The "Ferrous-Oxy" Intermediate in the Reaction of Dioxygen with Fully Reduced Cytochromes aa_3 and bo_3[†]

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ABSTRACT: We have studied the reactions with oxygen of two terminal oxidases, cytochrome *c* oxidase from mitochondria and cytochrome bo_3 from *Escherichia coli*. In each case, flow-flash methodology was used to react the fully reduced enzyme with a high concentration of oxygen (1 mM), and absorbance changes were recorded for a number of separate wavelengths in the α-band (visible) region. In both enzymes, an early kinetic phase could be resolved, corresponding to the binding of oxygen to produce a ferrous-oxy heme intermediate. In cytochrome *c* oxidase, this intermediate appears with a time constant of 10 μs; its spectrum has a peak at 595 nm (relative to the unliganded reduced enzyme). In cytochrome bo_3, the ferrous-oxy intermediate, resolved by optical absorbance spectroscopy for the first time, appears with a time constant of 11 μs and has a broad maximum near 570 nm.

Cytochrome *c* oxidase and related heme-copper oxidases[1] are probably responsible for more than 90% of all oxygen consumption by living organisms. These enzymes can operate effectively even in environments where oxygen concentrations are low, and so it seems initially surprising that binding of oxygen to these enzyme is relatively weak. Instead of binding the oxygen molecule tightly, however, the enzymes have developed an alternative that is energetically less costly: oxygen is bound weakly and reversibly to one of the hemes and is then caught by a kinetic trap, which relies on extremely fast electron transfer reactions (Verkhovsky et al., 1994, 1996).

This is beneficial for the organism, because it maximizes the amount of energy from the reduction of oxygen to water, available for conservation, but it complicates the task of experimentalists who wish to study the process of oxygen binding. Not only is the oxygen-bound species present in low abundance but, because of the kinetic trapping, it is also present for only a very short time.

Oxygen is believed to enter the enzyme by binding transiently to Cu_B (Woodruff, 1993; Oliveberg & Malmström, 1992; Verkhovsky et al., 1994), but this intermediate has yet to be established spectroscopically. The initial oxygen-bound heme species was first observed by Chance et al. (1975) in mitochondria, using a method known as "triple trapping" in which the reaction of the fully reduced enzyme with oxygen is initiated at low temperature by the photolysis of CO. At about −100 °C the reactions which follow oxygen binding are slow enough that the oxygen-bound intermediate can be trapped for further study by lowering the temperature. This intermediate was designated "compound **A**."[2] At low temperature its spectrum has a peak 591 nm and a trough at 611 nm, relative to the photolysis product (the fully reduced enzyme).

The reaction of the enzyme with oxygen at room temperature is much more rapid. Under these conditions, when fully reduced bovine cytochrome *c* oxidase encounters dioxygen, the slowest redox phase in the reaction takes place with a characteristic time of about 1.2 ms (Oliveberg et al., 1989), within the dead time of conventional mixing methods. In order to reveal kinetic detail in the reaction, microsecond resolution is needed. One way to achieve this is the "flow-flash" technique, originally introduced by Gibson and Greenwood in 1963 and developed steadily since then (below). This technique, which is used in the present work, employs CO to cage the fully reduced enzyme while it is mixed with oxygen-containing buffer (in this respect it is similar to triple-trapping). After mixing, the reaction of the enzyme with oxygen can be initiated very rapidly by photolyzing the CO with a flash of light.

The first observation of the ferrous-oxy intermediate (**A**) at ambient temperature was made in flow-flash experiments on the reaction of the half-reduced CO-mixed valence form of the enzyme with oxygen (Hill & Greenwood, 1983). The species formed in this way has relatively long lifetime, disappearing with a time constant of about 170 μs (Hill & Greenwood, 1983). Its spectrum (relative to the fully reduced enzyme) has a peak at about 595 nm. The longer lifetime is apparently related to the fact that in the mixed-valence enzyme there is no electron on the low-spin heme

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[1] Nomenclature: In cytochrome bo_3, the low-spin heme is known as heme *b* or Fe_b (the chemical entity is heme **B**). The oxygen-binding heme is known as heme o_3 or Fe_{o3} (chemically heme **O**). The corresponding hemes of cytochrome aa_3 are heme *a* (Fe_a) and heme a_3 (Fe_{a3}), both chemically hemes **A**. In both enzymes, the copper ion of the oxygen-reduction site is known as Cu_B. Cytochrome *c* oxidases contain an additional copper site known as Cu_A, which serves as the initial electron acceptor from cytochrome *c*. Cu_A is not found in the quinol oxidases (Puustinen et al., 1991). Cytochrome *c* oxidase (aa_3) is EC 1.9.3.1.

[2] Abbreviations: **A**, the ferrous-oxy intermediate of cytochrome *c* oxidase (Chance et al., 1975); DM, *n*-dodecyl β-D-maltoside; *k*, rate constant; PMT, photomultiplier tube; SVD, singular value decomposition; τ, time constant ($t_{1/e}$).

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so that the only electrons available to trap oxygen are on the two metals of the oxygen reduction site (Fe_{a3} and Cu_B).

In the reaction of the fully-reduced enzyme with oxygen, the ferrous-oxy intermediate (**A**) has a shorter lifetime and is hence more difficult to observe. Orii (1984, 1988a,b) reported time-difference optical absorbance spectra indicative of oxygen binding prior to the first redox phase of the reaction. This process was first kinetically resolved by Oliveberg et al. (1989), who reported that the initial electron transfer phase was preceded by a yet faster phase.

A ferrous-oxy intermediate has been identified in this reaction by means of resonance Raman spectroscopy (Han et al., 1990a,b; Ogura et al., 1990; Varotsis et al., 1989) and shown to be maximally populated at a time of 30−50 μs (Han et al., 1990a; Babcock & Varotsis, 1993). Its presence has also been inferred from photosensitivity measurements (Blackmore et al., 1991; Varotsis & Babcock, 1995).

Oxygen binding in the ferrous-oxy intermediate is relatively weak. It is the subsequent step, a redox process, which traps the oxygen molecule in the enzyme, in effect irreversibly. In the fully reduced enzyme, this trapping reaction crucially involves oxidation of the low-spin heme of the enzyme, the heme *not* in contact with oxygen. Hill and Greenwood (1984) first concluded that a significant subpopulation of the low-spin heme became oxidized during this phase of the reaction (which was the fastest phase they resolved). This was subsequently confirmed by the resonance Raman studies of Han et al. (1990a,b) which clearly demonstrated that oxygen binding precedes the oxidation of Fe_a [see also optical studies of Hill (1991)].

Optical absorption measurements in the Soret region have allowed the kinetics of the first two phases of the reaction of oxygen with the fully reduced enzyme to be clearly resolved. The ferrous-oxy intermediate (**A**) is formed with a time constant of 8 μs ($[O_2] = 1$ mM) and subsequently decays with a time constant of 32 μs (Verkhovsky et al., 1994). These results led us to formulate a detailed kinetic model for this process in which initial oxygen binding is described as an equilibrium of several states (see below).

In the related enzyme, cytochrome bo_3 from *Escherichia coli*, the corresponding oxygen bound species has thus far proved elusive. Although a ferrous-oxy intermediate has been detected in resonance Raman measurements (Hirota et al., 1994), it has not been observed by optical absorbance. This has led to speculation that there might not be enough difference between the rate of oxygen binding and the rate of the redox reaction which traps oxygen for this intermediate to be significantly populated (Svensson & Nilsson, 1993; Orii et al., 1994, 1995; Puustinen et al., 1996). In this paper, however, we report kinetic and spectral evidence for a ferrous-oxy intermediate (**A**) in the reaction of fully reduced cytochrome bo_3 with oxygen, together with the first visible (α-band) spectra of the corresponding intermediate in the reaction of fully reduced mammalian cytochrome aa_3 with oxygen at room temperature.

MATERIALS AND METHODS

Bovine heart cytochrome *c* oxidase was prepared by a modification of the method of Hartzell and Beinert (1974). During enzyme preparation, the pH was kept above 7.8 (Baker et al., 1987). No ethanol was used to remove the Triton X-114 following 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).

Cytochrome bo_3 from *E. coli* was prepared as described in Puustinen et al. (1996). A "histidine tag" has been genetically added to this enzyme in order to facilitate isolation (Jon N. Rumbley and Robert B. Gennis, personal communication). The variant of the isolation procedure which employs Triton X-100 was used. It has been shown that enzyme isolated in this way has a capacity of three reducing equivalents and lacks bound ubiquinone (Puustinen et al., 1996).

Catalase was Sigma type C-30 (19 000 units mL^{-1}), and *n*-dodecyl β-D-maltoside (DM) was from Anatrace (Maumee, OH).

Flow-flash kinetics were measured as described in previous papers (Verkhovsky et al., 1994; Puustinen et al., 1996). The instrument is a split-beam, dual-PMT spectrophotometer with a xenon camera flash as the probe light source. Light is passed through a monochromator before entering the sample and the PMT's are shielded from excitation light by slits and colored glass filters. One PMT measures the light level before the sample, and another measures the light which passes through the sample. A frequency-doubled YAG laser is used to initiate the reaction. Samples for flow-flash measurements were made as described by Puustinen et al. (1996); enzyme was first made anaerobic using a vacuum line and argon exchange, then reduced by addition of a small amount (approximately 200 μM) of dithionite, after which CO was added. Mixing ratio: 1:5 (enzyme solution: oxygen saturated solution). Typically, 10−12 transients of 2000 time points were measured at each wavelength.

Data analysis was done using MATLAB (The MathWorks, South Natick, MA) and Graphic Interactive Management (Alexander Drachev, Tempe, AZ).

SVD Analysis. The SVD routine in Matlab was used. Since the pre-flash absorbance defines the zero level for the raw data surface, the reference state is defined as the CO-bound fully reduced enzyme. Oxygen reaction begins with the unliganded fully reduced enzyme—the photolysis product—which is thus the correct reference state for the analysis. For this reason, the photolysis spectrum was subtracted from each line in the data surface before the SVD analysis. The first 200 μs of the data surface (17 or 13 × approximately 1000 points, pre-trigger data removed) was used in this analysis.

Kinetic Model Fit. The following two-step kinetic model was used:

$$\mathbf{0} \xrightarrow{k_1} \mathbf{I} \xrightarrow{k_2} \mathbf{II} \tag{1}$$

The absorbance values for the three intermediates at each wavelength as a function of time are given by

$$A_0 = a_0 e^{-k_1 t} \tag{2}$$

$$A_{\mathbf{I}} = b_0 \frac{k_1}{k_1 - k_2}(e^{-k_2 t} - e^{-k_1 t}) \tag{3}$$

$$A_{\mathbf{II}} = c_0\left(\frac{k_2}{k_1 - k_2}e^{-k_1 t} - \frac{k_1}{k_1 - k_2}e^{-k_2 t} + 1\right) \tag{4}$$

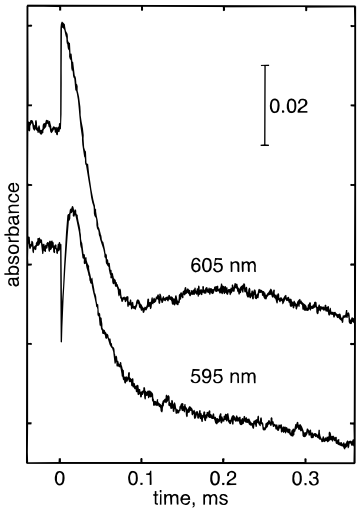

FIGURE 1: Flow-flash reaction of fully reduced cytochrome *c* oxidase with oxygen, kinetic traces. Zero on the time scale is the point at which the laser was fired. Concentrations after mixing: cytochrome *c* oxidase, 6.4 μM; dioxygen, 1 mM; MES, 100 mM (pH 6.0); DM, 0.1%; catalase, catalytic amount.

where coefficients a_0, b_0, and c_0 represent the products of extinction coefficient and concentration for each intermediate. The data surface was decomposed using a basis set defined by $A_{\mathbf{I}}$, $A_{\mathbf{II}}$ (above), and a constant term, by means of the minimization (Nelder−Mead simplex algorithm), and Gaussian elimination functions of MATLAB, to give best values of k_1 and k_2 together with the corresponding component spectra. In the case of the cytochrome bo_3 data, the complete surface (13 × approximately 1800 points, pre-trigger data removed) was used. In the case of cytochrome aa_3, only the first 200 μs of data (17 × 1000 points) were used.

RESULTS

Our aim of the present study was to confirm that the initial product of the reaction of the fully reduced enzyme with oxygen at room temperature (i.e., the product of the 8 μs phase) is really the same as the distinctive species found in the low-temperature reaction and the reaction of the mixed valence enzyme with oxygen, which has an absorbance peak at or near 595 nm.

To this end the flow-flash reaction between fully reduced cytochrome *c* oxidase and dioxygen was recorded at 17 separate wavelengths between 575 and 630 nm to create a surface of absorbance values on a time/wavelength plane. The data for two example wavelengths are shown in Figure 1. At the point where the traces begin the CO inhibited enzyme has already been mixed with oxygen-containing buffer, but since there is very little light the CO remains in place and no reaction occurs. At $t = 0$ CO is removed by the laser flash; at both wavelengths there is a jump in absorbance followed by rapid changes as oxygen binds and begins to take electrons from the enzyme. At 605 nm, which is an absorbance peak for the low-spin heme, the data are clearly dominated by the heme oxidation process, although it appears to be preceded by a lag. The data at 595 nm reveal not a lag but a rising phase at the time when oxygen binding has been shown to occur.

These data are consistent with the conclusion from the earlier Soret study (Verkhovsky et al., 1994), suggesting that this early part of the reaction consists of two principal kinetic processes. In order to study this further, the kinetic data surface was subjected to SVD analysis (see Materials and Methods). SVD also indicates that there are only two major components[3] (Figure 2; the left panel shows the time courses of the components and the right panel the corresponding spectra). Component 1 clearly increases in the first few microseconds of the reaction and decreases at the time of the first heme redox event, very much as would be expected for a ferrous-oxy intermediate. Component 2 shows an initial lag, after which there is a decrease in absorbance, on a time scale consistent with heme oxidation.

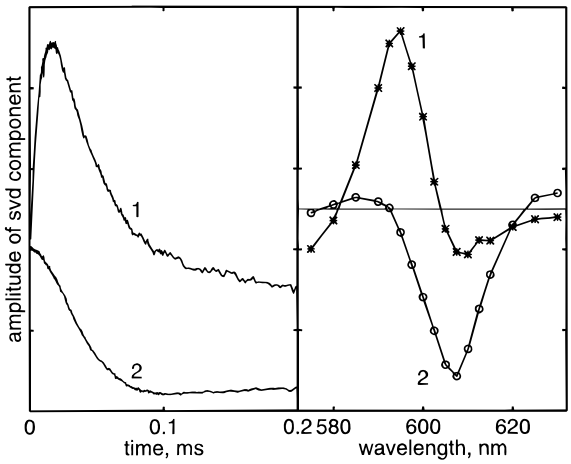

FIGURE 2: Flow-flash reaction of fully reduced cytochrome *c* oxidase with oxygen, SVD components (experimental conditions as in Figure 1). The left panel shows the two major kinetic components (1, 2); the right panel shows the *corresponding* spectral components (1, 2)[3]. The spectrum and time course labeled 1 correspond to the second most significant component from the SVD; the spectrum and time course labeled 2 correspond to the most significant component. The first five values from the diagonal of the SVD "S" matrix, which gives the weights of the components, are 2.072, 0.681, 0.055, 0.028, and 0.026.

In order to obtain spectra and rate constants, we fit the data using a simplified two-step kinetic model (see Materials and Methods).

$$\mathbf{0} \xrightarrow{k_1} \mathbf{I} \xrightarrow{k_2} \mathbf{II} \qquad (1)$$

This was chosen for mathematical simplicity as the minimal kinetic scheme which can account for the two-exponential relaxation kinetics observed. The results will be interpreted below in terms of our earlier, more detailed model (see Discussion).

Figure 3 shows the results of the model fit. The results are similar to those of the SVD. The two rate constants are $k_1 = 1.02 \times 10^5\ s^{-1}$ and $k_2 = 2.8 \times 10^4\ s^{-1}$ ($\tau_1 = 10\ \mu s$; $\tau_2 = 36\ \mu s$). The spectrum of component **I** (circles) has an absorbance maximum at 595 nm, consistent with its being the ferrous-oxy intermediate. The spectrum of component **II** (asterisks) has a minimum close to 605 nm, consistent with an intermediate in which a large amount of heme oxidation has taken place. This intermediate is expected to have a peroxy species at the oxygen binding site (Verkhovsky et al., 1994), but the formation of the peroxy intermediate

[3] In the text and figures these components are labeled 1 and 2 to indicate that they appear to contain faster and slower kinetic components, respectively. This does not correspond to ordering of the components in the SVD, which is based on weight in the solution (see figure captions).

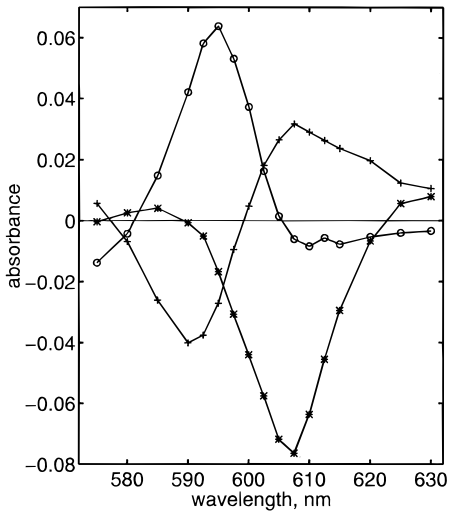

FIGURE 3: Flow-flash reaction of fully reduced cytochrome *c* oxidase with oxygen, spectra of intermediates as determined by a two-step reaction model fit. Circles, intermediate **I**; asterisks, intermediate **II**; crosses, constant term (experimental conditions as in Figure 1).

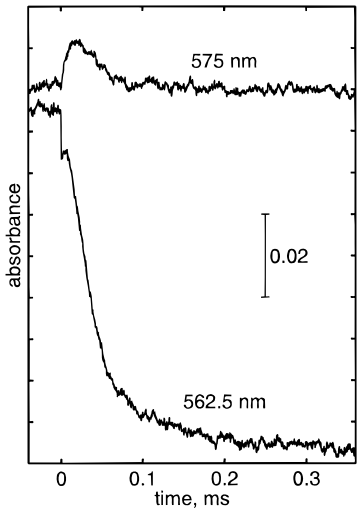

FIGURE 4: Flow-flash reaction of fully reduced cytochrome bo_3 with oxygen, kinetic traces. Zero on the time scale is the point at which the laser was fired. Concentrations after mixing: cytochrome bo_3, 6.2 μM; dioxygen, 1 mM; MOPS, 100 mM (pH 7.0); DM, 0.05%; catalase, catalytic amount.

does not appear as a distinct feature. This is presumably because, in this spectral region, the difference between the ferrous-oxy intermediate and the peroxy intermediate is relatively small compared to the reduced-minus-oxidized spectrum of the low-spin heme.

In order to have an orthogonal basis set for the fit, intermediate **0** could not be included explicitly, but the constant term from the fit (crosses) presumably reflects this intermediate to a large degree. This spectrum, with a minimum at 590 nm and a maximum near 608 nm, appears to be due to the dissociation of CO from the oxygen-binding heme. This is consistent with intermediate **0** being the unliganded fully reduced enzyme, produced by photolysis of the fully reduced CO-bound enzyme. Thus, the spectra of components **I** and **II** are difference spectra with respect to the unliganded fully reduced enzyme.

These results serve to confirm the existence of the ferrous-oxy compound (**A**) as an intermediate in the room temperature reaction of fully-reduced cytochrome *c* oxidase with oxygen, and that this species has a spectrum similar to that obtained at low temperature (Chance et al., 1975), and when the two-electron reduced CO-mixed valence enzyme reacts with oxygen (Hill & Greenwood, 1983). The two time constants found, $\tau_1 = 10$ μs; $\tau_2 = 36$ μs, agree well with our earlier results from measurements in the Soret region, $\tau_1 = 8$ μs; $\tau_2 = 32$ μs (Verkhovsky et al., 1994).

In the case of cytochrome bo_3 of *E. coli*, the ferrous-oxy intermediate proved more elusive. In this enzyme the binding of oxygen to the fully reduced enzyme had never been resolved by optical absorbance spectroscopy at room temperature. Reversible oxygen binding had been reported in low-temperature reactions (Poole et al., 1979a,b), and a ferrous-oxy species had been observed in flow-flash resonance Raman measurements (Hirota et al., 1994). However, several groups, including our own, studying the flow-flash reaction of fully-reduced cytochrome bo_3 with oxygen monitored by optical absorbance, were unable to resolve any events prior to the reoxidation of the hemes (Svensson & Nilsson, 1993; Orii et al., 1994, 1995; Puustinen et al., 1996). Svensson and Nilsson (1993) who worked in the visible (α-band) region reported "a small variation of the observed rate

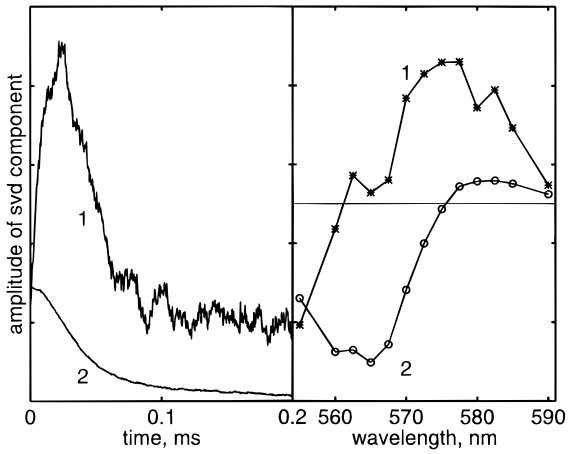

FIGURE 5: Flow-flash reaction of fully reduced cytochrome bo_3 with oxygen, SVD components, (experimental conditions as in Figure 4). The left panel shows the two major kinetic components (1, 2); the right panel shows the *corresponding* spectral components (1, 2).[3] The spectrum and time course labeled 1 correspond to the second most significant component from the SVD; the spectrum and time course labeled 2 correspond to the most significant component. The first five values from the diagonal of the SVD "S" matrix, which gives the weights of the components, are 6.018, 0.257, 0.077, 0.059, and 0.049.

constants with the observation wavelength... which may indicate the presence of an additional, unresolved component."

To study this further, we carried out a flow-flash experiment on the bo_3 enzyme, similar to the one on cytochrome aa_3, measuring the flow-flash kinetics at 13 wavelengths between 555 nm and 590 nm. Figure 4 shows data for two example wavelengths. 562.5 nm is near the absorbance maximum for low-spin heme *b* (the heme which does not bind oxygen), and at this wavelength the first 50 μs of the reaction are dominated by heme oxidation. At 575 nm, however, there is a small rising phase at the time when oxygen binding might be expected to be seen.

Here again, SVD analysis of the data surface reveals only two major components,[3] shown in Figure 5. The similarity of the kinetic components to those from cytochrome *c* oxidase (Figure 2) is striking.

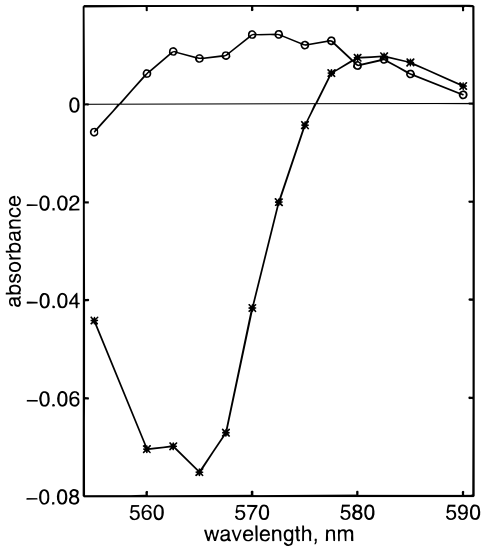

FIGURE 6: Flow-flash reaction of fully reduced cytochrome bo_3 with oxygenspectra of intermediates as determined by a two-step reaction model fit. Circles, intermediate **I**; asterisks, intermediate **II**; the spectrum of the photolysis even was subtracted from the entire surface before the fit; thus, the constant term is not shown (experimental conditions as in Figure 4).

The same two step model kinetic fit was applied to the cytochrome bo_3 data surface (Figure 6). The two rate constants are $k_1 = 9.2 \times 10^4\ s^{-1}$ and $k_2 = 4.1 \times 10^4\ s^{-1}$ ($\tau_1 = 11\ \mu s$; $\tau_2 = 24\ \mu s$). In this case, in order to obtain a good fit, the CO photolysis spectrum (measured using an identical sample without oxygen) was subtracted from the surface, and thus the constant term has zero amplitude and is therefore not shown. The spectrum of component **II** (asterisks) is again consistent with an intermediate in which a large amount of heme oxidation has taken place. Component **I** (circles), which has a broad absorbance peak centered at about 570 nm, is almost certainly the bound oxygen intermediate in cytochrome bo_3, seen here for the first time by optical absorbance spectroscopy in a room temperature reaction. Component **II** clearly shows oxidation of the low-spin heme *b*, which has an absorbance maximum at 563 nm (Puustinen et al., 1992).

DISCUSSION

We have previously studied the early phases of the flow-flash reaction of fully reduced cytochrome *c* oxidase with oxygen by measurements in the Soret region (Verkhovsky et al., 1994). On the basis of that work and other published data (Orii 1984, 1988b; Svensson & Nilsson, 1993; Morgan et al., 1993) we concluded that (1) oxygen binding to the heme is weak; at an oxygen concentration of 1 mM the ferrous-oxy intermediate accounts for only 50%−60% of the enzyme; (2) this binding process is followed by a redox process which traps oxygen, in effect irreversibly; (3) both of these processes are dependent on the oxygen concentration and show saturation kinetics; (4) this suggests that before binding to the heme, oxygen binds transiently to another site in the enzyme, probably Cu_B; (5) the rate of the redox trapping reaction depends on rate of inter-heme electron transfer as well as the oxygen concentration.

In order to account for these findings, in particular that the rate of the oxygen trapping reaction is dependent on *both* the oxygen concentration and the rate of electron transfer between the hemes of the enzyme, we proposed a detailed model for this process [Verkhovsky et al., 1994; see also a model developed by Han et al. (1990b)]. In the model, the first phase of the reaction results in an equilibrium of four states: a residual population of unliganded enzyme (**R**), oxygen bound to Cu_B (**B**), the ferrous-oxy heme species (**A**), and a high-energy, low-occupancy, peroxy- or superoxy-heme species (**P**/**S**). The second phase of the reaction was proposed to involve electron transfer from the low-spin heme trapping the **P**/**S** species as a ferric peroxy species (**P**). Since **R**, **B**, **A**, and **P**/**S** would be in fast equilibrium, this would lead to conversion of the entire enzyme population into the ferric peroxy (**P**) form.

On the basis of the oxygen concentration dependence of the rates of the two phases and on their spectra we concluded (Verkhovsky et al., 1994) that, in the equilibrium system produced by oxygen binding, **A** was present in between 50% and 60% of the enzyme, while **P**/**S** was present in about 10% of the enzyme. The other equilibrium states (**R** and **B**) are not expected to contribute to the spectrum because **R** is the reference state, and **B** is unlikely to be significantly different from **R** in the visible region.

Thus, according to the model and the Soret band data (Verkhovsky et al., 1994), in the reaction of cytochrome *c* oxidase with oxygen, the product of the fast phase of the reaction with cytochrome *c* oxidase with oxygen (intermediate **I**; Figure 3, circles) would correspond to a mixture of **A** and **P**/**S** with a population of between 60% and 70% of the enzyme molecules. In this mixture **A** would dominate. In the simplified kinetic model used earlier in this paper, it is assumed that no other intermediates exist in equilibrium with **I**. Thus, extinction coefficients calculated from **I** (Figure 3, circles) would have to be multiplied by roughly 1.5 to take into account the lower actual population of the ferrous-oxy intermediate (actually **A** plus **P**/**S**).

In the case of cytochrome bo_3, the amplitude of the intermediate **I** spectrum (Figure 6, circles) is very small compared to the corresponding spectrum from cytochrome *c* oxidase (Figure 3, circles), even though the spectra for the subsequent heme oxidation phase are similar in amplitude (Figures 3 and 6, asterisks). This small amplitude probably reflects a smaller extinction coefficient. Although the rate constants for formation and decay of **A** in the two enzymes are slightly different, the kinetic model is built in to the fitting method (Materials and Methods), so that the results should give absorbance values corresponding to 100% occupancy of the intermediate. Preliminary evidence (A. Garcia-Horsman, unpublished) suggests that in cytochrome bo_3 the overall binding of oxygen may be slightly weaker than in cytochrome aa_3 but that this difference is less than a factor of 2, which is too small to account for the present results. One possibility is that in this enzyme the relative populations in the initial equilibrium of states are different since according to the model, the rate of the forward reaction actually depends on the population of the **P**/**S** intermediate rather than on **A** directly.

CONCLUSION

The oxygen-bound species in cytochrome bo_3 is formed and then decays with time constants of 11 μs and 24 μs ($[O_2]$ = 1 mM), very close to those found for the ferrous-oxy intermediate of cytochrome *c* oxidase ($\tau_1 = 10\ \mu s$; $\tau_2 = 36$

μs). This intermediate is apparently the heme o_3 ferrous-oxy species. Its spectrum (difference with respect to reduced enzyme) has a broad maximum in the range 570−575 nm (Figure 6, circles), while the binding of CO to this enzyme produces a peak at 570 nm (Morgan et al., 1993). In the case of cytochrome *c* oxidase, the oxygen binding spectrum has a maximum at 595 nm, while the CO binding spectrum has a maximum at 590 nm (Figure 3). The oxygen binding hemes of cytochrome bo_3 and cytochrome aa_3 are different (Puustinen et al., 1991, 1992), so that peak positions for corresponding compounds are expected to be different, but in both of these enzymes the peak associated with oxygen binding is within a few nanometers of the CO-binding peak.

A complete description of intermediate **A** of cytochrome bo_3 will require more experimental work, but the present data confirm that a ferrous-oxy intermediate exists, and can be resolved by optical absorbance spectroscopy in the reaction of fully reduced enzyme with oxygen at room temperature. This can now be added to the growing list of properties shared by cytochrome bo_3 and the mammalian cytochrome aa_3.

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