The Room Temperature Reaction of Carbon Monoxide and Oxygen with the Cytochrome *bd* Quinol Oxidase from *Escherichia coli*†

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ABSTRACT: When grown under O_2-limited conditions, *Escherichia coli* expresses a cytochrome *bd* quinol oxidase that has an unusually high affinity for O_2. We have studied the reaction of cytochrome *bd* with CO and O_2 by rapid-reaction spectrophotometry. The reduced enzyme forms a photosensitive ferrocytochrome *d*−CO complex, and following photolysis, CO recombines with the reduced enzyme with a bimolecular rate of 8×10^7 M^{-1} s^{-1}. Reaction of CO-bound enzyme with O_2 gives a CO off-rate of 1.6 s^{-1}. The O_2 reaction is followed by a flow−flash procedure in which CO-ligated enzyme is mixed with O_2, and the reaction commenced by photolysis of cytochrome *d*−CO. In the presence of O_2, two processes are resolved on a time−scale of 300 μs. The absorbance at 645 nm first increases at a rate that is dependent on O_2 concentration with a value of 2×10^9 M^{-1} s^{-1}. The second phase results in decreased absorbance at 645 nm and increased absorbance at 680 nm. The rate of the second process is independent from O_2 concentration above 50 μM O_2 and reaches a first-order limit of 1×10^4 s^{-1}. A model for the reaction of the cytochrome *bd* quinol oxidase with O_2 is proposed in which an initial ferrocytochrome *d*−oxy adduct forms, and then decays to a ferryl−oxo species. The oxidation of the low-spin cytochrome *b* component of the oxidase, monitored at 560 nm, occurs at the same time as the ferryl species forms. We suggest that the suitability of the cytochrome *bd* quinol oxidase to function at low O_2 concentration is conferred by its rapid rate of binding O_2.

Aerobically grown *Escherichia coli* produces two respiratory quinol oxidases, cytochrome bo_3 and cytochrome *bd* complexes. The cytochrome *bd* quinol oxidase predominates when the bacterium grows in an environment with low oxygen tension (Rice & Hempfling, 1978; Anraku & Gennis, 1987). It has been demonstrated in nongrowing whole cells that the *bd*-type oxidase has an unusually high affinity for oxygen (K_M = 24 nM) relative to cytochrome bo_3 (K_M = 240 nM) (Rice & Hempfling, 1978) and to cytochrome *c* oxidase from mitochondria (K_M = 1 μM; Petersen et al., 1976). The *bd*-type oxidase is also unusual as an O_2 reductase in that it forms stable oxygen adducts at the oxygen reduction site (Lorence & Gennis, 1989; Kahlow et al., 1991). The air-oxidized enzyme, as isolated, is a mixture of different oxygenated states.

The cytochrome *bd* complex contains three metal centers: cytochrome b_{558}, cytochrome b_{595}, and cytochrome *d*, in a protein matrix composed of two subunits with molecular weights of 57K and 43K (Miller & Gennis, 1983). The stoichiometry of the centers is proposed to be 1:1:1 from quantitative electron paramagnetic resonance (EPR)[1] studies (Meinhard et al., 1989), but CO binding experiments have been interpreted to indicate two heme *d* moieties per enzyme molecule (Lorence et al., 1986). However, FTIR shows a single, uniform CO environment consistent with a single heme *d* group per enzyme molecule (Hill et al., 1993). Photolysis of ferrocytochrome *d*−CO at low temperature (i.e., 20 K) leads to occupancy of two other sites by CO: (1) a weak nonmetallic coordination site on the surface of the protein within the heme binding pocket and (2) a second heme site distinct from heme *d* and suggested to be the heme of cytochrome b_{595}. Thus, cytochrome b_{595} has been assigned a transient ligand binding role analogous to that proposed for the Cu_B center in mitochondrial cytochrome *c* oxidase (Hill et al., 1993).

Poole and co-workers (Poole et al., 1983) have studied the reaction of O_2 with cytochrome *bd* in membranes from O_2-limited cultures of *E. coli*, over the temperature range from 140 to 185 K. These workers were able to resolve two intermediates in the reaction with O_2 with peaks at 650 and 675 nm. In this study, we have characterized the reaction kinetics at room temperature of detergent-solubilized cytochrome *bd* by flash photolysis of its CO adduct in the absence and presence of O_2. The CO binding site in the cytochrome *bd* quinol oxidase is kinetically distinct from mitochondrial cytochrome *c* oxidase. It reacts much faster with both CO and O_2, and we conclude that this contributes significantly to the higher affinity for both of these ligands exhibited by the *bd*-type oxidase relative to cytochrome *c* oxidase from mitochondria.

MATERIALS AND METHODS

Cytochrome *bd* was purified from plasma membranes of *E. coli* as described by Miller and Gennis (1983). The isolated enzyme was dissolved in 67 mM potassium phosphate, pH 7.5, with 0.01% Sarkosyl and 0.1% Tween-20.

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[1] Abbreviations: EPR, electron paramagnetic resonance; FTIR, Fourier transform infrared; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

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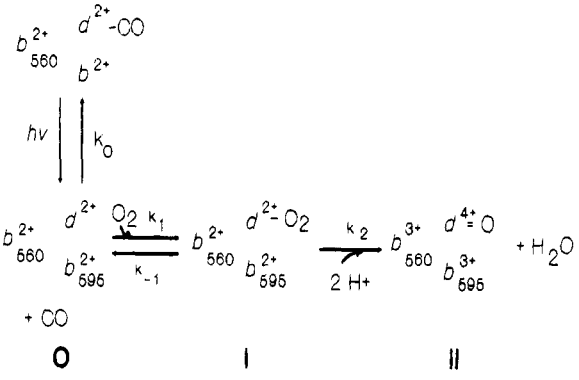
Scheme 1



The enzyme was prepared for kinetic experiments as follows. Stock enzyme was diluted to a concentration of 10−20 μM in the buffer described above. The enzyme concentration was determined by using an extinction coefficient of 18 mM^{-1} cm^{-1} at 560−577 nm for the reduced−oxygenated difference spectrum. The enzyme was reduced with 4 mM sodium ascorbate plus 2 μM TMPD. The reduced enzyme was then placed in a gas-tight Hamilton syringe previously flushed with N_2. CO was added to the reduced enzyme by addition of a small aliquot of CO-saturated buffer. The reduced, CO-bound enzyme was transferred to a Bio-Logic stopped-flow mixing block. The enzyme was mixed with varying concentrations of either CO or O_2 by use of the three-syringe mixing capability of the Bio-Logic mixer. Syringe 1 contained reduced enzyme, syringe 2 contained N_2-equilibrated buffer, and syringe 3 contained either CO- or O_2-saturated buffer. In the case of the CO concentration studies, the sample was allowed to sit for a few minutes between each mix to ensure that the sample was anaerobic prior to laser photolysis.

Reaction kinetics were measured using a kinetic spectrometer constructed as described previously (Hill, 1991). Data were acquired using a Phillips PM3323 digital oscilloscope and then transferred and analyzed using the Asystant GPIB software package running on a Dell personal computer. Simulation of intermediate time courses was done by numerical analysis of a system of linear differential equations shown below as derived from the model outlined in Scheme 1.

$$d[0]/dt = -k_1[O_2][0] + k_{-1}[I]$$

$$d[I]/dt = k_1[O_2][0] - (k_{-1} + k_2)[I]$$

$$d[II]/dt = k_2[I]$$

0, I, and II signify the intermediate forms of the oxidase, and k_1, k_{-1}, and k_2 are the elementary rate constants in their interconversion. A fourth-order Runge−Kutta method was used for this analysis. Static spectra were recorded on a Hewlett-Packard diode-array (Model 8452A) spectrophotometer.

RESULTS

Figure 1 is a set of spectra of purified cytochrome *bd* as isolated from *E. coli* plasma membranes. Panel A shows three absolute spectra corresponding to the enzyme as isolated, the enzyme reduced with ascorbate plus TMPD, and the reduced enzyme exposed to CO. The enzyme as isolated has a major absorption band centered at 646 nm with a distinct shoulder at 680 nm, and these are due to oxygenated states of the cytochrome *d* center. When the enzyme is reduced, the shoulder at 680 nm disappears, the 646 nm band shifts to 629 nm, and new bands appear at 595 and 560 nm. These features are due to the reduction of cytochrome *d*, cytochrome b_{595}, and cytochrome b_{560}. Addition of CO induces a shift in the band assigned to cytochrome *d* from 629 nm to 636 nm. Panel B shows the difference spectrum between the reduced enzyme with and without CO. This spectrum shows clearly the shift in the cytochrome *d* spectrum without any accompanying change at 560 nm. Whereas cytochrome *bd* in the membrane binds CO at only one site (i.e., cytochrome *d*), some preparations of purified enzyme display additional CO binding to the cytochrome b_{560} heme [e.g., see Kita et al. (1984)]. Such heterogeneity explains the high values obtained previously for the number of CO binding sites in purified cytochrome *bd* (Lorence et at., 1986). The detailed origin of this

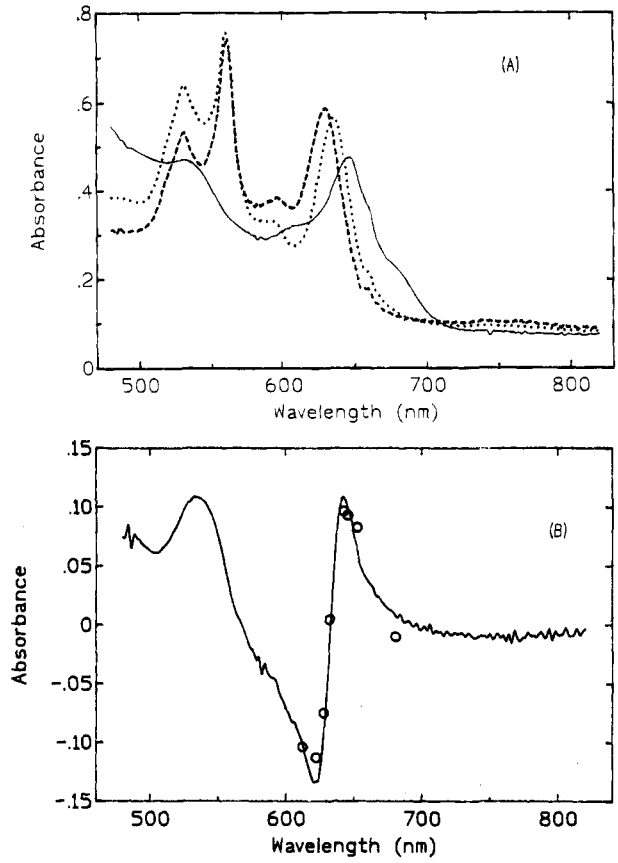


FIGURE 1: Visible region spectra of different states of detergent-solubilized *E. coli* cytochrome *bd* oxidase. (A) Absolute spectra. The enzyme was dissolved at a concentration of 19 μM in 67 mM potassium phosphate, pH 7.5, plus 0.01% sarkosyl and 0.1% Tween 20. The enzyme was reduced with 3.5 mM sodium ascorbate plus 3.5 μM TMPD. The CO-bound spectrum was obtained with 1 atm of CO. The spectra are indicated as follows: (—) isolated; (- - -) reduced; (•••) reduced plus CO. (B) CO difference spectra. The solid line indicates the difference spectrum obtained by subtracting the reduced plus CO spectrum minus the reduced spectrum. The conditions are the same as those given above. The open circles are absorbance amplitudes obtained from flash photolysis of the CO complex. These kinetic data were obtained under the conditions given in the legend to Figure 2A. The amplitudes have been normalized for comparison to the static difference spectrum.

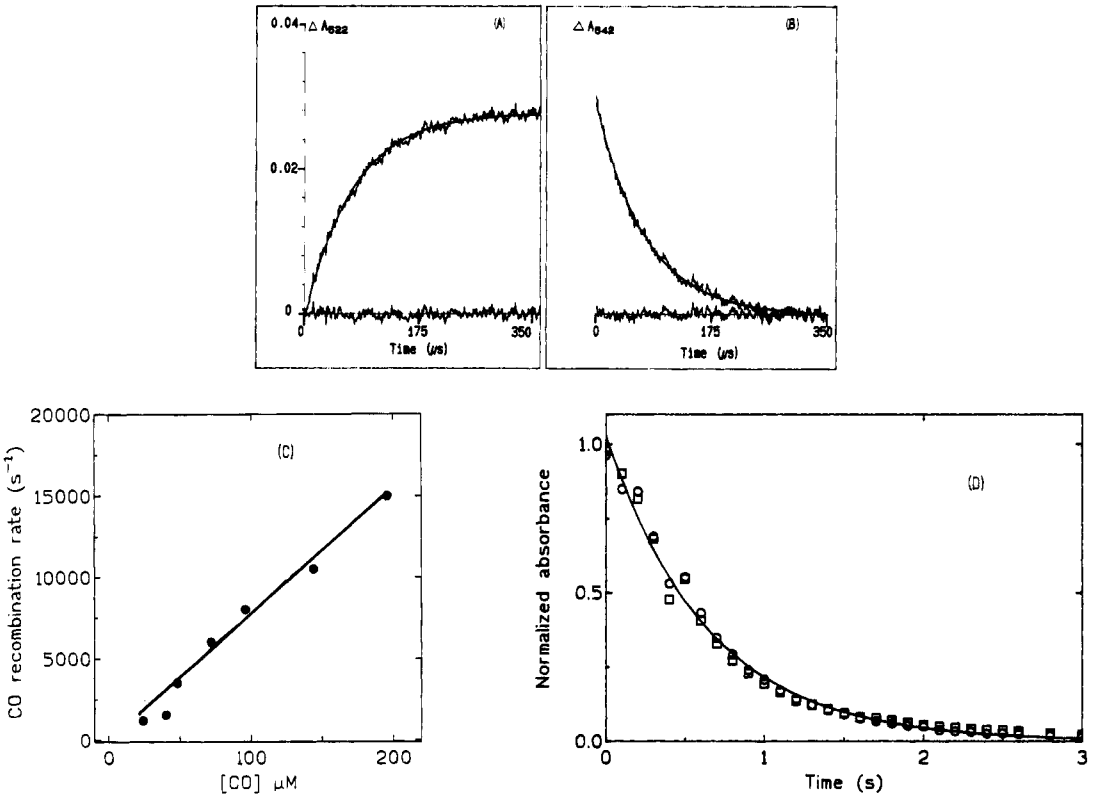


FIGURE 2: Kinetics of CO binding and dissociation to reduced cytochrome *bd* oxidase. (A) Absorbance at 622 nm following flash photolysis of the CO complex. The enzyme was dissolved in the same buffer described above, reduced with ascorbate plus TMPD as above, and reacted with 160 μM CO. The temperature was 20 °C. The noisy line of increasing absorbance is the experimental trace, and the smooth line through these data is a fit to a single-exponential curve. The noisy line centered around 0 ΔA is the distribution of residuals between the experimental data and the fitted line. (B) Absorbance at 642 nm following flash photolysis of the CO complex. The data were obtained under the same conditions as in panel A. The figure shows the experimental data, a single-exponential fit to these data, and the distribution of residual error between the experimental and fitted data. (C) Concentration dependence of the rate of CO recombination. The observed rate of CO recombination was obtained by fitting recombination profiles to a single-exponential expression. The profiles were measured at 622 nm, and the CO concentration was varied by adding aliquots of CO-saturated buffer. All measurements were made at 20 °C. (D) Rate of CO dissociation. The cytochrome *bd* oxidase was dissolved in the same buffer described in the legend to Figure 1 at a concentration of 7.9 μM. The enzyme was reduced with 2.5 mM sodium ascorbate plus 5 μM TMPD and then placed under 1 atm of CO. The CO atmosphere was displaced with air and the sample stirred for 1 s to commence the reaction. Spectra were collected at 0.1 s intervals, and the time course shown in the change in absorbance at 634−648 nm (○) and at 560−580 nm (□). The line through the data is a fit to a single-exponential decay.

Table 1: Kinetics and Thermodynamics of Ligand Binding by Different O_2 Reductases and O_2 Binding Proteins

	ligand					
	CO			O_2		
protein	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (μM)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	k_D (μM)
cytochrome *bd*	8×10^{7}[a]	1.6[a]	0.02	2×10^{9}[a]	50[b]	0.025
cytochrome aa_3	7×10^{4}[c]	0.02[c]	0.29	5×10^{7}[d]	2000[d]	40
cytochrome bo_3	6×10^{4}[e]	0.10[e]	1.7	1.6×10^{8}[f]		
myoglobin	5×10^{5}[g]	0.17[g]	0.34	1.4×10^{7}[g]	10.5[g]	0.75
leghemoglobin	1.3×10^{7}[g]	0.02	0.0015	1.2×10^{8}[g]	4.4[g]	0.037

[a] This paper. [b] J. J. Hill, Rolfe, Hille, and R. B. Gennis, unpublished result. [c] Gibson & Greenwood (1963). [d] Hill (1994). [e] Cheeseman et al. (1993). [f] Svensson & Nilsson (1993). [g] Mims et al. (1983).

heterogeneity is not known; however, it was minimal in the preparations used in the current work and had no influence on the interpretation of the results.

Figure 2A shows the time course of CO binding following photolysis at 642 and 622 nm. The time courses are well-fit by a single-exponential expression at all wavelengths and all CO concentrations used. The observed rate of CO recombination is proportional to CO concentration with a bimolecular rate of 8×10^7 M^{-1} s^{-1} (see Figure 2B). Figure 2C shows the time course of O_2 reacting with the CO-ligated enzyme in the dark. In this reaction, oxidation of the enzyme is independent of oxygen concentration with a rate of 1.6 s^{-1}, which we assign to the rate of CO dissociation. Thus, the value of K_{eq} for CO binding by cytochrome *bd* oxidase is 5×10^7 M^{-1}. This is 10-fold higher than the mitochondrial oxidase and is distinguished by both faster on- and off-rate constants (see Table 1). The data points on the spectrum in panel B of Figure 1 correspond to the kinetic difference observed upon photolysis, and illustrate that there is no detectable intermediate, at least at room temperature, unique to the photolytic process. In addition, we have measured the relative quantum yield of the fully reduced cytochrome

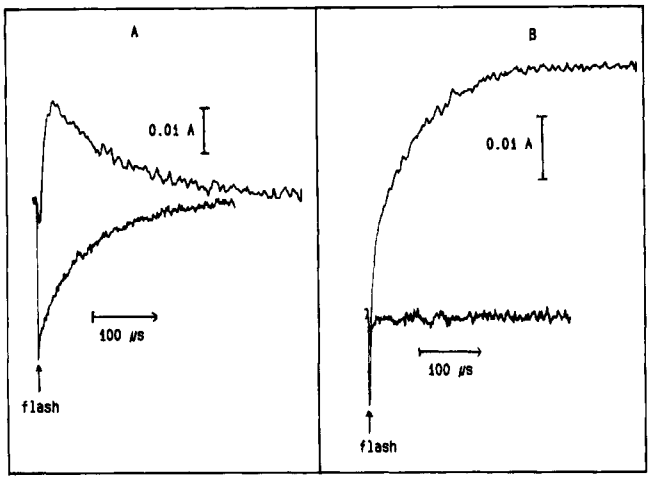


FIGURE 3: Absorbance time courses for the reaction of cytochrome *bd* oxidase with oxygen. The enzyme was at a final concentration of 7.4 μM in the same buffer described in the legend to Figure 1. The CO concentration was 100 μM, and the O_2 concentration was 55 μM. In panel A, the observation wavelength was 645 nm, and in panel B, it was 680 nm. The temperature was 20 °C.

bd−CO complex and find it close to the CO complex of mammalian cytochrome oxidase, which is near unity (Hill & Marmor, 1991).

The rapid nature of the CO interaction with cytochrome *bd* does represent more of a technical challenge for the flow−flash photolysis method than the mitochondrial oxidase. This approach relies on two criteria: (1) a slow CO dissociation rate relative to the time taken for mixing and photolysis, and (2) a fast O_2 combination rate relative to CO rebinding. To meet the first requirement, the stopped-flow apparatus used here had a mixing time of 3 ms, and the flash fired 5 ms after the flow stopped. In order to meet the second criterion, CO was kept at low concentration to diminish its competition with O_2. Figure 3 shows time courses at 650 and 680 nm when the CO adduct of reduced cytochrome *bd* was photolyzed in the absence and presence of O_2. In the absence of O_2, the standard photolysis and recombination reaction is observed for CO at 650 nm, whereas there is no reaction apparent at 680 nm because CO binding does not perturb the spectrum in this region. The spike at zero time in these traces is an electrical artifact arising from the laser pulse. When the reduced, CO-bound form of cytochrome *bd* is photolyzed in the presence of oxygen, two new transients are osberved on a time scale of a few hundred microseconds. These data show that the flow−flash method results in efficient ligand exchange (i.e., O_2/CO) and that this approach is useful for studying the reaction of the *bd*-type oxidase with oxygen. In the presence of 53 μM O_2 (Figure 3A), the absorbance at 650 nm first increases ($\Delta A = 0.032$) with an observed rate of 1.3×10^5 s^{-1}, and then decreases ($\Delta A = -0.028$) at a rate of 1.3×10^4 s^{-1}. Both of these kinetic components are also observed at 680 nm (Figure 3B), where the absorbance increases over both phases ($\Delta A = 0.005$ for the fast phase) but is dominated by the second slower phase ($\Delta A = 0.027$).

Observed rates and preexponential factors were obtained at these two wavelengths for a number of O_2 concentrations by fitting these time courses to a two-exponential expression: $\Delta A = B(e^{-k_1 t}) + C(e^{-k_2 t}) + D$, where ΔA is the total overall absorbance change observed at a particular wavelength and the terms B and C are the preexponential

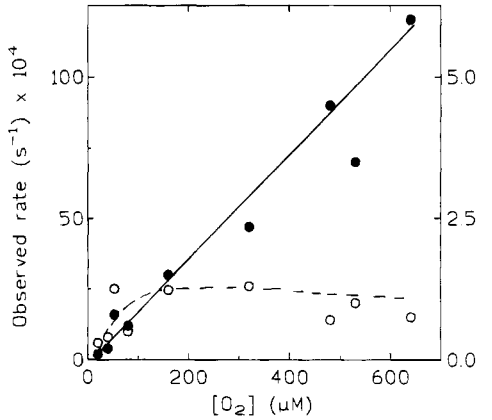


FIGURE 4: Concentration dependence for the observed rates of the reaction of cytochrome *bd* oxidase with oxygen. The first phase rates are indicated by the closed circles and are plotted on the left-hand scale. The second phase rates are indicated by the open circles and are plotted according to the right-hand scale. The conditions are the same as those outlined in the legend to Figure 3. The O_2 concentration was varied by changing the volume of O_2-saturated buffer added to a fixed total reaction volume.

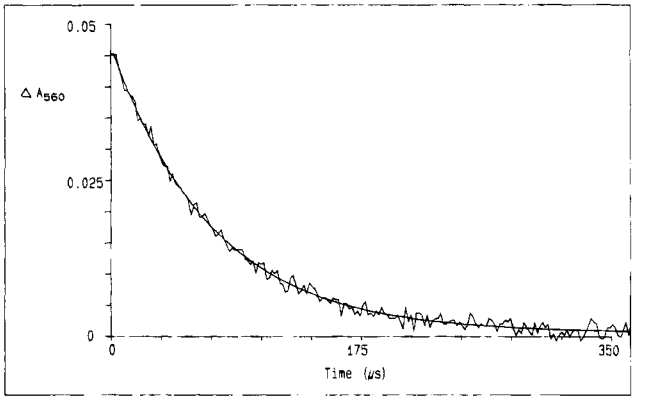


FIGURE 5: Time course of cytochrome b_{560} oxidation during the oxygen reaction of cytochrome *bd* oxidase. The conditions were the same as those outlined in the legend to Figure 3. The oxygen concentration was 530 μM. The smooth line through the data is a double-exponential fit.

factors which represent the absorbance contribution of the kinetic species changing at the observed rates k_1 and k_2. The term D allows for any error in the end point absorbance to be fit independently.

The dependence of the observed rates upon O_2 concentration is plotted in Figure 4. The rate of the first phase is proportional to O_2 concentration up to an observed rate of 1.2×10^6 s^{-1} at an O_2 concentration of 650 μM. The slope of this line is 1.9×10^9 M^{-1} s^{-1} and corresponds to the bimolecular reaction rate constant of reduced cytochrome *bd* with O_2. This rate is close to the diffusion-controlled limit for O_2 reacting with a slowly diffusing macromolecule such as the cytochrome *bd* complex. The rate of the second phase is independent of the O_2 concentration above 50 μM and is limited at a first-order value of 1×10^4 s^{-1}.

Figure 5 shows the time course of cytochrome *b* oxidation as measured at 560 nm. The observed time course is fit with a two-exponential expression as above, but the faster phase is given a small negative contribution and thus the time course begins with an initial lag period followed by an absorbance decrease at a rate of 1×10^4 s^{-1}. This rate is independent of O_2 concentration over the range from 50 to 650 μM O_2.

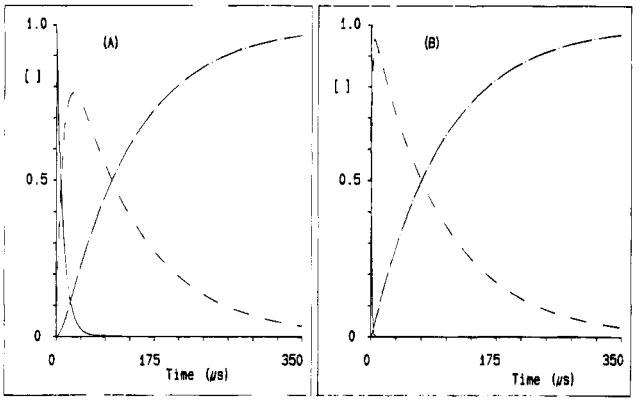


FIGURE 6: TIme courses for postulated intermediates in the reaction of cytochrome *bd* oxidase with O_2. The time courses are generated from the model in Scheme 1 and the rate constants k_1, k_{-1}, and k_2 of 2×10^9 M^{-1} s^{-1}, 50 s^{-1}, and 1×10^4 s^{-1}, respectively. Panel A was simulated using an oxygen concentration of 53 μM and panel B 530 μM.

DISCUSSION

Scheme 1 depicts the photolysis of CO-ligated cytochrome *bd* and its subsequent reaction with oxygen, and summarizes the results reported in this paper. In Scheme 1, free ferrocytochrome *d* (i.e., intermediate 0) is generated by photolysis, and this species reacts directly with O_2 to form the initial oxy-ferrocytochrome *d* (i.e., intermediate I). The rate of this reaction is very fast and proportional to $[O_2]$. The binding reaction is followed by electron transfer to form a second oxygenated species (i.e., intermediate II). It is difficult to be certain of the nature of this second species. The loss in absorbance at 645 nm coupled with the increase at 680 nm is consistent with the decay of an oxy species and the formation of a ferryl−oxo species at cytochrome *d*. Such species have also been observed by addition of peroxide to the "as isolated", oxygenated enzyme and designated as peroxy states (Lorence & Gennis, 1989). However, resonance Raman spectra using mixed-isotope O_2 have demonstrated that this "peroxy species" and the product of aerobic oxidation of fully reduced cytochrome *bd* are ferryl−oxo derivatives (Kahlow et al., 1991). Thus, we have proposed that intermediate II is a ferryl−oxo species (see Scheme 1). Transient-state resonance Raman, O_2 isotope experiments will be useful to tell if the ferryl-oxo species is formed at the stage shown in Scheme 1.

Figure 6 shows simulations of the time courses of intermediates 0, I, and II at two different O_2 concentrations, 53 μM in panel A and 530 μM in panel B. These have been obtained using values for k_1, k_{-1}, and k_2 of 2×10^9 M^{-1} s^{-1}, 50 s^{-1}, and 1×10^4 s^{-1}, respectively. The value used for k_{-1} is obtained from a ligand-exchange experiment. The one-electron-reduced *bd* oxidase binds O_2 reversibly, and the dissociation rate of this complex was determined by measuring its reactivity with CO (Hill, Rolfe, Hille, and Gennis, unpublished result). The simulation of the time course of the oxy species (ie.., intermediate I) reproduces the time course of the absorbance at 645 nm, indicating that the oxy species is the major contributor at this wavelength. Raising the oxygen concentration raises the occupancy level of the oxy species, but has little effect on its decay, or the appearance of the ferryl state (see Figure 6).

The rate of oxygen binding is extremely fast in this enzyme, and we suggest it is the primary reason for its higher O_2 affinity. Analysis of steady-state respiration rates in nongrowing whole *E. coli* cells, where the rate of reduction of the enzyme by quinol may be rate-limiting, gives a K_M for O_2 of 24 nM (Rice & Hempfling, 1978), close to the K_D for O_2 reported here (see Table 1). Assays of purified cytochrome *bd* under conditions where the quinol substrate is not limiting give a much higher value of K_M (i.e., 0.38 μM) (Kita et al., 1984), as expected. This compares to a steady-state K_M for O_2 of 1 μM (Petersen et al., 1976) and a K_D of 40 μM (Hill, 1994) for mitochondrial cytochrome *c* oxidase. In the case of mitochondrial oxidase, the relationship between K_M and K_D for O_2 is more complex than for cytochrome *bd* presumably due to at least one additional step of rapid intramolecular electron transfer between O_2 binding and enzyme turnover. Rapid electron transfer following O_2 binding raises the apparent affinity (i.e., lowers the steady-state K_M) for O_2 relative to a weak K_D. In general, the mitochondrial enzyme and presumably the bo_3-type oxidase of *E. coli* are designed to kinetically trap O_2 by reducing it by two electrons to a peroxy species (Verkhovsky et al., 1994),[2] whereas the *bd*-type oxidase is designed for rapid bimolecular O_2 binding and is essentially a thermodynamic trap for O_2. In a competition between two such oxidases at low O_2 tension, the *bd* type of oxidase would win due its rapid and efficient O_2 binding. The reaction of cytochrome *bd* oxidase with O_2 approaches the diffusion limit which implies that a higher percentage of encounters lead to product formation (i.e., oxy−ferrocytochrome *d*) than would be the case for the mitochondrial enzyme.

This scheme resembles that proposed for the cytochrome aa_3 ubiquinol oxidase from *Bacillus subtilis* (Hill, 1993) in which the fully reduced enzyme, at pH 7.4, proceeds via ferro−oxy and ferryl−oxo species in its single-turnover reaction with oxygen. Such a scheme is distinct from that proposed for mitochondrial cytochrome *c* oxidase in which a ferric−peroxy state precedes the ferryl species (Varotsis et al., 1993). At least at pH 7.4, in these quinol oxidases there is no accumulation of the ferric−peroxy state.

In the case of both of the ubiquinol oxidases (i.e., *bd*-type and aa_3-600), the enzymes are equipped with only three of the four centers found in the conventional cytochrome *c* oxidase. This suggests that there might be a special role for a tightly bound quinol or semiquinone molecule to provide extra, kinetically accessible reducing equivalents to return the ferryl−oxo species to a ferrous or ferric state. However, in the current experiments, there is no evidence that the reaction proceeds beyond the ferryl−oxo species, predicted to be the end product from the three electrons available in the three hemes of the *bd*-type oxidase.

Scheme 1 begins with the CO adduct of fully reduced cytochrome *bd* oxidase. The monophasic nature of CO binding supports the view that there is a single heme *d* moiety per enzyme complex as suggested from EPR (Meinhardt et al., 1989) and FTIR (Hill et al., 1993) studies. The finding that the CO recombination rate is proportional to CO

[2] In the mechanism proposed for mitochondrial cytochrome *c* oxidase by Verkhovsky et al. (1994), an O_2 complex with Cu_B (K_D = 8 mM) precedes the combination of O_2 with ferrocytochrome a_3. The overall K_D for O_2 binding to cytochrome a_3 would be 0.3 mM, still much higher than the steady-state K_M.

concentration implies at room temperature there are no kinetically-resolved intermediates distinguishable from the ligand-free and -bound states. These results do not contradict the finding from recent FTIR studies in which ligation of CO to cytochrome b_{595} was observed following photolysis of the ferrocytochrome *d*−CO complex at low temperature (Hill et al., 1993). At room temperature, the binding of CO to cytochrome *d* is highly favored, and the transfer from cytochrome b_{595} to cytochrome *d* must be very fast. Furthermore, the low-temperature studies (Hill et al., 1993) do not demonstrate that cytochrome b_{595} is an obligate kinetic intermediate binding site for CO destined for the cytochrome *d* moiety.

The kinetics of CO binding by cytochrome *bd* are unusual relative to mitochondrial cytochrome *c* oxidase (Gibson & Greenwood, 1963). For instance, the rate constant for CO binding is 1000-fold faster than for the heme−Cu binuclear center of mitochondrial cytochrome *c* oxidase, and the CO dissociation rate is 100-fold faster (see Table 1). The O_2 on-rate is faster, whereas the off-rate is slower for the *bd*-type oxidase relative to the aa_3-type oxidase. Although the cytochrome *d* and cytochrome b_{595} components may form a binuclear center analogous to the heme−Cu center in cytochrome *c* oxidase (Hill et al., 1993), the dynamics of ligand binding are different in the two classes of oxidase, and this may be reflected in the relative disposition of the two metals and the local environment in the two types of binuclear center. The role of Cu_B in binding ligands appears to be much more pronounced than that of cytochrome b_{595} (Dyer et al., 1994). The CO binding kinetics to the *bd*-type oxidase are also distinct from myoglobin and leghemoglobin (see Table 1). The *E. coli* enzyme is faster at binding and releasing CO than either of the oxygen binding proteins.

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