Flow-Flash Study of the Reaction between Cytochrome *bo* and Oxygen†

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Received January 26, 1993

ABSTRACT: The reaction between reduced cytochrome *bo* from *Escherichia coli* and oxygen has been studied using flash photolysis of the CO complex of the reduced protein after rapid mixing with oxygen. Absorbance changes were monitored in the α and Soret spectral regions. Two kinetic phases taking place at catalytically competent rates could be detected. The apparent rate constant obtained for both the first and second phase showed a hyperbolic dependence on the oxygen concentration. For the first phase, we obtained limiting first- and second-order rate constants at saturating and low oxygen concentrations of 4.5×10^4 s^{-1} and 1.6×10^8 M^{-1} s^{-1}, respectively. The corresponding values for the second phase were 5×10^3 s^{-1} and 1.7×10^7 M^{-1} s^{-1}. The first phase accounted for 30% of the total absorbance change in the Soret band (430 nm) and 15% of the total absorbance change in the α band (555 nm). These reactions are followed by a very slow phase with a lifetime of about 1 s. We have also studied the interaction between the fully oxidized enzyme and hydrogen peroxide, and we have found that peroxide binding induces an absorbance increase in the α band and a red shift of the Soret band. A consideration of the magnitude of the absorbance changes taking place during the first phase suggests that this reaction includes at least partial oxidation of the low-spin cytochrome *b*.

The final step in the respiratory chain of *Escherichia coli* is the oxidation of ubiquinol by molecular oxygen. When the bacterium grows under highly aerobic conditions, this reaction is carried out by an *o*-type terminal oxidase, which also couples the redox reaction to proton translocation across the cell membrane (Puustinen et al., 1989). In contrast to the proton-translocating oxidase of mitochondria, a cytochrome aa_3, this enzyme contains only three redox-active prosthetic groups: the low-spin cytochrome *b*, the high-spin cytochrome *o*, and the copper ion Cu_B. Cytochrome *o* and Cu_B form together the active site for binding and reduction of oxygen, whereas cytochrome *b* has been suggested to serve as an intermediate electron reservoir between ubiquinol and the oxygen-binding site (Gennis, 1991). In the following, we will refer to the functional enzyme as cytochrome *bo* and to its low- and high-spin heme groups as cytochrome *b* and cytochrome *o*, respectively.

Despite their different sets of redox-active groups, the cytochromes *bo* and aa_3 have been shown to be closely related in terms of sequence homology (Chepuri et al., 1990), and both enzymes catalyze redox-linked proton translocation. Extensive sequence homology among terminal oxidases present in a variety of prokaryotes led these authors to suggest that the proteins form a superfamily of proton-translocating terminal oxidases. All members of this family contain three redox-active sites: two heme groups and a copper ion equivalent to Cu_B. In addition, the cytochrome *c* oxidases contain a fourth site, Cu_A. For recent reviews on the similarities and differences among the proton-translocating terminal oxidases, see Gennis (1991) and Saraste et al. (1991).

The course of oxygen reduction in the mitochondrial cytochrome *c* oxidase has been studied extensively by the flow-flash method pioneered by Gibson and Greenwood (1967). In a recent review, Babcock and Wikström (1992) summarize a wealth of results obtained using the flow-flash method in different configurations. A mechanism for the reduction of oxygen consistent with available data is also proposed. Given the similarities between the different terminal oxidases in terms of protein structure and of the overall reaction, one would expect also a similar course of oxygen reduction in all these enzymes. However, detailed kinetic studies have been carried out only on the bovine cytochrome *c* oxidase.

In the members of the oxidase family that utilize quinol as substrate, the Cu_A site is absent. Consequently, only three electron equivalents are available in their fully reduced states. In the absence of additional electron donor, the end product in the reaction between a reduced quinol oxidase and water is therefore expected to be a species where oxygen is reduced to the three-electron level. During turnover, water formation would then take place only after input of an additional electron from the reducing substrate. The availability of three electrons in the fully reduced state of quinol oxidases makes them particularly attractive for investigating the generality of reaction schemes such as that suggested by Babcock and Wikström (1992). Also, the absence of the Cu_A site is likely to result in a simpler time course of the reaction.

In the present paper, we have investigated the reaction between the reduced form of the quinol oxidase cytochrome *bo* from *E. coli* and oxygen at room temperature using the flow-flash method with spectrophotometric detection. In contrast to the complex kinetics of the corresponding reaction in the mitochondrial cytochrome *c* oxidase, we observed only two kinetic phases. From the magnitude of the rate constants and their dependence on the oxygen concentration, these kinetic phases seem to correspond to the first and intermediate phases observed in mitochondrial cytochrome *c* oxidase by Hill and Greenwood (1984). The magnitude of the observed absorbance changes and their spectral dependence are consistent with substantial oxidation of the low-spin cytochrome *b* during the first phase. The extrapolated first-order rate constant at

† This work was supported by grants from the Procordia Research Foundations, the Knut and Alice Wallenberg Foundation, the Sven and Ebba-Christina Hagberg Foundation, and the Swedish Natural Science Research Council.

0006-2960/93/0432-5442$04.00/0

saturating oxygen concentration for this phase was 4.5×10^4 s^{-1}. A preliminary account of some of the results was presented at the 7th EBEC Conference (Svensson & Nilsson, 1992).

MATERIALS AND METHODS

Materials. DEAE-Sepharose CL-6B was obtained from Pharmacia (Uppsala, Sweden) and Bio-Gel HT from Bio-Rad (Hercules, CA). Urea (sequenal grade) was from Pierce (Rockford, IL) and hydrogen peroxide (Suprapur; 30%) from Merck (Darmstadt, Germany). All other chemicals were of analytical grade. Cytochrome *bo* was isolated from the *E. coli* strains RG145 (Au & Gennis, 1987) and GO103 (Oden et al., 1990). Both strains, in which the cytochrome *d* gene has been deleted from the chromosome, were generous gifts from Prof. Robert Gennis, University of Illinois. Cells were grown in a 20-L fermenter (Chemap) in the medium described by Georgiou et al. (1988) containing 1% sodium lactate as the carbon source instead of glucose. Maximal aeration was used, and cells were harvested at an OD (550 nm) of about 5 (Zeiss PMQII spectrophotometer). Cell membranes were isolated as described by Georgiou et al. (1988) and stored frozen at −70 °C. Cytochrome *bo* was solubilized by Triton X-100 and (*N*-dodecyl-*N*,*N*-dimethylammonio)propanesulfonate (Sulfobetaine-12) after the membranes were prewashed with Triton X-100, as suggested by Krantz and Gennis (1983) using the following procedure. Membranes from about 150 g of wet cells were suspended in 50 mM Tris-HCl, pH 8, at 50 mg of protein/mL [the concentration was determined using the biuret method as described by Hartzell et al. (1978)]. Then, Triton X-100 was added to a final concentration of 1% (20% stock solution); the suspension was stirred for 30 min at 4 °C and ultracentrifuged at 160000*g* for 90 min. After homogenization of the pellet with Tris buffer as above, Triton X-100 and Sulfobetaine-12 were added to final concentrations of 1%. The suspension was stirred for 30 min at 4 °C and ultracentrifuged at 160000*g* for 90 min. The supernatant was chromatographed on DEAE-Sepharose (5 × 30 cm column) as described by Kita et al. (1986). The red fraction eluting at about 0.15 M NaCl was concentrated by ultrafiltration (Amicon YM-50 membrane) and applied to a hydroxyapatite column (Bio-Gel HT; 5 × 15 cm) equilibrated with 5 mM potassium phosphate, pH 7.5, containing 0.05% sodium *N*-dodecylsarcosinate. The column was washed with one volume of equilibration buffer, and then the enzyme was eluted with 0.7 M potassium phosphate, pH 7.5, containing 0.05% sodium *N*-dodecylsarcosinate. The red eluate was concentrated as above to 50–100 μM, frozen, and stored in liquid nitrogen. Analysis of the purified protein by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate showed the presence of four bands, and the purity of the preparation was judged comparable to that obtained in the recent work of Minghetti et al. (1992). The concentration of cytochrome *bo* in stock solutions was estimated using the pyridine hemochrome method as described by Puustinen et al. (1991), with the extinction coefficient given by these authors [$\Delta\Delta\epsilon$(red-ox, 553–535 nm) = 24 mM^{-1} cm^{-1}]. Optical spectra were recorded on a Shimadzu UV-3000 or a Cary 4 spectrophotometer.

Flow-Flash Experiments. Samples of the fully reduced CO complex of cytochrome *bo* were prepared by one of the following two procedures. The enzyme stock solution was diluted with 0.1 M Hepes[1]–KOH, pH 7.5, containing 0.05% sodium *N*-dodecylsarcosinate. 2,3-Dimethoxy-5-methylbenzoquinone was added to a concentration of 10 μM, and the sample was made anaerobic by repeated evacuation and flushing with purified nitrogen. Ascorbate was then added anaerobically to a final concentration of 5 mM and the sample incubated on ice overnight. Alternatively, phenazine methosulfate (PMS) at a concentration of 5 μM was used as mediator, in which case reduction was complete after 1–2 h at room temperature. After the formation of the fully reduced enzyme was complete, the atmosphere in the cuvette was changed to CO. Formation of the fully reduced enzyme and its CO derivative was verified with optical spectroscopy, and the final concentration of the CO complex was calculated from the absorbance difference induced by CO binding to the reduced enzyme and a differential extinction coefficient of 287 mM^{-1} cm^{-1} at 416–430 nm (Puustinen & Wikström, 1991). Flow-flash experiments were carried out as described by Hallén and Nilsson (1992). Enzyme and oxygenated buffer were mixed in a ratio of 1:5 in the rapid-mixing apparatus in order to access a wide range of oxygen concentrations.

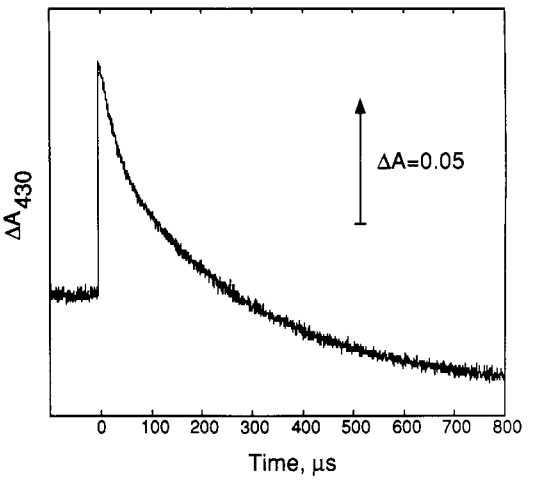

FIGURE 1: Absorbance changes in the Soret band (430 nm) after flash photolysis of the CO compound of fully reduced cytochrome *bo* in the presence of oxygen. The concentrations of enzyme and oxygen after mixing were 0.75 μM and 1 mM, respectively. The trace shown is the average of three individual transients.

RESULTS

Figure 1 shows the time course of absorbance changes in the Soret band after flash photolysis of the CO complex of the fully reduced cytochrome *bo* in the presence of oxygen, detected at 430 nm. Kinetic analysis of this trace revealed the presence of two phases, with apparent rate constants of 3.2×10^4 s^{-1} and 3.4×10^3 s^{-1}. The contributions of the first and second phases to the total amplitude were 30% and 70%, respectively. Two phases with similar rate constants were observed also when the reaction was followed at 555 nm in the α band (Figure 2). At this wavelength, the contribution of the first phase to the total amplitude was 15%. The wavelength dependences of the amplitudes of the two phases are shown as kinetic difference spectra in Figure 3. For comparison, this figure also displays the total amplitude of the two phases, and the absorbance change caused by the initial CO photodissociation at the different wavelengths.

The traces shown were obtained using enzyme isolated from the RG145 strain. With enzyme obtained from the GO103 strain, similar rate constants were obtained in an experiment with detection in the Soret band. However, the relative amplitudes of the two phases were different, with a larger relative contribution from the rapid phase in enzyme isolated

[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMS, phenazine methosulfate.

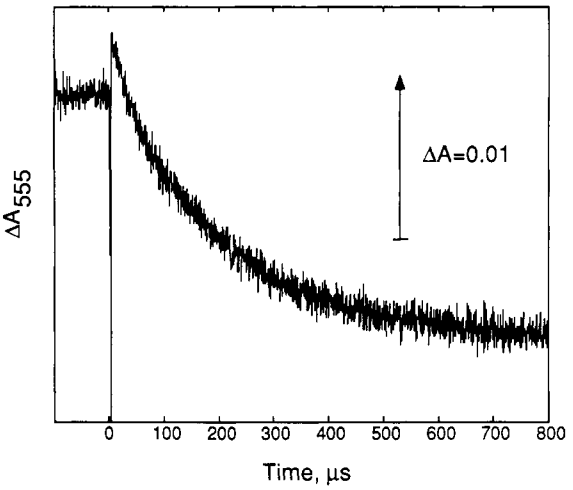

FIGURE 2: Absorbance changes in the α band (555 nm) after flash photolysis of the CO compound of fully reduced cytochrome *bo* in the presence of oxygen. The concentrations of enzyme and oxygen after mixing were 1.6 μM and 1 mM, respectively. The trace shown is the average of 12 individual transients.

from the GO103 strain (not shown). The method used for sample preparation did not affect the time course monitored in the Soret band. The results shown in the following were obtained with enzyme isolated from the RG145 strain.

The rate constants obtained for both the first and the second phases were found to depend on the concentration of oxygen. Figure 4 shows the apparent rate constants as functions of the oxygen concentration. After an initial linear increase, both rate constants appear to approach a limiting value. The data were found to be well described by a hyperbolic function

$$k_{obs} = \frac{kk'[O_2]}{k + k'[O_2]}$$

where k and k' are the limiting first- and second-order rate constants obtained when the oxygen concentration approaches infinity or zero, respectively. From curve fitting, we obtain $k = 4.5 \times 10^4\ s^{-1}$ and $k' = 1.6 \times 10^8\ M^{-1}\ s^{-1}$ for the first phase. The corresponding values for the second phase were $k = 5 \times 10^3\ s^{-1}$ and $k' = 1.7 \times 10^7\ M^{-1}\ s^{-1}$. The curve fits are shown as solid lines in the figure.

In addition to the rapid reactions described above, a very slow component with a lifetime of about 1 s was observed. To eliminate the possibility that this phase reflects thermal CO dissociation in a subpopulation of the CO complex present after incomplete photolysis, we examined the effect of additional laser pulses during the course of the reaction. This did not affect the time course. The reaction was also present in a stopped-flow variant of the experiment, where reduced enzyme in the absence of CO was allowed to react with oxygen without photolysis. The possibility that this slow phase reflects a reaction between CO and the species present after the two rapid phases was investigated by including CO in the oxygenated buffer. No effect of the presence of CO was, however, seen (not shown). Variation of the concentration of mediator (PMS) used for reduction of the enzyme in the range 5–25 μM did not affect the observed time course.

A possible intermediate in the oxygen reaction contains peroxide bound to ferric cytochrome *o* (Babcock & Wikström, 1992). In order to help the assignment of the time-resolved absorbance changes, we have therefore also investigated the spectral changes induced by the reaction between the oxidized enzyme and hydrogen peroxide by static difference spectroscopy. Addition of a small excess of hydrogen peroxide to the oxidized enzyme resulted in increasing absorbance in the α band and a red shift of the Soret band. These spectral changes are shown in the difference spectrum in Figure 5. From this spectrum, we estimate a differential extinction coefficient of 2.6 $mM^{-1}\ cm^{-1}$ at 555 nm for peroxide binding.

DISCUSSION

Two kinetic phases taking place at catalytically competent rates could be detected in the reaction between reduced cytochrome *bo* and oxygen at room temperature. The rate constants obtained are similar to those of the first and intermediate phase of the oxygen reaction in the mitochondrial cytochrome *c* oxidase observed by Hill and Greenwood (1984). With the latter enzyme, a third component with a rate constant of about 700 s^{-1} is also present. This reaction has been attributed to transfer of the fourth electron from cytochrome a/Cu_A to the oxygen-binding site (Babcock & Wikström, 1992). The absence of a corresponding kinetic phase in the reaction of cytochrome *bo* and oxygen is consistent with this interpretation. The reaction of reduced cytochrome *bo* in whole *E. coli* cells with oxygen at cryogenic temperatures has been studied earlier by Poole et al. (1979a,b) using optical spectroscopy. At −105 °C, only photoreversible oxygen binding could be detected by these authors (Poole et al., 1979b). At somewhat higher temperatures (>−70 °C), this reaction became unmeasurably fast, and instead two phases interpreted as cytochrome oxidation appeared. This behavior is similar to that observed here with the purified enzyme at room temperature.

The first phase observed by Hill and Greenwood in the bovine cytochrome *c* oxidase at room temperature was later resolved into two components with apparent rate constants (at 1 mM O_2) of $9 \times 10^4\ s^{-1}$ and $3 \times 10^4\ s^{-1}$ (Oliveberg et al., 1989; Oliveberg & Malmström, 1992). In the present study we have not been able to detect a faster component in the rapid phase despite the use of a high oxygen concentration. We have, however, observed a small variation of the observed rate constants with the observation wavelength (not shown), which may indicate the presence of an additional, unresolved component.

The rate constant obtained for the first phase observed in the present study depends on the oxygen concentration in a hyperbolic manner. From the data shown in Figure 4, we obtain a limiting first-order rate constant of $4.5 \times 10^4\ s^{-1}$ at saturating oxygen concentration. The limiting second-order rate constant at low oxygen concentration was $1.6 \times 10^8\ M^{-1}\ s^{-1}$. Both values are somewhat higher than those observed with the bovine enzyme by Hill and Greenwood (1984), and the latter is consistent with the combination of the reduced protein with oxygen being limited by diffusion. From the limiting first- and second-order rate constants, an apparent K_M value for oxygen of 0.28 mM may be calculated, which is close to that found for the bovine cytochrome *c* oxidase (Hill & Greenwood, 1984).

For the mitochondrial cytochrome *c* oxidase, it was suggested originally by Hill and Greenwood (1984) that substantial oxidation of the low-spin cytochrome *a* takes place during the $2.5 \times 10^4\ s^{-1}$ phase. This was later confirmed in studies using time-resolved resonance Raman spectroscopy to monitor the progress of the reaction (Han et al., 1990; Varotsis & Babcock, 1990). However, it has been suggested that this rapid cytochrome *a* oxidation is a short-circuit reaction unique to the fully reduced cytochrome *c* oxidase (Babcock & Wikström, 1992). We shall, therefore, consider also two alternative

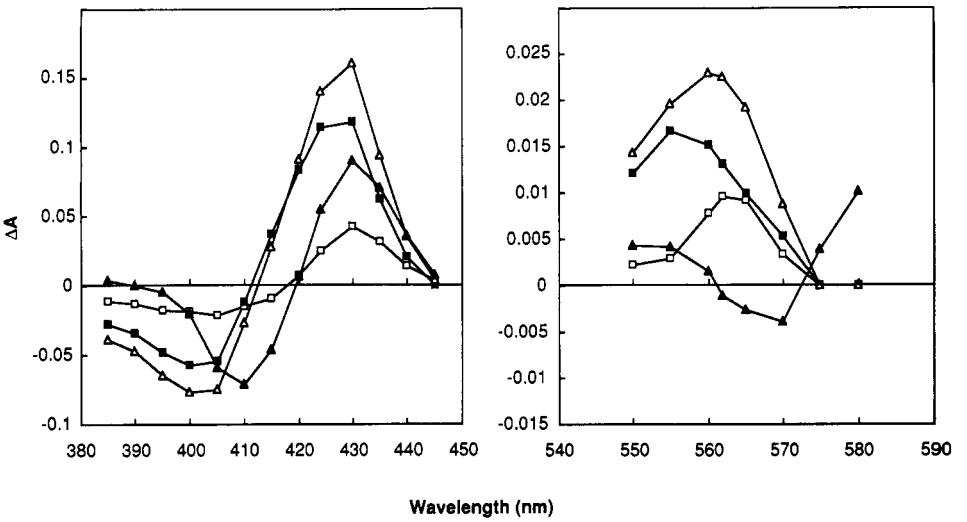

FIGURE 3: Kinetic difference spectra for the first and second phase of the reaction, for the overall reaction, and for the initial CO dissociation from the reduced CO complex. In the Soret band (left panel) the reaction was monitored under the same conditions as in Figure 1, whereas the conditions given with Figure 2 were used for detection in the α band (right panel). Symbols: (▲) absorbance changes induced by CO dissociation, given as the absorbance immediately after the flash *minus* the absorbance before the flash; (□) amplitude of the first phase, given as absorbance of species present before the reaction *minus* that of the species after the reaction; (■) amplitude of the second phase, with the same sign convention as for the first phase; (△) total amplitude of the two phases.

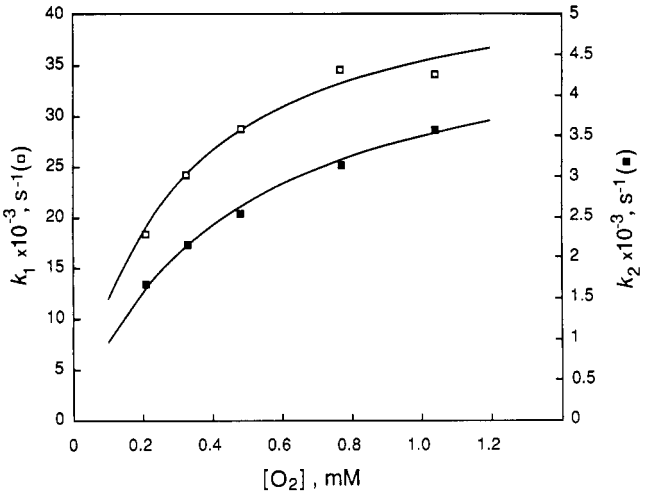

FIGURE 4: Effect of oxygen concentration on the apparent rate constants for the first and second phases: (□) first phase (k_1; left axis); (■) second phase (k_2; right axis). The solid lines are hyperbolic fits as described in the text.

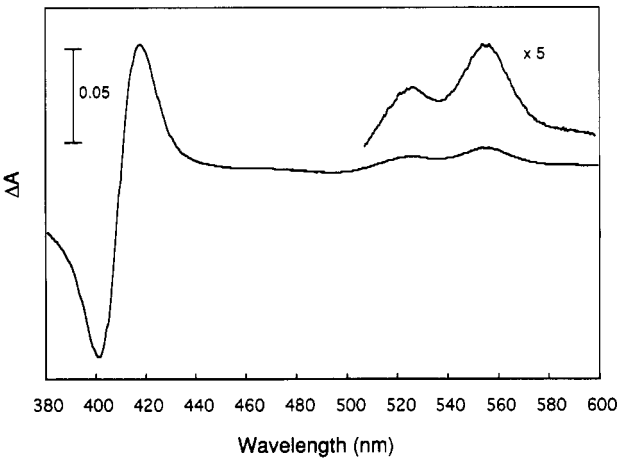

FIGURE 5: Difference spectrum obtained by subtracting the spectrum of the oxidized enzyme from that of the hydrogen peroxide adduct. Cytochrome *bo* was diluted to a concentration of 3.75 μM (this concentration is based on the pyridine hemochrome assay) with 0.1 M Hepes–KOH, pH 7.5, containing 0.05% sodium *N*-dodecylsarcosinate, and a spectrum of the oxidized enzyme was recorded. Then H_2O_2 was added (final concentration 10 μM), and the spectrum of the H_2O_2 adduct was recorded after 20 min.

possibilities for the nature of the first phase observed in cytochrome *bo*: the formation of a ferrous cytochrome *o*–dioxygen adduct and the formation of a compound at the oxidation level of ferric–cupric peroxide at the oxygen-binding site, with cytochrome *b* still reduced. In the low-temperature study by Poole et al. (1979b), an initial phase of photoreversible oxygen binding with a difference spectrum closely resembling that for CO photodissociation was identified at the lowest temperatures. In the present case, however, the difference spectra for CO photodissociation and the first phase of the oxygen reaction are clearly different (Figure 3). We hold it therefore less likely that the rapid phase observed in the present study corresponds to the reversible oxygen binding observed at low temperature. The second alternative was suggested in a preliminary account of this work (Svensson & Nilsson, 1992). However, this interpretation can also be argued against on spectral grounds. The total absorbance change at 555 nm in a static reduced minus oxidized difference spectrum corresponds to a $\Delta\epsilon$ of 20.5–24 mM^{-1} cm^{-1} (Puustinen et al., 1991; Minghetti et al., 1992). These authors have demonstrated that the major part of the absorbance change is due to cytochrome *b* oxidoreduction. From the data provided, it can be calculated that the differential extinction coefficient for cytochrome *o* oxidoreduction is less than 2 mM^{-1} cm^{-1}. On the other hand, binding of hydrogen peroxide to the fully oxidized enzyme results in an absorbance increase, corresponding to a $\Delta\epsilon$ of 2.6 mM^{-1} cm^{-1} at 555 nm (Figure 5). This means that oxidation of cytochrome *o* only, followed by binding of peroxide to the oxidized species, would be expected to produce little absorbance decrease, or even absorbance increase at 555 nm. Since we observe a substantial absorbance decrease at 555 nm in the rapid phase (Figure 2), formation of a ferric–cupric peroxide compound without cytochrome *b* oxidation appears less likely. These considerations leave, as the one most viable, the notion that the first phase does include cytochrome *b* oxidation. It appears thus that the oxygen reactions of cytochrome *bo* and cytochrome *c* oxidase are very similar and, consequently, that rapid oxidation of the low-spin cytochrome can take place also in a three-electron system. The analogy is further supported by our observation of proton

uptake after the first phase (S. Hallén, M. Svensson, and T. Nilsson, unpublished observation). The species present after the first phase may then be expected to correspond to the ferric–cuprous peroxide intermediate 4 of Babcock and Wikström (1992).

The second phase of the reaction also showed a hyperbolic dependence on the oxygen concentration (Figure 3). A hyperbolic fit to the data shown gave limiting first- and second-order rate constants of 5×10^3 s^{-1} and 1.7×10^7 M^{-1} s^{-1}, respectively. The value for the limiting first-order rate constant is similar, but somewhat lower than that found for the intermediate phase [7×10^3 s^{-1}; Hill and Greenwood (1984)]. These authors also noted that the apparent rate constant of their intermediate phase was dependent on the oxygen concentration. It is not clear why the rate constant for the second phase observed in the present work, and that of the intermediate phase of Hill and Greenwood, is dependent on the oxygen concentration. In a linear sequence of irreversible reactions where only the first step includes oxygen [such as the scheme suggested for the bovine cytochrome aa_3 by Babcock et al. (1992)], only one rate constant is expected to be oxygen dependent. This may indicate the occurrence of reversible steps in the early part of the reaction (Orii, 1988).

If the species present after the first phase is a ferric–cuprous peroxide intermediate, the subsequent reaction is likely to be cleavage of the oxygen–oxygen bond with the formation of a species corresponding to the oxoferryl intermediate (compound F) of cytochrome aa_3. The question then arises whether such a reaction can account for all the spectral changes observed in the second phase. In the bovine cytochrome aa_3, conversion of the peroxy form (compound P) to compound F is accompanied by absorbance decrease in most of the α band (Vygodina & Konstantinov, 1988). In the Soret band, however, the spectra of compounds P and F are nearly identical. In the present work, the spectral changes taking place in the second phase are absorbance decrease in the α band and a blue shift of the Soret band (Figure 3). Assignment of the second phase solely to a conversion from a peroxy to an oxoferryl state requires therefore, in contrast to what is observed in the mitochondrial enzyme, that the Soret band of the latter species is blue shifted relative to the former. Although this appears possible, we cannot rule out the alternative that the observed spectral changes in the second phase also contain contributions from cytochrome *b* oxidation. In the latter case, cytochrome *b* oxidation would be kinetically heterogeneous.

Kinetic heterogeneity in the oxidation of the corresponding site in the bovine enzyme, cytochrome *a*, was observed by Hill and Greenwood (1984). An attractive explanation for this behavior was put forward by Hill (1991). He suggested that the two phases observed reflect two different steps in oxygen reduction and that both these result in cytochrome *a* oxidation because cytochrome *a* is partially rereduced by Cu_A after the first step at a rate intermediate between the two redox steps. However, this line of reasoning requires the presence of a reduced Cu_A site that can donate electrons rapidly to cytochrome *a*. Kinetic heterogeneity in cytochrome *b* oxidation during the oxygen reaction of cytochrome *bo* (which lacks the Cu_A site) would suggest, rather, that the behavior is intrinsic to the three conserved metal sites. Clearly, the contribution of cytochrome *b* oxidation to the two phases needs to be further investigated with methods that more directly can probe the oxidation states of cytochrome *b* and cytochrome *o*.

In the absence of additional electrons, a species at the ferryl level of oxidation should be the stable end product. This is in accord with our observation that subsequent reaction only takes place at a rate more than 3 orders of magnitude slower than the first two phases. With reported turnover numbers around 700 e^-/s (Puustinen et al., 1992), this reaction is clearly too slow to be of catalytic significance and probably reflects decay of the species present after the two rapid phases into the fully oxidized enzyme. The nature of the slow reaction is not clear. The observed rate was dependent neither on the mediator concentration nor on the presence of CO, which suggests that no additional electron input is required.

In conclusion, the results obtained in the present work are consistent with a mechanism for oxygen reduction in cytochrome *bo* analogous to that suggested for the bovine cytochrome *c* oxidase. This further strengthens the notion that the quinol and cytochrome *c* oxidases are related functionally despite their different sets of redox-active components. Furthermore, we have demonstrated the applicability of the flow-flash method to this class of oxidases. In future work, we will apply this technique to the study of proton transfers during the oxygen reaction of cytochrome *bo*.

ACKNOWLEDGMENT

We thank Prof. Robert B. Gennis for generously providing the *E. coli* strains RG145 and GO103, Mr. Stefan Hallén and Prof. Bo G. Malmström for useful discussions, and Mrs. Ann-Cathrine Smiderot and Ms. Ann Kjellström for help with the protein purification.

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