Internal Electron Transfer in Cytochrome *c* Oxidase from *Rhodobacter sphaeroides*†

Pia Ädelroth, Peter Brzezinski, and Bo G. Malmström*

Department of Biochemistry and Biophysics, Göteborg University, Medicinaregatan 9C, S-413 90 Göteborg, Sweden

Received September 16, 1994; Revised Manuscript Received November 23, 1994®

ABSTRACT: Absorbance changes following CO dissociation by flash photolysis from mixed-valence aa_3 cytochrome oxidase from *Rhodobacter sphaeroides* have been followed in the Soret and α regions. They reflect internal electron transfer in the partially reduced enzyme, and the kinetics of the reactions has been determined. As with the bovine enzyme, three kinetic phases are found with relaxation time constants at neutral pH of about 3 μs, 35 μs, and 1 ms. The first reaction phase represents electron transfer from cytochrome a_3 to cytochrome *a*, and the extent of this reaction is about 3 times larger compared to the bovine enzyme. The energetics of the reaction has been analyzed on the basis of measurements of its temperature dependence. The reorganization energy is close to 120 kJ mol^{-1}, and it is suggested that this rather high value is the result of changes in solvation at the cytochrome a_3-Cu_B site. The subsequent electron transfer between cytochrome *a* and Cu_A, with a time constant of 35 μs, is almost activationless and has a very low reorganization energy. The final phase, with a time constant close to 1 ms at neutral pH, represents a further shift in the equilibrium between cytochrome a_3 and cytochrome *a*, and it is limited by proton-transfer reactions. The pK_a values of the groups involved are significantly shifted in the bacterial oxidase compared to the bovine one. The total extent of electron transfer in the three backflow reactions has also been determined by a comparison of the CO-recombination rates in the mixed-valence and fully reduced enzymes. The difference between these two rates is larger in the bacterial compared to the bovine oxidase, consistent with the greater extent of electron transfer.

Cytochrome *c* oxidase is a redox-linked proton pump [for a review, see Malmström (1993)]. One approach to the elucidation of the mechanism for the coupling between electron transfer and proton translocation is to study the kinetics of internal electron transfer in different redox and protonation states of the oxidase. This can be done by dissociation by flash photolysis of CO bound to the reduced cytochrome a_3-Cu_B site in the partially reduced enzyme. Since CO binds exclusively to reduced cytochrome a_3, its dissociation leads to a drop in the apparent reduction potential of this site. This, in turn, results in a backflow of electrons from cytochrome a_3 to other redox sites (Boelens et al., 1982; Brzezinski & Malmström, 1987).

With the bovine oxidase it has been well established that the electron backflow on CO photolysis of the partially reduced enzyme involves at least three kinetic phases with time constants at neutral pH of around 3 μs, 35 μs, and 1 ms, respectively (Oliveberg & Malmström, 1991; Hallén et al., 1994). The two fastest phases represent electron transfer from cytochrome a_3 to cytochrome *a* and from cytochrome *a* to Cu_A [see also Morgan et al. (1989)]. Recently it has been shown that the millisecond phase involves electron transfer between cytochrome a_3 and cytochrome *a* as well, but that the rate is limited by a proton-transfer reaction (Hallén et al., 1994).

To illuminate the internal electron- and proton-transfer reactions further, we have initiated investigations of mutant forms of the aa_3 cytochrome oxidase from *Rhodobacter sphaeroides* (Hosler et al., 1993). As a first step in this direction, we here report studies of these reactions in the wild-type bacterial enzyme. We found that the fastest phase in electron backflow experiments has an amplitude which is 3 times larger than that observed in the bovine enzyme, but the relaxation rate, which is the sum of the rate constants for the forward and reverse reactions[1] in the electron-transfer equilibrium between cytochrome *a* and cytochrome a_3, is approximately the same. There are major differences in the pH dependence of the millisecond phase compared to the bovine oxidase, showing that the pK_a values of the proton-transfer groups are different in the two enzymes.

MATERIALS AND METHODS

Cytoplasmic membranes from *Rhodobacter sphaeroides* CY91 were prepared by the procedure of Hosler et al. (1992). Cytochrome oxidase was solubilized and isolated from the membranes by a modification of the method of Hosler et al. (1992). The hydroxyapatite chromatography was eliminated, and the purification by FPLC was performed by two runs on a RESOURCE Q 6-mL column (Pharmacia LKB Biotechnology). Peak fractions were collected and stored at −80 °C. The optical and EPR spectra of the isolated enzyme were identical with those described by Hosler et al. (1992), except for the fact that the contribution from Mn(II) in the EPR spectrum was smaller.

Bovine cytochrome oxidase was purified by the method of Brandt et al. (1989).

Mixed-valence bovine and bacterial oxidase were prepared as described by Brzezinski and Malmström (1985). Optical absorption spectra were recorded on a Cary 4 spectropho-

† This work was supported by the Swedish Natural Science Research Council, the Commission of the European Communities, The Royal Society of Arts and Sciences in Gothenburg, and Wilhelm och Martina Lundgrens Vetenskapsfond.

® Abstract published in *Advance ACS Abstracts,* February 1, 1995.

[1] Here and in the following, "forward" and "reverse" (or "back") refer to the normal physiological directions of electron transfer.

0006-2960/95/0434-2844$09.00/0

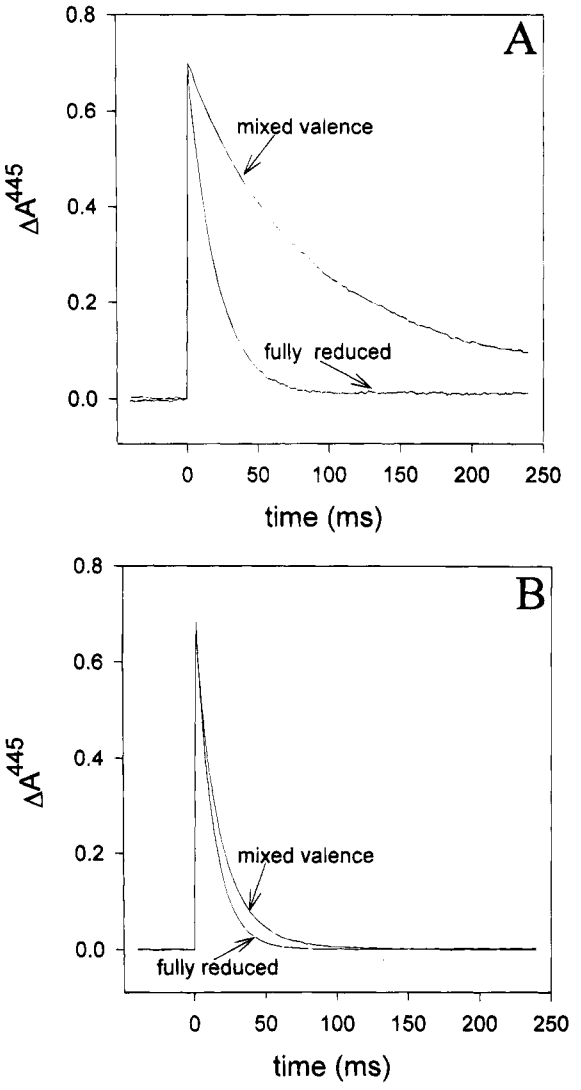

FIGURE 1: Kinetics of CO recombination at pH 7.2 and 22 °C, monitored at 445 nm, in mixed-valence and fully reduced cytochrome oxidase from *Rhodobacter sphaeroides* (A) or bovine heart (B). The buffer was 100 mM Hepes−KOH with 0.1% dodecyl D-maltoside. The enzyme and CO concentrations were 10 μM and 1 mM, respectively.

tometer (Varian). The buffers used in the study of the pH dependence of the electron-transfer kinetics were the same as those used by Hallén et al. (1994).

The excitation laser and observation equipment have been described in detail earlier (Hallén & Brzezinski, 1994). Amplitudes and time constants of the exponential components in the kinetic traces were determined by the use of a curve-fitting program, based on the Levenberg-Marquardt algorithm, written by Dr. Örjan Hansson at this Department.

RESULTS

The absorbance changes associated with CO dissociation and recombination in mixed-valence and fully reduced bacterial and bovine cytochrome oxidase are compared in Figure 1. Upon CO dissociation from the mixed-valence oxidase, electrons are transferred from cytochrome a_3 to cytochrome a and Cu_A, and the CO recombination rate is determined by the fraction of the oxidase having reduced cytochrome a_3 during the CO recombination [see Verkhovsky et al. (1992)]:

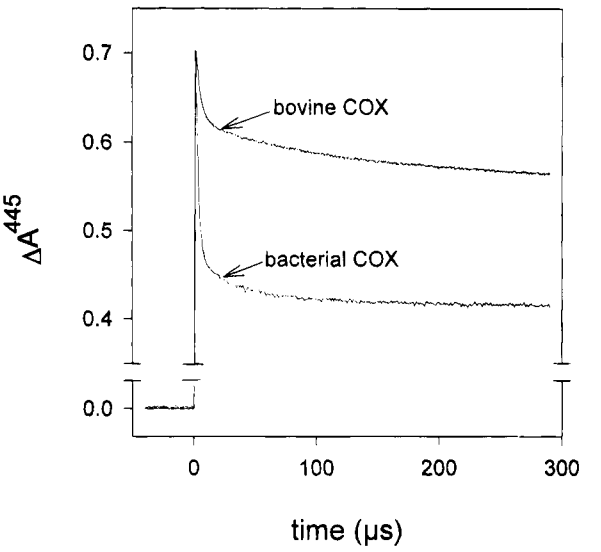

FIGURE 2: Comparison of the amplitude of the 3-μs phase in mixed-valence bacterial and bovine cytochrome oxidase, monitored at 445 nm. The experimental conditions were as in Figure 1.

$$x^- a_3^{3+} \underset{\text{rapid}}{\longleftrightarrow} x a_3^{2+} \xrightarrow[\text{slow}]{+CO} x a_3^{2+}{\cdot}CO \qquad (1)$$

In eq 1, x is cytochrome a, Cu_A, or both, and the minus superscript represents a reduced site. The observed recombination rate is

$$k_{obs} = k_{CO} \frac{[x a_3^{2+}]}{[x a_3^{2+}] + [x^- a_3^{3+}]} \qquad (2)$$

The parameter k_{CO} in eq 2 is the CO recombination rate in molecules with cytochrome a_3 reduced. It can be determined from fully reduced oxidase, in which cytochrome a_3 stays reduced after CO dissociation, and consequently $k_{obs} = k_{CO}$. With the fully reduced enzymes, the recombination kinetics is monophasic, and it has rates of 50 and 80 s^{-1}, respectively, in the bacterial and bovine oxidases. Due to internal electron transfer from cytochrome a_3 to cytochrome a and Cu_A, the recombination becomes slower in the mixed-valence forms, with rates of 15 and 50 s^{-1}, respectively, in the bacterial and bovine enzymes. With the bacterial oxidase, however, the kinetics becomes biphasic with a small, slower component. The rate of the main component in the bacterial enzyme is only 30% of the rate with the fully reduced enzyme, whereas in the bovine enzyme the rate is 63% of the rate obtained on full reduction. This demonstrates that there is a greater extent of electron transfer in the bacterial oxidase.

The larger degree of electron transfer in the bacterial oxidase compared to the bovine enzyme is also demonstrated more directly in Figure 2, which compares the absorbance changes at 445 nm associated with electron transfer on a shorter time scale. The absorbance at 445 nm decreases on oxidation of both cytochromes, but more for cytochrome a_3 compared to cytochrome a (Vanneste, 1966). Thus, electron transfer from cytochrome a_3 to cytochrome a, or from cytochrome a to Cu_A, results in a net decrease in absorbance. In the first phase with the bacterial oxidase at pH 7.2, about 45% of cytochrome a is reduced with an observed time

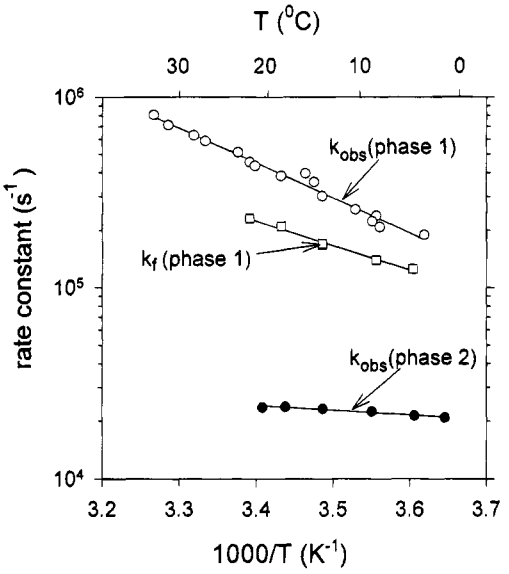

FIGURE 3: Temperature dependence of the observed rate constant of the 3- and 35-μs phases in the bacterial oxidase at pH 6.5, monitored at 445 nm, and of the forward rate constant for the fast phase. Due to some reduction past the mixed-valence level at higher temperatures, these data were not used in the calculations of the forward rate constant. The buffer used was 100 mM Mes−KOH with 0.1% dodecyl D-maltoside.

constant of 3 μs. The rate and amplitude of this phase are not significantly dependent on pH. The reduction of Cu_A in the second phase, with an observed time constant of 35 μs, is at most 10%, assuming that the changes in molar absorption coefficients ($\Delta\epsilon$) on electron transfer are the same in the bacterial as in the bovine enzyme (Vanneste, 1966). At this pH, maximally 1% electron transfer from cytochrome a_3 occurs in the slowest phase with a time constant of about 1 ms (not shown in Figure 2).

Figure 3 gives the temperature dependence of the rates for the two fastest phases in the bacterial enzyme. To assure that only the mixed-valence form (defined by its optical absorption spectrum) of cytochrome oxidase was present, experiments were performed at low pH (6.5) and started at short times after addition of CO [cf. Brzezinski and Malmström (1985)]. The temperature dependence of the forward rate for the first phase was calculated from the temperature dependences of the observed rate (k_{obs}) and amplitude $(\Delta A)^{445}_{3\mu s}$:

$$k_{obs} = k_f + k_b \quad (3)$$

$$\Delta A^{445}_{3\mu s} = \epsilon^{445}_{a_3 \rightarrow a} C_{MV} \frac{k_b}{k_f + k_b} \quad (4)$$

where k_f and k_b are the forward and backward rates, respectively, $\epsilon^{445}_{a_3 \rightarrow a}$ is the absorption coefficient for the electron transfer from cytochrome a_3 to cytochrome a at 445 nm, and C_{MV} is the concentration of mixed-valence cytochrome oxidase (determined from the CO-dissociation absorbance change) in a 1-cm cuvette.

Table 1 summarizes the values calculated from the experimental data for the forward rate constant (k_f), the activation energy (E_a), and the free energy (ΔG^0) and enthalpy changes (ΔH^0) for the electron transfer from cytochrome a to cytochrome a_3. The amplitude of the second phase was not easily determined because it is small and strongly

Table 1: Kinetic and Thermodynamic Parameters for the Electron Transfer between Cytochrome a and Cytochrome a_3 at pH 6.5 and 25 °C[a]

enzyme	k_f x 10^{-5} (s^{-1})	E_a (kJ mol^{-1})	$\Delta G°$ (kJ mol^{-1})	$\Delta H°$ (kJ mol^{-1})
bacterial	1.2 ± 0.1	25 ± 2	−0.01 ± 1	−15 ± 2
bovine	2.0 ± 0.2	20 ± 2	−4.2 ± 0.2	−3.3 ± 0.5

[a] The errors are standard deviations based on 5−9 measurements.

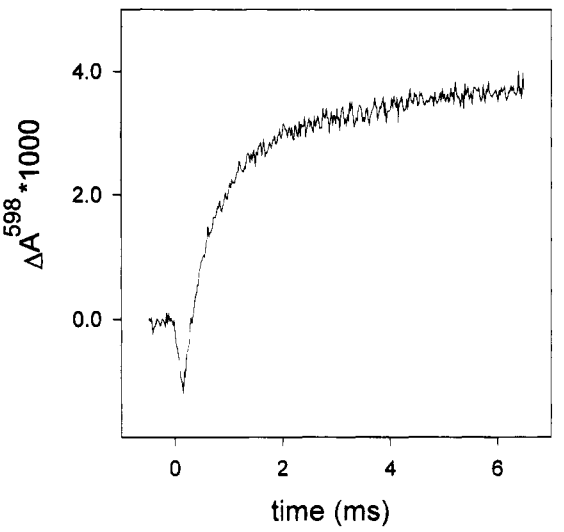

FIGURE 4: Millisecond phase of the bacterial oxidase at pH 7.9 and 22 °C, monitored at 598 nm. The buffer used was 100 mM Tris-HCl with 0.1% dodecyl D-maltoside. The enzyme and CO concentrations were as in Figure 1.

dependent on the reduction level of the enzyme; hence its temperature dependence could not be determined. However, since only a small fraction (<10%) of Cu_A is reduced in this phase, the observed rate and its temperature dependence are approximately equal to those of the forward rate from Cu_A to cytochrome a (cf. eqs 3 and 4). With this approximation, the rate constant for electron transfer from Cu_A to cytochrome a in the bacterial enzyme was found to be 2.5×10^4 s^{-1} and the activation energy, 5.2 kJ mol^{-1}.

The slowest of the three reaction phases, representing a further shift in the electron equilibrium between cytochrome a_3 and cytochrome a, is illustrated in Figure 4. The reaction was here monitored at 598 nm, where an absorbance increase is mainly due to cytochrome a reduction. In addition, this wavelength is isosbestic for the CO recombination reaction, so that this does not interfere with the rate determination. As in the bovine enzyme (Hallén et al., 1994), both the rate and the amplitude of the slow phase are strongly dependent on pH, which is shown in Figure 5. There are, however, significant differences between the oxidases from the two species. In the bovine oxidase, the amplitude at high pH (~10) corresponds to almost complete oxidation of cytochrome a_3, whereas in the bacterial enzyme the amplitude indicates 12% oxidation only; this corresponds to approximately 25% of the electrons remaining on cytochrome a_3 after the first two reaction phases. In addition, the amplitude does not reach a plateau at high pH.

DISCUSSION

Following pulsed illumination of the mixed-valence cytochrome oxidase−CO complex, electrons are transferred

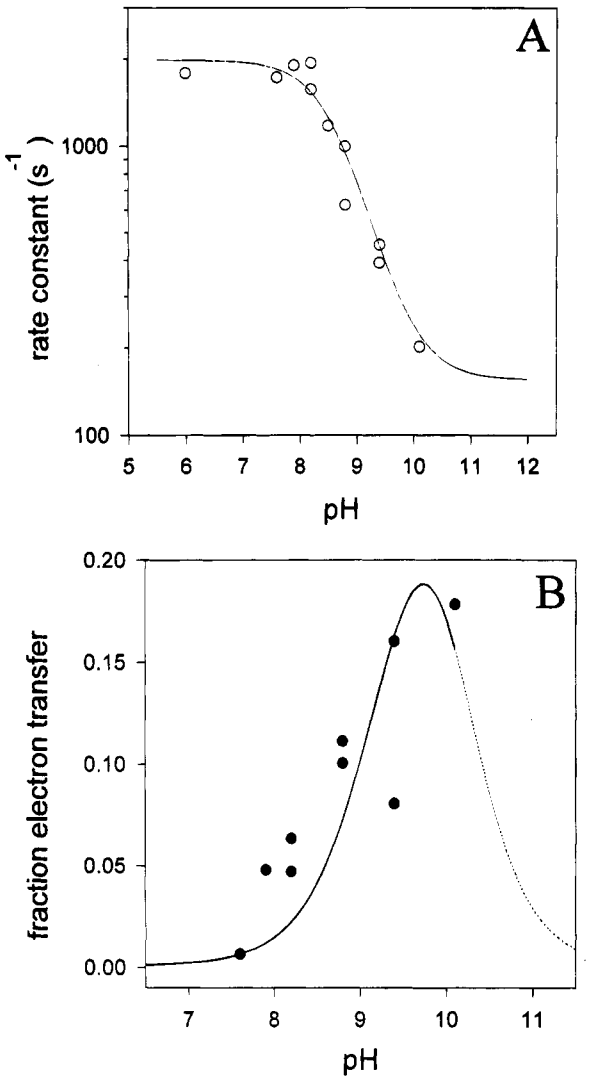

FIGURE 5: pH dependence of the rate (A) and fraction of electron transfer (B) calculated from the amplitude of the millisecond phase in bacterial cytochrome oxidase at 22 °C. The solid line in panel A is a least-square fit of eq 10 to the experimental data, using k_{on} = 2000 s^{-1}, k_{off} = 150 s^{-1}, and pK_a = 8.7. The value of k_{off} could not be determined uniquely, but an upper limit was found to be 150 s^{-1}. The solid/dashed line in panel B is a least-square fit of eq 16 to the experimental data, using $pK_{a_3^{2+}} \cong 10.3$ and $pK_{a_3^{3+}} \cong 9.1$.

rapidly from cytochrome a_3 to cytochrome a/Cu_A. From the measured recombination rate the fraction of electrons which have been removed from cytochrome a_3 was calculated (see Results). This fraction is 0.7 for the bacterial oxidase compared to 0.4 for the bovine enzyme. These values are approximately 30% larger than those obtained from the amplitudes of the kinetic phases (Figure 2). This discrepancy could be due to uncertainties in the values of $\Delta\epsilon$ associated with the electron-transfer reactions, but these are unlikely to be as large as 30%. Two more probable possibilities are that the CO recombination is limited by another step than the binding to reduced cytochrome a_3 or that site interactions lead to different conformations of the bimetallic site in the mixed-valence compared to the fully reduced enzyme. It is still quite clear, however, that the difference in reduction potential between the two cytochromes must be smaller in the bacterial oxidase compared to the bovine one.

Despite the smaller driving force for electron transfer from cytochrome a to cytochrome a_3, the observed rates are nearly the same in the two oxidases. This means that the sum of the rate constants for the forward and back reactions is unchanged. Since we find that the forward rate constant is 1.2×10^5 s^{-1} in the bacterial oxidase compared to 2.0×10^5 s^{-1} in the bovine enzyme (Table 1), the rate of the back reaction must be larger in the bacterial than in the bovine enzyme. With the thermodynamic parameters also listed in Table 1, it is possible to analyze the energetics of the electron-transfer reactions in greater detail.

Table 2: Marcus Parameters for the Electron Transfer between Cytochrome a and Cytochrome a_3 at pH 6.5 and 25 °C[a]

enzyme	ΔG^* (kJ mol^{-1})	λ (kJ mol^{-1})	d (Å) $\beta = 1.4$ $Å^{-1}$	d (Å) $\beta = 1.0$ $Å^{-1}$
bacterial	30 ± 2	119 ± 6	8 ± 1	10 ± 1
bovine	17 ± 2	76 ± 8	11 ± 1	14 ± 1

[a] The only significant contribution to the errors in λ, ΔG^*, and d is from the error in E_a.

According to Marcus theory [reviewed in Marcus and Sutin (1985)], the rate constant for electron transfer, k_{ET}, is the product of a nuclear frequency factor, ν_n, an electronic factor, Γ, and an exponential factor containing the free energy of activation, ΔG^*:

$$k_{ET} = \nu_n \Gamma \exp(-\Delta G^*/RT) \quad (5)$$

where R is the gas constant and T is the absolute temperature. The frequency factor is generally taken to be 10^{13} s^{-1}, and the electronic factor falls off exponentially with the distance, d, between the electron donor and acceptor corrected by the van der Waals distance, d_0:

$$\Gamma = \exp[-\beta(d - d_0)] \quad (6)$$

The free energy of activation, ΔG^*, depends on the driving force for the reaction, $-\Delta G^0$, and the reorganization energy, λ:

$$\Delta G^* = \frac{\lambda}{4}\left(1 + \frac{\Delta G^\circ}{\lambda}\right)^2 \quad (7)$$

The Arrhenius activation energy, E_a, is approximately equal to the enthalpy of activation (ΔH^*):

$$E_a \cong \Delta H^* = \frac{\lambda}{4} + \frac{\Delta H^\circ}{2}\left(1 + \frac{\Delta G^\circ}{\lambda}\right) - \frac{(\Delta G^\circ)^2}{4\lambda} \quad (8)$$

With eqs 5–8, using d_0 = 3 Å and β = 1.4 $Å^{-1}$ or 1.0 $Å^{-1}$, we have calculated the values of ΔG^*, λ, and d for the two enzymes, as listed in Table 2. According to this analysis, it is not only the decreased driving force that causes the decrease in k_{ET} for the intraprotein electron transfer from cytochrome a to cytochrome a_3 in the bacterial oxidase but also an increase in λ. The value for λ may seem unusually high for electron transfer between two natural partners. For example, in the electron transfer between cytochrome c and Cu_A, λ has been estimated to be 55 kJ mol^{-1} (Brzezinski et al., 1995). In this case, however, both redox sites are buried inside the proteins in the electrostatic complex between the oxidase and cytochrome c, whereas cytochrome a_3-Cu_B is known to communicate with the solvent. This is necessary for the uptake of the scalar protons involved in dioxygen reduction, and there is also evidence for a proton channel connecting an acid–base group close to this site with the solvent (Hallén et al., 1994). Thus, the high λ that we

observe may be related to changes in solvation or other structural changes at cytochrome a_3-Cu_B.

According to Moser et al. (1992), β in eq 4 should be 1.4 Å^{-1}, but the use of this value with our data gives too short a distance between the two cytochromes in the bacterial oxidase (Table 2). EPR measurements (Ohnishi et al., 1982; Brudvig et al., 1984; Goodman & Leigh, 1987) have put the distance between 12 and 20 Å, and the shorter of these values agrees with a current structural model (Hosler et al., 1993). Earlier, Woodruff (1993), using $\beta = 1.4$ Å^{-1}, had found a good agreement between the distance in the model and our measured rate with the bovine enzyme (Oliveberg & Malmström, 1991), but his calculation assumed that the reaction is activationless, whereas we find a substantial temperature dependence of this electron transfer (Figure 3). There is evidence that β for long-distance electron transfer in proteins can be smaller than 1.4 Å^{-1}. For example, theoretical calculations show that in a very tightly coupled system λ may be as small as 0.7 Å^{-1} (Broo & Larsson, 1990). Work with cytochrome *c* (Wuttke et al., 1992), on the other hand, indicates a less tight coupling with a β of 1.0 Å^{-1}, and applying this value to our data gives distances of 14 and 10 Å for the bovine and bacterial enzymes, respectively. The apparent discrepancy may be due to different proteins having different average β values (Beratan et al., 1992).

The 35-μs phase has a very low activation energy (Tables 1 and 2). With the bovine enzyme, the value of λ has recently been estimated to be 11.5 kJ mol^{-1} (Winkler et al., 1994). As the reaction is almost activationless also in the bacterial enzyme (Tables 1 and 2), and the rates in the two enzymes are rather close, a similar λ value should apply in this case. Thus, there is probably very little structural change around cytochrome *a* and Cu_A on electron transfer between these redox centers, which is in agreement with the rack mechanism for electron-transfer metalloproteins (Gray & Malmström, 1983; Malmström, 1994).

Even if the turnover rate of the bacterial oxidase is faster than that of the bovine enzyme (Hosler et al., 1992), the rate constants for the two fastest phases are both too high for these internal electron-transfer steps to be rate-determining. The slowest phase, on the other hand, does have a rate constant close to the turnover number of the enzyme. The pH dependence of the rate of the millisecond phase (Figure 5A) has recently been interpreted in terms of an acid−base group associated with a proton channel (Hallén et al., 1994). The pK_a of this channel can be estimated from the following equations:

$$\alpha = \frac{1}{1 + 10^{\mathrm{pH}-\mathrm{p}K_a}} \tag{9}$$

$$k_{obs} = \alpha k_{on} + k_{off} \tag{10}$$

where α is the degree of saturation of the proton channel, and k_{on} and k_{off} are the proton-transfer rates to and from the group. The curve in Figure 5A is calculated with pK_a = 8.7. This value is 1 pH unit higher than that found with the bovine enzyme (Hallén et al., 1994). The maximal rate is about the same in both oxidases (about 2×10^3 s^{-1}), and since this is close to the maximum turnover of the enzyme, it is possible that proton transfer to this group is rate-limiting. This would agree with the fact that k_{cat} increases with decreasing pH (Wilms et al., 1980; Thörnström et al., 1984; Gregory & Ferguson-Miller, 1988) in the same pH range as the rate of the millisecond phase.

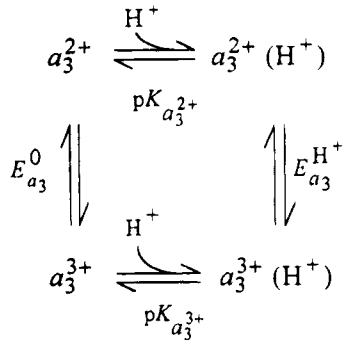

FIGURE 6: Scheme for the shift in the pK_a value of a group L, close to cytochrome a_3, on oxidation or reduction of the cytochrome. (H$^+$) represents protonated L (LH), and $E^{H^-}_{a_3} = E^0_{a_3} + \Delta E_{L\rightarrow LH}$ (see text).

The amplitude changes of the millisecond phase (Figure 5B) are due to a group close to cytochrome a_3, whose pK_a decreases when the cytochrome becomes oxidized, as illustrated in Figure 6. This leads to a shift in equilibrium by electrostatic interactions, which results in further electron transfer from cytochrome a_3 to cytochrome *a*. Qualitatively, the bacterial and the bovine enzyme behave in the same way, but there are marked quantitative differences. In particular, the maximum amplitude in the bacterial oxidase corresponds to about 20% oxidation of cytochrome a_3 (Figure 5B), whereas there is almost complete oxidation in the bovine enzyme (Hallén et al., 1994). To explain this behavior, we have to assume that the shift in pK_a in the scheme in Figure 6 ($pK_{a_3^{2+}} - pK_{a_3^{3+}}$) is smaller in the bacterial compared to the bovine oxidase, as demonstrated by the following detailed model.

The interaction energy between cytochrome a_3 and L is given by eq 11:

$$\Delta E_{L\rightarrow LH} = \frac{RT\ln(10)}{F}(pK_{a_3^{2+}} - pK_{a_3^{3+}}) \tag{11}$$

where $\Delta E_{L\rightarrow LH}$ is the change in the cytochrome a_3 reduction potential when the protonation state of L changes from fully unprotonated to fully protonated; $pK_{a_3^{2+}}$ and $pK_{a_3^{3+}}$ are the pK_a values of L in the presence of reduced and oxidized cytochrome a_3, respectively; and *F* is the Faraday constant.

With CO bound to cytochrome a_3^{2+}, its apparent reduction potential increases by 200−400 mV (Boelens et al., 1982). Consequently, assuming that with CO bound cytochrome a_3 stays reduced independently of the protonation state of L, and that the reduction potential of cytochrome a_3 is proportional to the fraction of protonated L, the apparent reduction potential of cytochrome a_3, E_{a_3}(pH), is given by:

$$E_{a_3}(\mathrm{pH}) = E^0_{a_3} + \Delta E_{CO} + \frac{1}{1 + 10^{(\mathrm{pH}-pK_{a_3^{2+}})}}\Delta E_{L\rightarrow LH} \tag{12}$$

where $E^0_{a_3}$ is the reduction potential of cytochrome a_3 in the absence of CO and ΔE_{CO} is the change in its reduction potential upon binding of CO.

After CO dissociation, at times ≪1 ms, i.e., much shorter than the time constant for equilibration of L with protons,

the reduction potential of cytochrome a_3 is given by:

$$E_{a_3,1}(\mathrm{pH}) = E_{a_3}(\mathrm{pH}) - \Delta E_{CO} = E^0_{a_3} + \frac{1}{1 + 10^{(\mathrm{pH}-\mathrm{p}K_{a_3^{2+}})}}\Delta E_{L\rightarrow LH} \quad (13)$$

After L has equilibrated with protons, at times much shorter than the CO recombination time constant, the reduction potential of cytochrome a_3 is given by:

$$E_{a_3,2}(\mathrm{pH}) = \frac{RT}{F}\ln\left(\frac{[a_3^{2+}(\mathrm{H}^+)] + [a_3^{2+}]}{[a_3^{3+}(\mathrm{H}^+)] + [a_3^{3+}]}\right) = \frac{RT}{F}\ln\left(\frac{a_3^{2+}}{a_3^{3+}}\right) + \frac{RT}{F}\ln\left(\frac{10^{\mathrm{p}K_{a_3^{2+}}-\mathrm{pH}} + 1}{10^{\mathrm{p}K_{a_3^{3+}}-\mathrm{pH}} + 1}\right) = E^0_{a_3} + \frac{RT}{F}\ln\left(\frac{10^{\mathrm{p}K_{a_3^{2+}}-\mathrm{pH}} + 1}{10^{\mathrm{p}K_{a_3^{3+}}-\mathrm{pH}} + 1}\right) \quad (14)$$

The equilibrium constants for electron transfer between cytochrome a and cytochrome a_3 before and after the millisecond phase are K_1(pH) and K_2(pH):

$$K_1(\mathrm{pH}) = \exp\left(\frac{(E_{a_3,1}(\mathrm{pH}) - E_a)}{RT/F}\right) \qquad K_2(\mathrm{pH}) = \exp\left(\frac{(E_{a_3,2}(\mathrm{pH}) - E_a)}{RT/F}\right) \quad (15)$$

where E_a is the reduction potential of cytochrome a. We assume that it is independent of pH in the range studied (see Results).

The change in the reduction level of cytochrome a in the millisecond phase, Δa^{2+}(pH), is calculated from the difference in the fraction of reduced cytochrome a before and after the millisecond phase:

$$\Delta a^{2+}(\mathrm{pH}) = \frac{1}{1 + K_2(\mathrm{pH})} - \frac{1}{1 + K_1(\mathrm{pH})} = \frac{K_1(\mathrm{pH}) - K_2(\mathrm{pH})}{(1 + K_1(\mathrm{pH}))(1 + K_2(\mathrm{pH}))} \quad (16)$$

At pH $\ll \mathrm{p}K_{a_3^{2+}}, \mathrm{p}K_{a_3^{3+}}$, the potentials $E_{a_3,1}(\mathrm{pH}) \cong E_{a_3,2}(\mathrm{pH})$ are both pH independent. In this pH region we found the difference in the reduction potentials of cytochrome a and cytochrome a_3 to be 10 mV. From a least-square fit of the experimental data in Figure 5B to eq 16, we obtained $\mathrm{p}K_{a_3^{2+}} \cong 10.3$ and $\mathrm{p}K_{a_3^{3+}} \cong 9.1$. The lower value is close to that in the bovine oxidase (8.7), whereas the value with reduced cytochrome a_3 is much lower. This has the result that the amplitude should decrease again at high pH, whereas a plateau is reached in the experimentally available pH range with the bovine enzyme. Unfortunately, it has not been possible to verify this decrease directly for experimental reasons (precipitation of the enzyme around pH 11).

The changed $\mathrm{p}K_a$ value with reduced cytochrome a_3 in the bacterial enzyme may be related to the particular conditions under which the chemiosmotic mechanism has to operate in the natural habitat of the bacterium, since we have earlier argued that the proton-transfer reactions associated with the slow electron transfer involve the uptake of either scalar or vectorial protons (Hallén et al., 1994). We are currently testing this idea further by investigating internal electron transfers and their pH dependence in mutant forms, in which potential proton-transfer groups have been modified.

ACKNOWLEDGMENT

We are grateful to Professor Robert B. Gennis for providing the bacterial strain used for the isolation of cytochrome oxidase and to Dr. J. Arturo Garcia-Horsman for advice concerning the purification of the enzyme. We have had helpful discussions with Dr. Stefan Hallén. Ann Sörensen and Ann-Cathrine Smiderot have provided valuable technical assistance.

REFERENCES

Beratan, D. N., Onuchic, J. N., Winkler, J. R., & Gray, H. B. (1992) *Science 258*, 1740−1741.
Boelens, R., Wever, R., & van Gelder, B. F. (1982) *Biochim. Biophys. Acta 682*, 264−272.
Brandt, U., Schägger, H., & von Jagow, G. (1989) *Eur. J. Biochem. 182*, 705−711.
Broo, A., & Larsson, S. (1990) *Chem. Phys. 148*, 103−115.
Brudvig, G. W., Blair, D. F., & Chan, S. I. (1984) *J. Biol. Chem. 259*, 11001−11009.
Brzezinski, P., & Malmström, B. G. (1985) *FEBS Lett. 187*, 111−114.
Brzezinski, P., & Malmström, B. G. (1987) *Biochim. Biophys. Acta 984*, 29−38.
Brzezinski, P., Sundahl, M., Ädelroth, P., Wilson, M. T., El-Agez, B., Wittung, P., & Malmström, B. G. (1995) *Biophys. Chem.* (in press).
Goodman, G., & Leigh, J. S., Jr. (1987) *Biochim. Biophys. Acta 890*, 360−367.
Gray, H. B., & Malmström, B. G. (1983) *Comments Inorg. Chem. 2*, 203−209.
Gregory, L. C., & Ferguson-Miller, S. (1988) *Biochemistry 27*, 6307−6314.
Hallén, S., & Brzezinski, P. (1994) *Biochim. Biophys. Acta 1184*, 207−218.
Hallén, S., Brzezinski, P., & Malmström, B. G. (1994) *Biochemistry 33*, 1467−1472.
Hosler, J. P., Fetter, J., Tecklenburg, M. M. J., Espe, M., Lerma, C., & Ferguson-Miller, S. (1992) *J. Biol. Chem. 267*, 24264−24272.
Hosler, J. P., Ferguson-Miller, S., Calhoun, M. W., Thomas, J. W., Hill, J., Lemieux, L., Ma, J., Georgiou, C., Fetter, J., Shapleigh, J., Tecklenburg, M. M. J., Babcock, G. T., & Gennis, R. B. (1993) *J. Bioenerg. Biomembr. 25*, 121−136.
Malmström, B. G. (1993) *Acc. Chem. Res. 26*, 332−338.
Malmström, B. G. (1994) *Eur. J. Biochem. 223*, 711−718.
Marcus, R. A., & Sutin, N. (1985) *Biochim. Biophys. Acta 811*, 265−322.
Morgan, J. E., Li, P. M., Jang, D.-J., El-Sayed, M. A., & Chan, S. I. (1989) *Biochemistry 28*, 6975−6983.
Moser, C. C., Keske, J. M., Warncke, K., Farid, R. S., & Dutton, P. L. (1992) *Nature 355*, 796−802.
Ohnishi, T., LoBrutto, R., Salerno, J. C., Bruckner, R. C., & Frey, T. G. (1982) *J. Biol. Chem. 257*, 14821−14825.
Oliveberg, M., & Malmström, B. G. (1991) *Biochemistry 30*, 7053−7057.
Thörnström, P.-E., Soussi, B., Arvidsson, L., & Malmström, B. G. (1984) *Chem. Scr. 24*, 230−235.
Vanneste, W. H. (1966) *Biochemistry 5*, 838−848.
Verkhovsky, M. I., Morgan, J. E., & Wikström, M. (1992) *Biochemistry 31*, 11860−11863.
Wilms, J., van Rijn, J. L. M. L., & van Gelder, B. F. (1980) *Biochim. Biophys. Acta 593*, 17−23.
Winkler, J. R., Malmström, B. G., & Gray, H. B. (1995) *Biophys. Chem.* (in press).
Woodruff, W. H. (1993) *J. Bioenerg. Biomembr. 25*, 177−188.
Wuttke, D. S., Bjerrum, M. J., Winkler, J. R., & Gray, H. B. (1992) *Science 256*, 1007−1009.

BI942192E