Observation and Assignment of Peroxy and Ferryl Intermediates in the Reduction of Dioxygen to Water by Cytochrome *c* Oxidase†

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ABSTRACT: The reaction of fully reduced cytochrome *c* oxidase with oxygen has been studied in flow-flash experiments at −25 °C. Under these conditions the time course of the reaction at 445 nm is qualitatively similar to that recorded at room temperature. In addition to heme redox events, three intermediates in the oxygen reaction are observed: a ferrous−oxy species (**A**), a 607-nm species (**P**), and a 580-nm ferryl species (**F**). Formation of **A** is not resolved. Conversion of the ferrous−oxy intermediate (**A**) into the 607-nm species (**P**) takes place at the same time that an electron is transferred from the low-spin heme to the oxygen reduction center ($k \approx 1500$ s^{-1}). Subsequently, **P** decays into the 580-nm species **F** at the same time that the low-spin heme becomes partially re-reduced by Cu_A ($k \approx 280$ s^{-1}). Although the 607-nm species (**P**) has been produced in other reactions of the enzyme, this is the first time that it has been observed as a transient in the forward reaction of the fully reduced enzyme with its natural substrate, demonstrating that it is a true catalytic intermediate. The structures of both **P** and **F** are discussed in the light of these findings.

Cellular respiration is an energy transduction process in which oxygen is reduced to water and the energy is conserved as a electrochemical membrane proton gradient ($\Delta\mu_{H^+}$), which is used as the energy source for synthesis of ATP. In eukaryotic organisms the catalyst of oxygen reduction is cytochrome *c* oxidase, an enzyme which resides in the inner mitochondrial membrane. In this enzyme a binuclear heme−copper center (Fe_{a3} Cu_B)[1] forms the actual site of oxygen binding and reduction. The electrons for this reaction enter the enzyme via Cu_A and reach the oxygen reduction center by way of a low-spin heme (Fe_a).

The reduction of oxygen to water is a four-electron process. Definition of the steps in the enzymatic reaction is important for understanding several fundamental aspects of the enzyme mechanism [see Babcock and Wikström (1992)]: how the otherwise inert dioxygen molecule is activated, how the reduction of dioxygen is carried out safely, without release of intermediates which could be dangerous to the cell, and how energy released by the reaction is converted into $\Delta\mu_{H^+}$. The mechanism of energy transduction is of particular interest. The oxygen reaction generates $\Delta\mu_{H^+}$ directly (electrons and protons enter the enzyme from opposite sides of the membrane), but in addition the reduction of oxygen to water drives an electrogenic proton pump (Wikström, 1977).

A number of methods have been developed to produce or trap putative intermediates in the oxygen chemistry.

Partial Reversal of the Oxygen Reduction Reaction. Wikström (1981) found that the oxygen reduction reaction of the enzyme could be partially reversed by applying backward driving force in the form of a membrane potential and establishing a highly electron-accepting redox potential at cytochrome *c*, which is normally the electron donor to the enzyme. Reversing the reaction in this way led to two species one after the other. The first had a spectral maximum at 580 nm, and the second had a spectral maximum at 607 nm. It was inferred (Wikström, 1981; Wikström & Morgan, 1992) that these two transitions correspond to the last two one-electron steps in the reduction of oxygen to water, and the 607-nm spectrum was assigned as a peroxy species (**P**)[2] and the 580-nm spectrum as a ferryl species (**F**).

Trapping the Incomplete Forward Reaction. The **P** and **F** species can be observed when the reaction with O_2 is initiated in cytochrome *c* oxidase which contains less than four electrons, and cannot complete the reduction of O_2 to water. Chance et al. (1975) found **P** (607 nm) as the product of the reaction of O_2 with the two-electron reduced "CO-mixed valence" enzyme. (**P** is "compound C" in Chance's nomenclature). Witt et al. (1986) found that **F** (580 nm) could be produced by carefully adding 3 reducing equiv to

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[1] Nomenclature: In cytochrome *c* oxidase (cytochrome aa_3; EC 1.9.3.1), the low-spin heme is known as heme *a* or Fe_a and the oxygen-binding heme is known as heme a_3 or Fe_{a3} (in both, the chemical entity is heme **A**). The copper ion of the oxygen-reduction site is known as Cu_B. An additional copper site known as Cu_A serves as the initial electron acceptor from cytochrome *c*. The related quinol oxidases do not contain Cu_A (Puustinen et al., 1991).

[2] Abbreviations: **A**, the ferrous−oxy compound of Fe_{a3}; $\Delta\mu_{H^+}$, electrochemical membrane proton gradient; **F**, compound of Fe_{a3} characterized by a 580-nm absorbance peak; *k*, rate constant; **O**, fully oxidized form of binuclear oxygen reduction site; **P**, compound of Fe_{a3} characterized by a 607-nm peak; $\mathbf{P}_R$, form of the binuclear site containing **P** intermediate in the reaction of fully reduced enzyme with oxygen; $\mathbf{P}_M$, form of the binuclear site containing **P** intermediate in the reaction of CO mixed-valence enzyme with oxygen; RT, room temperature; τ, time constant ($t_{1/e}$).

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cytochrome *c* oxidase and then reacting the enzyme with O_2. Lauraeus et al. (1993) and Puustinen et al. (1996) later confirmed this using related bacterial oxidases which only contain 3 reducing equiv when fully reduced.

Artificial Generation of Oxygen Intermediates. **P** and **F** can also be generated by treating the oxidized enzyme with hydrogen peroxide (Wrigglesworth, 1984). The substantial literature on these reactions was recently reviewed by Fabian and Palmer (1995; but see Discussion).

It is important to establish the identity of the transient oxygen species during actual catalytic turnover. The best way currently available to approach this is to study the reaction of the fully reduced enzyme with O_2. This can be done using a method known as "flow-flash" that allows the reaction to be started by a flash of light (Gibson & Greenwood, 1963), but observation of oxygen intermediates in this reaction is difficult. The reaction is fast, and the large absorbance changes which accompany the redox events at the metal centers tend to obscure the smaller changes due to the oxygen chemistry. In spite of these problems, some intermediates have been identified.

The reaction involves at least four different kinetic phases. Oxygen binds first, forming a ferrous−oxy intermediate (**A**). This was originally reported by Chance et al. (1975) in low-temperature studies where it appeared as a 591-nm absorbance peak. At room temperature, oxygen binds rapidly ($\tau = 8\ \mu$s; $k = 1.25 \times 10^5\ s^{-1}$; $[O_2] = 1$ mM; Oliveberg et al., 1989; Verkhovsky et al., 1994) and the resulting intermediate has an absorbance peak at 595 nm (Hill & Greenwood, 1984; Verkhovsky, M. I., Morgan, J. E., Puustinen, A., and Wikström, M., unpublished). This intermediate has also been identified in time resolved resonance Raman and photosensitivity measurements (Varotsis et al., 1989; Ogura et al., 1990a; Han et al., 1990a; Blackmore et al., 1991; Varotsis & Babcock, 1995).

Oxygen binding is followed by a phase ($\tau = 32\ \mu$s; $k = 3.1 \times 10^4\ s^{-1}$; $[O_2] = 1$ mM; RT; Verkhovksy et al., 1994) in which the low-spin heme (Fe_a) becomes oxidized, leading to a state of the enzyme where three of the 4 redox equiv which will be needed for the reduction of oxygen to water are resident in the binuclear site, while the fourth electron is still on Cu_A (Hill & Greenwood, 1984; Han et al., 1990b; Hill, 1991, 1994). The resulting oxygen intermediate has not been captured by optical spectroscopy, but it has been proposed to be a peroxy species (Hill & Greenwood, 1984), and there is some evidence from time-resolved resonance Raman studies to support such an assignment (Varotsis et al., 1993; Ogura et al., 1993).

In the next phase ($\tau = 140\ \mu$s; $k = 7 \times 10^3\ s^{-1}$; RT; Hill & Greenwood, 1984), the main redox event observed is the partial oxidation of Cu_A as the fourth electron equilibrates between Cu_A and Fe_a (Hill, 1991). It seems likely that **F** is formed during this phase. Orii (1988), studying this reaction, reported peaks at 575 and 530 nm in a time-difference spectrum (100 μs minus 20 μs) and assigned them to the formation of **F**. Since **P** was not resolved, Orii concluded that **F** was either formed directly from the ferrous−oxy (**A**) intermediate or, if there was an intervening peroxy intermediate, it was converted to the **F** very rapidly and never achieved an observable population. Time-resolved resonance Raman studies have also indicated that an oxy−ferryl intermediate is present although there is not complete agreement as to the time in the reaction when it appears (Varotsis & Babcock, 1990; Ogura et al., 1993; Han et al., 1990c).

Finally, in a much slower process, the remaining electron, shared between Cu_A and Fe_a, migrates to the binuclear center resulting in the formation of water and the fully oxidized enzyme (Hill, 1991).

Thus, the ferrous−oxy species (**A**) is firmly established as an intermediate in the reaction of the fully reduced enzyme with O_2, and there is evidence to indicate that **F**, the 580-nm species, is present, but **P**, the 607-nm species, has not been found. We have studied this reaction at −25 °C and now report unequivocal evidence that both **P** and **F** are true intermediates in this reaction.

MATERIALS AND METHODS

Bovine heart cytochrome *c* oxidase was prepared by a modification of the method of Hartzell and Beinert (1974) as described previously (Verkhovsky et al., 1994). Catalase was Sigma type C-30 (19 000 units mL^{-1}), and *n*-dodecyl β-D-maltoside was from Anatrace (Maumee, OH).

Samples for flow-flash measurements were made as described by Puustinen et al. (1996); the enzyme was first made anaerobic using a vacuum line and argon exchange, and then reduced by addition of a small amount (approximately 100 μM) of dithionite, after which CO was added. Low-temperature flow-flash measurements were carried out using a diode array kinetic spectrophotometer made my Unisoku Instruments (Kyoto, Japan). The cuvette containing the CO-inhibited reduced enzyme was placed in the cuvette holder which was then cooled using a Lauda RCS thermostat and kept dry by a nitrogen flush. Oxygen-saturated buffer, also at low temperature, was mixed into the sample (ratio 1:1) and the reaction was started by a xenon camera flash. In order to prevent premature photolysis by the probe beam, a camera shutter was used to block the beam until a few milliseconds before the flash. In some cases, cutoff filters were also used to minimize probe light irradiation outside of the measurement region.

Data Analysis. Basic data matrix manipulations and presentation were done with Matlab (The Mathworks, South Natick, MA). Decomposition of data surfaces was done using SPLMOD (Provincer & Vogel, 1983) a global multiexponential fitting program; our implementation is described by Morgan et al. (1995).

RESULTS

In order to resolve and assign the various reaction intermediates it is helpful to follow the development of the entire spectrum during the reaction. At room temperature the reaction is extremely fast, and it is difficult to record spectra frequently enough to capture the process in real time. By lowering the temperature we were able to slow the reaction to the point that all but the most rapid phases were detectable even though we could only record spectra at a rate of 1000 per second. Working at a lower temperature has the additional advantage that the peaks of the heme absorption spectra become narrower. This can make it possible to distinguish spectral components which would not be resolvable at room temperature.

Thus, lowering the temperature improves both the temporal and the spectral resolution of the experiment. This concept

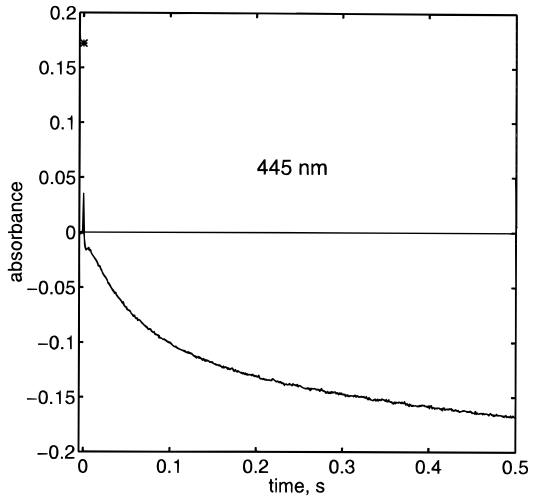

FIGURE 1: Reaction of fully reduced cytochrome *c* oxidase with O_2 at −25 °C followed at 445 nm. Zero absorbance corresponds to the CO-inhibited fully reduced enzyme; the reaction was initiated by a flash at time zero. The asterisk shows the absorbance of the unliganded, fully reduced enzyme, obtained by photolysis of the CO bound enzyme sample in the absence of oxygen (scaled to compensate for dilution). Cytochrome *c* oxidase, 3.5 μM; MOPS (pH 7.0), 50 mM; dodecyl maltoside, 0.05%; dithionite, 100 μM; a catalytic quantity of catalase; CO, saturated at room temperature; ethylene glycol, 50% (concentrations after mixing). Buffer containing MOPS (pH 7.0), 50 mM; ethylene glycol 50% was saturated with O_2 at −32 °C prior to mixing (ratio 1:1). Detector gating time, 1 ms.

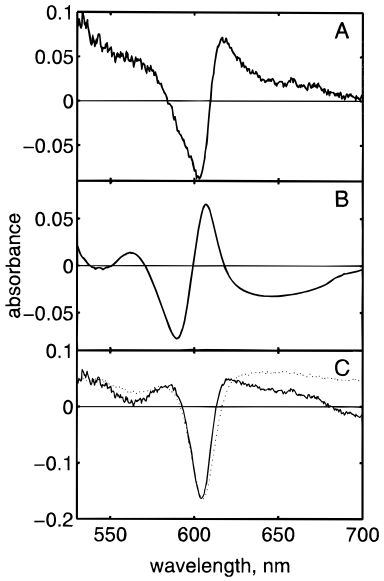

FIGURE 2: Kinetic component spectra from reactions of cytochrome *c* oxidase with O_2 at −25 °C. (A) Reaction of O_2 with fully reduced cytochrome *c* oxidase; component from global multiexponential fit (k = 1024 s^{-1}). (B) Reaction of O_2 with CO mixed-valence cytochrome *c* oxidase; component from global multiexponential fit (k = 322 s^{-1}). (C) Trace A minus trace B (offset by −0.025 absorbance units; solid line); spectrum of Fe_a from room temperature, arbitrarily scaled (dots). Cytochrome *c* oxidase, 5.5 μM; other conditions same as in Figure 1 except that CO mixed-valence enzyme sample contains no dithionite.

is not a novel one for work on cytochrome *c* oxidase (Chance et al., 1975; Clore et al., 1980a,b; Blair et al., 1985), but previous experiments have generally used samples frozen to cryogenic temperatures and there has been some doubt as to whether the reaction followed its normal course to completion (Chance et al., 1975; Blair et al., 1985; Witt et al., 1986). In the current experiments we avoided excessively low temperatures and freezing. The flow-flash reaction of cytochrome *c* oxidase with dioxygen can be carried out at −25 °C in the liquid phase (in the presence of 50% ethylene glycol), and under these conditions the reaction proceeds rapidly and uniformly to the fully oxidized form of the enzyme.

Figure 1 shows the time course of the low-temperature reaction of fully reduced enzyme with O_2 at 445 nm. This is a single line from the data surface (absorbance values on a time−wavelength plane) recorded by the diode array. This wavelength corresponds to the reduced-minus-oxidized absorbance maximum for both hemes of the enzyme and was chosen so that this trace could be compared to published data for the room temperature reaction (Hill & Greenwood, 1984; Oliveberg et al., 1989). The reaction is initiated with a light flash which photolyzes CO from the enzyme giving rise to the jump in absorbance at zero on the time scale. The measurement is not fast enough to resolve the earliest components in the reaction, but the absorbance of the photolysis product (an asterisk in the Figure) was obtained by photolyzing the sample in the absence of oxygen. What is important for present purposes is that this low-temperature reaction has a time course which runs parallel to the reaction at room temperature.

The data surface was analyzed using a global multiexponential fitting algorithm (see Materials and Methods). Figure 2A shows the spectrum of the first kinetic phase resolved by this method. With the time resolution of our system (1 ms per point), even at −25 °C, only the tail of the fast process was recorded (see Figure 1), but its rate can be estimated at approximately 1500 s^{-1} ($\tau \approx 0.6$ ms). It corresponds, in the sequence of events, to a phase in the room temperature reaction ($\tau = 32$ μs) in which Fe_a becomes oxidized, causing the conversion of the ferrous−oxy (**A**) intermediate into the subsequent oxygen species. The spectrum has a trough at 602 nm with a negative shoulder on its low-wavelength side and a distinct peak near 617 nm. The trough can be attributed to oxidation of Fe_a (605 nm) together with the disappearance of the ferrous−oxy compound **A** (595 nm). The location of the peak suggests that it could be due to formation of the 607-nm **P** species.

Whereas the 607-nm species has never been reported as an intermediate in this reaction, it has been known for some time that this compound is formed in the reaction of O_2 with the two-electron reduced CO mixed-valence form of the enzyme. Upon photolysis, the CO mixed-valence compound binds oxygen to form the ferrous−oxy species (**A**) and this is subsequently converted into the 607-nm **P** species (Hill & Greenwood, 1983; Han et al., 1990d). Figure 2B shows the spectrum of this **A** to **P** transition obtained in a similar low-temperature experiment. The trough near 590 nm and peak near 607 nm correspond to the disappearance of the ferrous−oxy (**A**) intermediate and the appearance of the 607-nm species (**P**), respectively. Note that this is the same transition of oxygen intermediates which we proposed earlier to explain the phase in the reaction of the fully reduced enzyme depicted in Figure 2A. If these assignments are correct, the only difference between the kinetic components in Figure 2A and B should be due to the oxidation of Fe_a, a process which can only take place in the fully reduced enzyme. The solid trace in Figure 2C is the result of subtracting these two spectra; the dotted trace is the reduced-minus-oxidized spectrum of Fe_a (inverted). The similarity

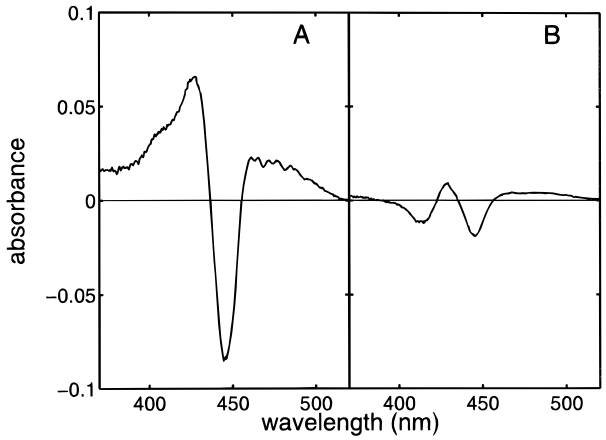

FIGURE 3: Kinetic component spectra from reactions of cytochrome *c* oxidase with O_2 at −25 °C. (A) Reaction of O_2 with fully reduced cytochrome *c* oxidase; component from global multiexponential fit (k = 1324 s^{-1}). (B) Reaction of O_2 with CO mixed-valence cytochrome *c* oxidase; component from a global multiexponential fit (k = 220 s^{-1}); cytochrome *c* oxidase, 3.5 μM; other conditions same as in Figure 1 except that CO mixed-valence enzyme sample contains no dithionite.

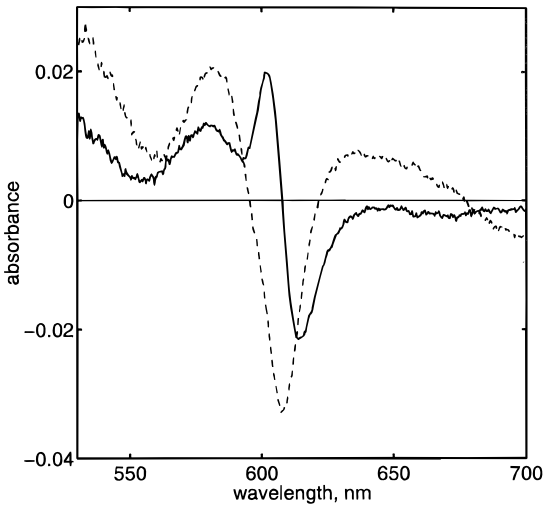

FIGURE 4: Kinetic component spectrum from reaction of fully reduced cytochrome *c* oxidase with O_2 at −25 °C. Component from global multiexponential fit (k = 279 s^{-1}) (solid line); same spectrum after subtraction of spectrum of Fe_a (arbitrarily scaled) from Figure 2C solid line (dashed line). Cytochrome *c* oxidase, 5.5 μM; other conditions same as in Figure 1.

between these spectra shows that oxidation of Fe_a is the only factor in Figure 2A which is not in Figure 2B. Thus, the same transition between oxygen intermediates takes place in both of these reactions, although in the case of the fully reduced enzyme, this transition is accompanied by oxidation of Fe_a.

The conversion of **A** to **P** in the CO mixed-valence enzyme is slower than in the fully reduced enzyme both at −25 °C ($\tau \approx$ 3.1 and 0.6 ms, respectively) and at room temperature ($\tau \approx$ 170 and 32 μs, respectively; Hill & Greenwood, 1983; Oliveberg et al., 1989; Verkhovksy et al., 1994). The presence of the electron on Fe_a clearly accelerates this process. In the reaction of the mixed-valence enzyme it seems that **A** is converted to **P** by the transfer of electrons from Fe_{a3} and Cu_B to oxygen, but in the case of the fully reduced enzyme there is reason to believe that the same two-electron transition is accomplished by the transfer of one electron from each heme and that the electron on Cu_B is a spectator during this phase of the reaction (Han, 1990b; Verkhovsky et al., 1994, 1996a; see Discussion).

Figure 3A and B show spectra from the Soret region corresponding to Figure 2A and B. In this spectral region there is very little difference between intermediates **A** and **P** (Figure 3B) despite the narrowing of absorption bands due to the low temperature. Therefore the oxidation of Fe_a completely dominates the Soret region absorbance changes at this point in the reaction of the fully reduced enzyme.

The next kinetic phase which we resolve in the reaction of the fully reduced enzyme with O_2 takes place at approximately 280 s^{-1} (at −25 °C). This time corresponds to the plateau between the fast and slow phases in the 445 nm trace (Figure 1). The kinetic spectrum of this phase is shown in Figure 4 (solid line). The trough near 617 nm and the peak near 580 nm suggest the disappearance of the 607-nm **P** species and formation of the 580-nm species (**F**), but the position of the trough is too far red-shifted and there is also a significant peak at 602 nm.

Room temperature studies have shown that, concurrent with the plateau at 445 nm, there is a significant increase in absorption at 830 nm (τ = 170 μs; $k = 6 \times 10^3$ s^{-1}, RT; Hill & Greenwood, 1984), indicating that Cu_A becomes partially oxidized at this time. This has been attributed to net electron redistribution from Cu_A to Fe_a when the electron, initially localized on Cu_A, becomes equilibrated between the two centers, partially filling the hole left by oxidation of Fe_a in the previous phase of the reaction (Hill, 1991). Re-reduction of Fe_a would cause an increase in absorbance at 605 nm, which could account for the 602-nm peak in Figure 4.

In order to check this possibility, the reduced-minus-oxidized spectrum of Fe_a (from Figure 2C) was subtracted from the kinetic component spectrum. After this subtraction we obtain a spectrum with a deep trough at 607.5 nm and a peak at 582 nm (Figure 4, dashed line), consistent with conversion of **P** into **F**. Note that the trough at 607.5 nm is about twice the size of the peak at 582 nm in agreement with specific absorptivities of 5.3 and 11 mM^{-1} cm^{-1} of compounds **F** and **P**, respectively (Wikström & Morgan, 1992).

Thus, in the reaction of fully reduced enzyme with dioxygen, the faster phase ($\tau \approx$ 0.7 ms; $k \approx$ 1500 s^{-1}, −25 °C) can be assigned to the conversion of intermediate **A** into intermediate **P**, together with oxidation of Fe_a. The second phase (τ = 3.6 ms; k = 280 s^{-1}, −25 °C) can be assigned to the subsequent conversion of intermediate **P** to intermediate **F**, together with the partial re-reduction of Fe_a by Cu_A.

The α-band peak of intermediate **P** (607 nm) is quite similar to that of Fe_a (605 nm). This, together with the fact that **P** is formed concurrently with oxidation of Fe_a and destroyed concurrently with partial re-reduction of Fe_a keeps the α-band absorbance changes during these phases relatively small and complicates separation of kinetic components, especially with data collected at room temperature.

The final monotonic absorption decrease at 445 nm (Figure 1) corresponds to oxidation of the remaining partially reduced Fe_a and Cu_A centers, and conversion of the enzyme from the **F** state to oxidized enzyme (not shown).

DISCUSSION

Wikström (1981) showed that the reaction catalyzed by cytochrome *c* oxidase can be partially reversed in mitochon-

dria by applying a reverse driving force to the energy conservation reaction. At high $\Delta\mu_{H^+}$ and high electron acceptor potential at cytochrome *c*, the originally ferric−cupric binuclear site of the oxidized enzyme (**O**) is converted sequentially into two new species with spectral maxima at 580 and 607 nm. These steps both titrated as one-electron oxidations and were attributed to conversion of the ferric oxidized enzyme into a ferryl species and subsequently into a peroxy species. The form of the enzyme which gave the 580-nm peak was named **F**, and the form with the 607-nm peak was named **P**.

On the basis of these findings Wikström (1981) proposed that the **F** and **P** species both correspond to intermediates of the forward reaction of the fully reduced enzyme with O_2. The present results confirm that this is the case. **P** forms in an early reaction phase and subsequently decays into **F**, which is the sequence of catalytic events proposed earlier (Wikström, 1981; Wikström & Morgan, 1992), except that it is now clear that electron transfer from Fe_a to the binculear site accompanies the conversion of **A** into **P** but not the conversion of **P** into **F**.

Although these measurements were carried out at −25 °C, the time course of the reaction at 445 nm (Figure 1) is qualitatively similar to the data for the room temperature reaction. This is a strong indication that the reaction sequence is the same at these two temperatures.

This is the first time that **P**, the 607-nm species, has been reported as an intermediate in the reaction of fully reduced cytochrome *c* oxidase with O_2. It is well-known that **P** is formed in the reaction of the CO mixed-valence enzyme with O_2 (Chance et al., 1975; Nicholls & Chanady, 1981; Han et al., 1990c). There might be some concern that **P** is observed in the current experiments only because the low-temperature conditions made the fully-reduced enzyme behave like the partially reduced enzyme by retarding electron transfer from Fe_a. This possibility can be ruled out because, in the reaction of the fully reduced enzyme with oxygen, we observe oxidation of Fe_a in the same phase in which formation of **P** takes place (Figure 2). This places **P** at a point in the reaction where three of the four electrons needed to reduce oxygen to water are already present in the oxygen binding site (the fourth electron is on Cu_A). In contrast, in the reaction of the CO-mixed valence enzyme with oxygen, **P** is formed with only 2 reducing equiv in the binuclear site. It seems clear that the actual chromophore which gives rise to the 607-nm spectrum is the same in both cases (see Figure 2), but in terms of the overall redox state of the binuclear center these intermediates are not the same. In the **P** intermediate formed in the reaction of fully reduced enzyme with O_2, the binuclear center contains one more reducing equivalent than in the **P** formed in the reaction of the CO mixed-valence enzyme with O_2. In the following discussion we will refer to these binuclear center species as $\mathbf{P}_R$ and $\mathbf{P}_M$ respectively.

We recently published a model for the early steps in the oxygen reaction (Verkhovsky et al., 1994) in which oxygen binds reversibly to Fe_{a3} and is only trapped when electron transfer from Fe_a and Fe_{a3} converts the bound oxygen species to a peroxy species where Cu_B was still reduced. This species, which was called **P1**, corresponds to $\mathbf{P}_R$ in the present discussion. We will use the name **P** to refer to the form of Fe_{a3} which gives rise to the 607-nm spectrum regardless of the overall state of the enzyme in which it is found.

The difference between $\mathbf{P}_R$ and $\mathbf{P}_M$ cannot be in the heme−oxygen moiety itself. The **P** and **F** species have also been studied in cytochrome bo_3 of *Escherichia coli*, where the oxygen binding moiety is heme **O** rather than heme **A**, and a comparison of the **P** and **F** spectra for the two enzymes shows that the features of these spectra must arise from the oxygen binding heme (Morgan et al., 1995). Any differences in the structures of the heme−oxygen moieties would have to be reflected in their spectra. This limits the possible differences between $\mathbf{P}_R$ and $\mathbf{P}_M$ to either an additional "invisible" reducing equivalent in $\mathbf{P}_R$, probably on Cu_B, or an additional "invisible" oxidizing equivalent in $\mathbf{P}_M$ (possibly some kind of radical).

We have recently re-investigated the redox relationships of intermediates **P**, **F**, and **O** (the oxidized enzyme) using a photoreduction technique [Verkhovsky et al., 1996b; cf. Wikström (1981)]. Injection of a one-electron equivalent converts $\mathbf{P}_M$ to **F**, and one more converts **F** to **O**. This places $\mathbf{P}_M$ at a redox level two reducing equivalents above the oxidized enzyme. Thus, $\mathbf{P}_M$ must be at the same formal redox level as the peroxy species:

$$\text{Fe(III)}-\text{O}^{-}-\text{O}^{-}\ \text{Cu(II)} \qquad (1)$$

Other possible structures at this redox level include (Cheesman et al., 1994; Watmough et al., 1994, Fabian & Palmer, 1995)

$$\text{porphyrin } \pi\text{-radical}(+)\ \text{Fe(IV)}{=}\text{O}\ \text{Cu(II)} \qquad (2)$$

$$\text{Fe(IV)}{=}\text{O}\ \text{Cu(III)} \qquad (3)$$

$$\text{protein radical}(+)\ \text{Fe(IV)}{=}\text{O}\ \text{Cu(II)} \qquad (4)$$

Structure 2 has been ruled out on the basis of MCD spectra (Fabian & Palmer, 1995), and structure 3 is very unlikely because the same 607-nm spectrum is observed in both $\mathbf{P}_R$ and $\mathbf{P}_M$ but not in **F**. If $\mathbf{P}_M$ would have the structure shown as in 3, then the structure of $\mathbf{P}_R$, which has one additional reducing equivalent, would probably be

$$\text{Fe(IV)}{=}\text{O}\ \text{Cu(II)} \qquad (5)$$

However, this is almost certainly the structure of intermediate **F** since there is evidence from resonance Raman studies that **F** is a ferryl species (Varotsis & Babcock, 1990; Ogura et al., 1990b). In this case, $\mathbf{P}_R$ and **F** would be the same species, which is impossible since they appear as distinct intermediates in the reaction of the fully reduced enzyme with oxygen. From another perspective, with these assignments the same difference in the redox state of Cu_B ($3^+/2^+$) would be called on to account for a marked difference between the spectra of $\mathbf{P}_M$ and **F** (607 nm/580 nm) as well as the "invisible" difference between $\mathbf{P}_R$ and $\mathbf{P}_M$.

In the case of structure 4 the difference between the spectra of **P** and **F** would be due to the influence of a protein radical. As discussed above, the spectral features of **P** and **F** arise from the oxygen binding heme itself, so that the **P** spectrum cannot be due to light absorption by a protein radical per se, but would have to be a secondary effect of the radical on the heme spectrum. Compound **I** of yeast cytochrome *c* peroxidase contains a ferryl−heme and a tryptophan radical which lie in van der Waals contact (Liu et al., 1994). Even with this proximity, reduction of the radical causes a change of less than 3 $mM^{-1}\ cm^{-1}$ (peak to trough) in the absorbance

spectrum (Ho et al., 1983). This can be compared to a change of 16 mM^{-1} cm^{-1} (peak to trough) for the **P** to **F** transition (Wikström & Morgan, 1992). It thus seems very unlikely that the influence of a protein radical would account for the difference between the **P** and **F** spectra.

We have recently shown (in experiments at room temperature) that the early steps in the reaction of the fully reduced enzyme with oxygen are not accompanied by charge translocation by the membrane-embedded enzyme (Verkhovsky, M. I., Morgan, J. E., Verkhovskaya, M. L., & Wikström, M., unpublished). The part of the reaction which corresponds to the formation of **P** in the current experiment, does not create a membrane potential. Breaking the O−O bond would necessarily require consumption of protons, which should be seen as a charge-translocating event. The fact that this is not observed is a further indication that the O−O bond is intact in **P**. The present data also agree very well with our earlier finding that "energized" partial reversal of the oxidase reaction yields **P** ($\mathbf{P}_M$), but that the reaction cannot be reversed further (Wikström, 1981; Wikström & Morgan, 1992).

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