as follows: Once the binuclear heme iron/copper site is reduced, it can bind O_2 reversibly to form a primary "oxy" intermediate (6–12). If the enzyme is fully reduced, then the bound dioxygen is "trapped" to the site by fast electron transfer from the low-spin heme (8, 13–15), as well as from the oxygen-binding heme itself, presumably yielding a "peroxy" intermediate (ref. 5 and references therein).† Subsequently, this intermediate is protonated, the O—O bond breaks, and a "ferryl" intermediate is formed (16–18). Arrival of a fourth electron via the low-spin heme finally reduces the ferryl heme iron to ferric, after which the cycle can begin again.

Two spectral forms of the binuclear heme/copper center in cytochrome-*c* oxidase, originally described in mitochondria, are believed to be intermediates in the O_2 reaction. Energization of the mitochondria with ATP creates an electrochemical proton gradient by reversal of the H^+-ATPase. Together with a high acceptor potential at cytochrome *c*, this was discovered to lead to a partial reversal of the oxygen reaction (19–22). In equilibrium experiments, the initially oxidized ferric/cupric binuclear site was shown to undergo two consecutive one-electron oxidation steps with ferricytochrome *c* as electron acceptor (19). At lower driving force, this yielded a species with a major peak at ≈580 nm ($\varepsilon_{580} - \varepsilon_{630} \approx 5.3$ $mM^{-1}\cdot cm^{-1}$) and a minor one at ≈530 nm, as observed from the difference spectrum relative to the oxidized enzyme. At higher driving force, this species was replaced by another with a major peak at 607 nm ($\varepsilon_{607} - \varepsilon_{630} \approx 11$ $mM^{-1}\cdot cm^{-1}$), and a minor one at ≈566 nm (20). Both compounds exhibit similar red-shifted Soret difference spectra relative to the oxidized enzyme (23). Equilibrium redox titrations (19) suggested that the overall redox state of the 580-nm species is 1 and that of the 607-nm species is 2 electron equivalents more oxidized than the ferric/cupric binuclear-site. Comparison of rates of formation of these compounds with the rate of reduction of cytochrome *c* during such electron backflow gave results consistent with this proposal (20).

Wikström (19) proposed that the "580 nm species" has an oxyferryl heme iron, and the "607 nm species," a ferric heme peroxide structure, and they were named **F** and **P**, respectively, on this basis (20–22).

There are also other routes of forming the **P** and **F** species. When the "CO mixed-valence" (two-electron reduced) enzyme reacts with O_2 a state originally called "compound C" is formed (6, 24), which is analogous to **P** on the basis of its optical spectrum. This is clearly consistent with **P** being a ferric peroxy state. The corresponding reaction between a three-electron reduced enzyme and O_2 has been shown to yield **F** (25–27). In addition, both **F** and **P** have been observed as products of the reaction of the oxidized enzyme with H_2O_2 (28–32). Low concentrations of peroxide typically yield largely **P**, thought to be simply due to binding of peroxide to the ferric heme, whereas high concentrations yield mostly **F**.

The structural and redox assignments of **F** and **P** have recently become matters of some controversy. **P** has been proposed to be a ferryl species (33, 34), and **F** a low-spin form of the oxidized ferric/cupric enzyme (33). Weng and Baker (35) and Watmough *et al.* (34) have suggested that both **F** and **P** are "ferryl" compounds, but with an additional free radical center in **P**. Similarly, Proshlyakov *et al.* (36, 37) have claimed that **P** must be a ferryl species. Recently, Fabian and Palmer (23) suggested that **F** and **P** have the same overall redox state, both 2 electron equivalents more oxidized than the ferric/cupric enzyme.

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Abbreviations: **F**, form of cytochrome-*c* oxidase with absorbance difference peak at 580 nm, putative ferryl intermediate; **P**, form of cytochrome-*c* oxidase with absorbance difference peak at 607 nm, putative peroxy intermediate; **O**, oxidized form of cytochrome-*c* oxidase; $Ru(bipy)_3$, tris(2,2′-bipyridyl)ruthenium(II); MCD, magnetic circular dichroism.

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†In the two-electron reduced enzyme the "oxy" intermediate decays much more slowly, and into a species called "compound C" (6, 9).

In this work we confirm the original assignment that **F** and **P** have 1 and 2 more oxidizing equivalents than the oxidized enzyme, respectively, and discuss their structures in the light of this finding and the recent literature.

MATERIALS AND METHODS

Reagents and Enzymes. Bovine heart cytochrome-*c* oxidase was prepared by a modification of the method of Hartzell and Beinert (38). During enzyme preparation, the pH was kept above 7.8 (39). No ethanol was used to remove the Triton X-114 after the red/green cut; instead, the green pellet was repeatedly resuspended in the preparation buffer and centrifuged, until the amount of detergent was significantly reduced, as judged by the extent of bubbling when the supernatant was shaken (usually three or four exchanges). To facilitate the binding of the photoreductant, the enzyme was dialyzed into a low ionic strength medium containing 2 mM Tricine, pH 8.5 (1000 vol, three repetitions). Catalase was Sigma type C-30 (19,000 units·ml^{-1}). Tris(2,2′-bipyridyl)ruthenium(II) chloride hexahydrate [Ru(bipy)$_3$] was obtained from Aldrich.

Sample Preparation. Preparation of the CO mixed-valence enzyme and its subsequent reaction with oxygen were performed essentially as described previously (40). **F** was formed by addition of 5 mM H_2O_2 to the enzyme followed by a 10-min incubation. During the last half of the incubation time, no major changes in absorbance were observed in the sample.

Photoreduction Measurements. Measurements on the enzyme during photoreduction were carried out with a kinetic spectrophotometer equipped with a diode array (Unisoku Instruments, Kyoto, Japan). The cuvettes used were 5 mm × 5 mm. A camera flash was used to photolyze CO from the enzyme, and later to excite the Ru(bipy)$_3$ photoreductant. Aniline was used as the sacrificial electron donor. Spectra were recorded at a rate of one per millisecond. In order for the prereaction spectrum to be recorded, data collection was started a few tens of milliseconds before the flash.

Data Analysis. The spectra of the changes brought about by electron injection into the enzyme were obtained by subtracting the prereaction spectrum from the spectrum immediately after the flash. The prereaction spectrum was obtained by averaging 48 sequential spectra from the preflash part of the data. The postflash spectrum was obtained by fitting the part of the data after the flash with a single-exponential function, using a global fitting program (40, 41) and extrapolating back to the time of the flash. Electron transfer from the photoexcited Ru(bipy)$_3$ into Cu_A and subsequent reduction of the low-spin heme and the oxygen intermediates are rapid and not resolved by our measuring system. However, we often observed slow reoxidation of the low-spin heme. This presumably reflects redistribution of electrons to the binuclear site in a population of enzyme molecules which do not have an active oxygen intermediate at that site. The extrapolation removes this slow component so that the fast processes can be studied. MATLAB (Mathworks, South Natick, MA) was used for data handling and presentation.

RESULTS

Injection of electrons from a photoreductant often provides a convenient way to initiate redox reactions within an enzyme. Gray and coworkers (42, 43) developed the use of the photoreductant Ru(bipy)$_3$ for the study of intramolecular electron transfer, and Nilsson (44) adapted this methodology for use with cytochrome-*c* oxidase, studying the rates of electron transfer in different states of the enzyme. Under low ionic strength conditions, Ru(bipy)$_3$ is bound electrostatically, and photoactivation results in fast electron transfer to the Cu_A center, which quickly equilibrates with the low-spin heme. This is essentially a pure one-electron reduction because electron transfer occurs only from Ru(bipy)$_3$ bound to the enzyme, and the average yield is only ≈0.03 electron per enzyme molecule (44). Hence it is very unlikely, using this technique, that any one enzyme molecule would receive 2 or more electrons.

We have now applied this technique to study the spectral changes which occur when a single electron equivalent is injected into the **P** and **F** intermediates of cytochrome-*c* oxidase.

The **F** form of cytochrome-*c* oxidase was prepared by adding an excess of H_2O_2 to the oxidized enzyme (Fig. 1*A*). Injection of an electron from Ru(bipy)$_3$ caused clear conversion of a small subpopulation of **F** back into the oxidized enzyme (**O** state), as shown by the difference spectrum of Fig. 1*B*. The efficiency of the photoreduction was here ≈1%, as judged from the ΔA at 580 − 550 nm in Fig. 1*A* relative to that in Fig. 1*B*. On the basis of the molar absorptivity of **F** (20), ≈80% of the enzyme was initially in this state after the H_2O_2 addition. Some enzyme that is present in the oxidized state in the H_2O_2-treated sample was converted into one electron-reduced enzyme (**E** state) on photoreduction. This is seen from the sharp peak at 605 nm (Fig. 1*B*) due to reduction of the low-spin heme. This is a minor event, however, and corresponds to less than 20% of the amount of **F** being converted into **O** (the molar absorptivity of the change at 605 nm is approximately 4 times that of **F** at 580 nm). Conversion of **F** to **O** is associated not only

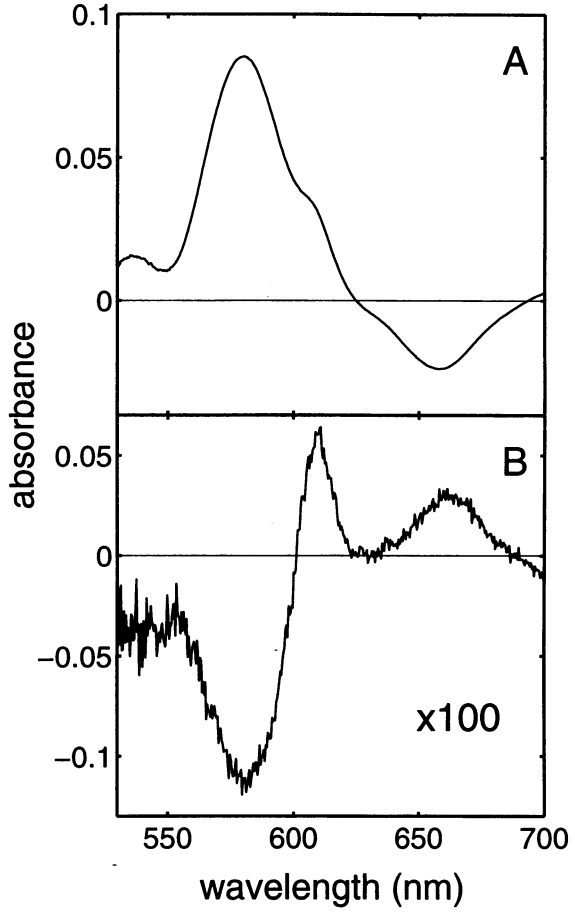

FIG. 1. Photoreductive injection of electrons from Ru(bipy)$_3$ into the **F** form of cytochrome-*c* oxidase that was formed by reaction with peroxide. (*A*) Difference spectrum (with respect to oxidized) showing the state of the enzyme prior to flash. (*B*) Spectrum of absorbance changes produced by one-electron photoreduction of a small subpopulation of the enzyme by Ru(bipy)$_3$. Bovine cytochrome-*c* oxidase, 46 μM (dialyzed to lower ionic strength); Ru(bipy)$_3$, 50 μM; aniline, 10 mM; Tricine, 2 mM (pH 8.5); dodecyl maltoside, 0.1%; H_2O_2, 5 mM; cuvette dimensions, 5 mm × 5 mm.

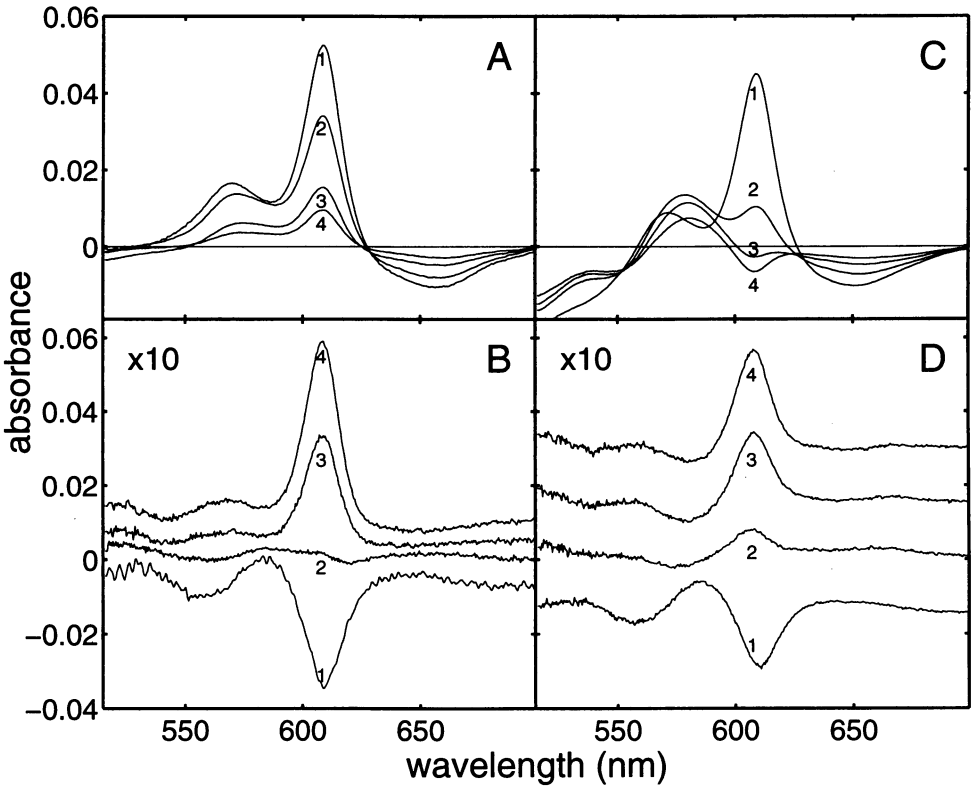

FIG. 2. Photoreductive injection of electrons from $Ru(bipy)_3$ into intermediates formed by reaction of CO mixed-valence cytochrome-*c* oxidase with oxygen at low and high pH. (*A* and *C*) Difference spectra (with respect to oxidized) showing the state of the enzyme at various times after reaction with oxygen. (*B* and *D*) Corresponding spectra of absorbance changes produced by one-electron photoreduction of a small subpopulation of enzyme by $Ru(bipy)_3$. *A* and *B*, pH 8.5; *C* and *D*, pH 6.5. Elapsed time between addition of oxygen and flash in *A* and *B*: 1, 0.5 min; 2, 3 min; 3, 9 min; 4, 16 min; in *C* and *D*: 1, 0.5 min; 2, 1.5 min; 3, 2.5 min; 4, 3.5 min. Bovine cytochrome-*c* oxidase, 13 μM (dialyzed to lower ionic strength); $Ru(bipy)_3$, 59 μM; aniline, 5.9 mM; CO, 40 μM; pH 6.5 buffer, 5 mM Mes; pH 8.5 buffer, 5 mM Tris; dodecyl maltoside, 0.1%; 25 μl of O_2-saturated water was injected to give $[O_2] \approx 50$ μM; cuvette dimensions, 5 mm × 5 mm.

with disappearance of the 580-nm band but also with appearance of an absorption band at 655 nm, which is a unique feature of the oxidized ferric/cupric binuclear site (45). This result confirms that **F** is 1 electron equivalent more oxidized than the ferric/cupric enzyme.

We then formed **P** by allowing the "CO mixed-valence" (two-electron reduced) enzyme to react with O_2, at both high and at low pH, as shown in Fig. 2 *A* and *C* (traces 1). **P** thus formed decays spontaneously into the oxidized enzyme via the **F** intermediate (Fig. 2*B*). This decay is much slower at high pH (Fig. 2*A*), which may be why **F** is not discerned as an intermediate in this case. We used photoreduction as before, at different stages of the decay process. At both low and high pH, electron injection early in the decay clearly converts the prevailing **P** into **F**, as seen from the 580-nm peak and 607-nm trough in the difference spectra of Fig. 2 *B* and *D* (traces 1). This confirms that **P** is indeed 1 electron equivalent more oxidized than **F**. Later in the decay, electron injection caused reduction of the low-spin heme in the increasing population of oxidized enzyme (**O**), which is seen as the sharp absorption increase at 605 nm (Fig. 2*B*, traces 3 and 4; Fig. 2*D*, traces 2–4). Reduction of **F** into **O** can be seen in the low-pH experiment of Fig. 2*D* (trace 3), although this is now somewhat obscured by the simultaneous reduction of the low-spin heme in the oxidized enzyme population. Conversion of **F** to **O** is clearly seen, however, after subtraction of the low-spin heme spectrum (Fig. 3; cf. Fig. 1*B*).

DISCUSSION

Our data provide definite evidence that the addition of one electron to cytochrome-*c* oxidase intermediate **P** converts it into **F**, and that addition of one electron into **F** yields the **O** state of the enzyme. Thus the overall redox states of **F** and **P** are 1 and 2 electron equivalents more oxidized than **O**. This is

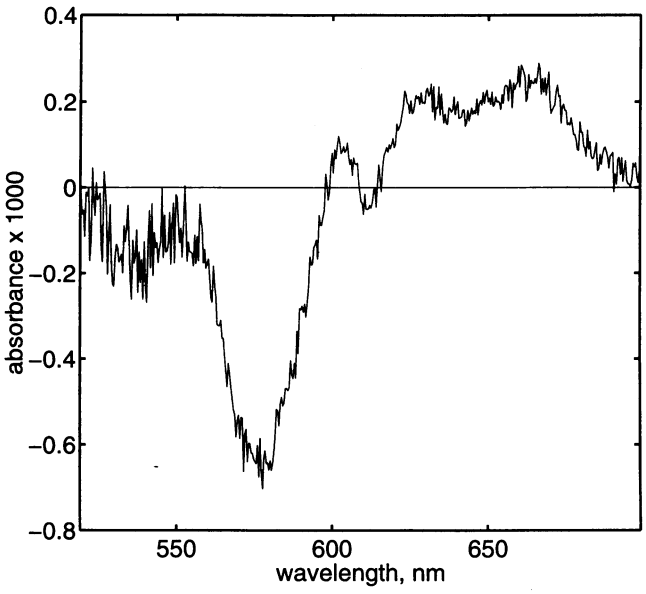

FIG. 3. Photoreductive injection of electrons from $Ru(bipy)_3$ into the **F** form of cytochrome-*c* oxidase. Data are from Fig. 2*D*, trace 3, with low-spin heme reduction component subtracted. The spectrum of low-spin heme reduction was obtained by photoreduction of the same sample with $Ru(bipy)_3$ after all oxygen intermediates had been allowed to decay.

consistent with a reaction of the enzyme where two-, three-, and four-electron reduction of enzyme-bound O_2 yields **P**, **F**, and **O**, respectively (5, 19, 20, 25, 26), and with the results of titrations of the **F** state by Witt (46).

It should be stressed that the **P** and **F** states have been defined on the basis of their optical signatures (see introduction). Comparison of **F** and **P** in the heme-copper oxidases of the cytochrome aa_3 and bo_3 type strongly suggests that this signature is due specifically to the oxygen-reactive heme. Thus the spectrum of **P** relative to oxidized enzyme is red-shifted ≈25 nm in the α-band, compared with that of **F**, irrespective of whether the heme is of A or O type (40). It is obvious, however, that the observed difference in overall redox state between **P** and **F** reflects a structural difference at the Fe_{a3} site unequivocally only if there are no redox state differences among the other redox-active sites in the enzyme, notably Cu_B. On the other hand, if redox differences at other sites would occur, they would have to explain the difference in optical spectrum between **F** and **P**. However, we should also emphasize that, in some conditions, **P** and **F** states may well be encountered in which the overall redox state of the binuclear site, or of the entire enzyme, does not differ by the amount reported here. For example, we have recently noted that the **P** state appears unstable in bovine cytochrome-*c* oxidase *specifically* when it is produced by using H_2O_2 at low pH. In such conditions an **F**-like state (with the 580-nm absorption peak) is also formed, but it may then have the same overall redox level as **P**, and the possible structure Fe^{4+}_{a3}=O Cu^{3+}_B (unpublished results). This might explain some findings suggesting that the H_2O_2-induced **F** can in some cases be 2 electron equivalents more oxidized than the **O** state (see especially 23).

Analogously, we have recently found (unpublished results) that the **P** state is formed transiently when the fully reduced enzyme reacts with O_2 and when the binuclear site has received three electrons. This **P** state relaxes into the **F** state without transfer of additional electrons into the binuclear center, and thus its overall redox state remains the same in this case.

Although we feel that the optical signatures of the **P** and **F** states primarily report the structure of the Fe_{a3} site, suggesting that they are structurally different, we cannot exclude the possibility that the optical and redox differences are due to changes at another adjacent site. There are several cases in the recent literature where the **P** state has been assigned an oxyferryl structure by magnetic circular dichroism (MCD) or resonance Raman spectroscopy. Keeping the overall redox difference to **F** in mind, this would mean that **P** contains an additional oxidizing equivalent, such as an amino acid radical or a Cu^{3+}_B state. Although we do not question these MCD and Raman assignments as such, there is reason to question whether they have truly been made for the **P** state. For example, Watmough *et al.* (34) studied the product of the reaction of cytochrome bo_3 with peroxide, and of the "CO mixed-valence" enzyme with O_2, using MCD spectroscopy. They assigned the single product as the equivalent of **P** with an oxyferryl structure. However, we recently showed (40) that Watmough's species is actually analogous to **F**, and that it is preceded in these reactions by another species which strongly resembles **P**.

Proshlyakov *et al.* (36, 37) treated oxidized bovine cytochrome-*c* oxidase with hydrogen peroxide in conditions designed to yield either mainly **P** or mainly **F**. They reported three Raman modes in these conditions, which were all ascribed to an oxyferryl structure. However, their optical spectra show that even at their maximum occupancy of the 607-nm **P** species, it represented only ≈30% of total enzyme with considerable occupancy of the 580-nm **F** species as well. At maximum **F** occupancy there was very little **P**, if any. Yet, all Raman modes were present in both maximum **P** and maximum **F** conditions (37), and were actually enhanced in the latter. Proshlyakov *et al.* (36) also ascribed an 804-cm^{-1} ferryl mode to **P** on the basis that it was observed by 607-nm excitation. However, since the absolute spectra of **F** and **P** are quite similar (A. A. Konstantinov, personal communication), and a considerable amount of **F** was present, it is again difficult to exclude that this resonance arose from **F** rather than from **P**. We suggest, instead, that all three resonance Raman modes reported by these workers may be attributed to a mixture of **F**-like states that give rise to the optical 580-nm band, rather than from the 607-nm **P** species.

The data presented here do not allow us to make conclusions about the electronic structure of **P** and **F**. However, we find it difficult to accept that Fe_{a3} in **P** would have an oxyferryl structure. Fe_{a3} in **F** is almost certainly an oxyferryl species (see above). If **P** had the same Fe_{a3} structure, its optical spectrum would have to be strongly perturbed by a close-lying amino acid radical, or by Cu^{3+}_B. MCD spectra already excluded a porphyrin radical in **P** (23). Interestingly, the MCD *difference* spectrum between **P** and oxidized enzyme in the 600-nm region is highly reminiscent of the absolute spectrum of the oxidized enzyme itself, both in shape and in intensity (see figure 7, upper panel, of ref. 23). Since the absolute MCD spectrum of the oxidized enzyme is largely due to the low-spin *ferric* heme in this spectral domain (47), this seems to be the most likely state of Fe_{a3} in **P**. We, therefore, disagree with Fabian and Palmer (23) that the MCD spectrum of **P** would resemble that of a low-spin *ferrous* heme A, the MCD intensity of which is much larger in this region (47). In contrast, the MCD spectrum of **F** is quite different and does not resemble a low-spin ferric heme A model at all (see lower panel of figure 7 in ref 23, and ref. 47).

We conclude that we find no compelling reason to modify the original assignment (19) of the **P** (607-nm) compound to a low-spin ferric peroxide state of Fe_{a3}. Compound **F**, which gives rise to the 580-nm band, almost certainly has an oxyferryl structure of Fe_{a3}. The one-electron difference in redox state reported here between these species is, therefore, ascribed to a one-electron difference at the Fe_{a3} site.

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