Internal Electron Transfer in Cytochrome *c* Oxidase Is Coupled to the Protonation of a Group Close to the Bimetallic Site†

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ABSTRACT: Absorbance changes following CO dissociation by flash photolysis from mixed-valence cytochrome oxidase have been followed in the Soret and α regions. Apart from CO dissociation and recombination, three kinetic phases with rate constants in the range 10^5–10^3 s^{-1} at pH 7.5 can be resolved in both spectral regions. The slowest one of these phases, which had earlier only been observed in the α region, has now been detected in the Soret region by the use of a low CO concentration to slow down the recombination reaction. This phase had been assigned to a structural change, but a kinetic difference spectrum demonstrates that it represents electron transfer from cytochrome a_3 to cytochrome *a*. A kinetic deuterium isotope effect of 2–3 at pH 7.5 suggests that it involves proton transfer as well. The temperature dependence of the reaction gives an Arrhenius activation energy of 42 kJ·mol^{-1}. The reaction is faster at low pH, and the equilibrium is shifted toward cytochrome *a* as the pH is raised. The rate and equilibrium changes can be described as involving acid–base groups with pK_a values of approximately 7.7 and 8.7, respectively. The kinetic results can be simulated on the basis of a model in which one acid–base group interacts with cytochrome a_3, so that its pK_a drops on oxidation of this center. The group is in proton equilibrium with the solvent via a proton pathway, suggested to be a proton channel. The rate of a shift in the redox equilibrium between the two cytochromes reaches a high limit at low pH, where the channel is saturated with protons. At high pH, the channel is empty, and the low rate is determined by the proton off-reaction. It is suggested that the proton-transfer reactions observed participate in the proton translocation by cytochrome oxidase.

Cytochrome oxidase is the terminal enzyme of energy transduction in mitochondria and some bacteria. It catalyzes a vectorial process in which electron transfer from cytochrome *c* to dioxygen is coupled to the translocation of protons across the energy-transducing membranes [for a review, see Babcock and Wikström (1992)]. In such redox-linked proton pumps, there must be structural control of the rates of electron and proton transfers. In attempts to illuminate this control, our group has investigated the kinetics of internal electron transfer in various states of the oxidase, and the effect of pH and D_2O on these kinetics [see Malmström (1993)].

Internal electron transfer in cytochrome oxidase can be followed after dissociation of CO bound to the mixed-valence enzyme by flash photolysis (Boelens et al., 1982; Brzezinski & Malmström, 1987). In this enzyme form, CO is bound to the reduced oxygen-reducing site, the bimetallic cytochrome a_3–Cu_B center, whereas the primary acceptors of electrons from cytochrome *c*, Cu_A and cytochrome *a*, are oxidized. On CO dissociation, the reduction potential of cytochrome a_3 drops, and this causes a back-flow of electrons from the bimetallic center to the primary electron acceptors. Three kinetic phases can be observed, and these have rate constants at pH 7.5 close to 10^5, 10^4, and 10^3 s^{-1}, respectively (Oliveberg & Malmström, 1991; Verkhovsky et al., 1992). The two fastest phases were assigned to electron transfer from cytochrome a_3 to cytochrome *a* and from cytochrome *a* to Cu_A, respectively, on the basis of the absorbance changes observed at different wavelengths. The slowest phase was originally assigned to an electron transfer between Cu_A and cytochrome *a* (Brzezinski & Malmström, 1987). Later it was, however, suggested not to involve electron transfer but rather a structural change, because the redox equilibria between the sites were thought to be established in the second phase already and, in addition, no absorbance changes were seen in the Soret region (Oliveberg & Malmström, 1991).

In this paper, we report the kinetic difference spectrum for the slowest electron-transfer phase. This demonstrates that the slowest reaction, like the fastest one, involves electron transfer from cytochrome a_3 to cytochrome *a*, because the kinetic difference spectrum corresponds to such a transfer on the basis of the absorbance changes associated with redox changes in these sites (Vanneste, 1966; Blair et al., 1982). We have, therefore, investigated this reaction (called the slow phase in the remainder of this paper) in greater detail, recording the effect of pH, D_2O, and temperature on the rate. The electron transfer observed displays a significant kinetic isotope effect at pH 7.5, and it depends on pH in a complex manner. The extent of the reaction increases with increasing pH, whereas the rate decreases. These observations can be explained on the basis of a model, in which there is an acid–base group associated with cytochrome a_3. This group is in proton equilibrium with the solvent via a proton channel. Proton-transfer reactions, with a similar pK_a as found here, have also been observed in the reoxidation of the fully reduced enzyme by dioxygen (Hallén & Nilsson, 1992). They are suggested to be important in the proton translocation by cytochrome oxidase.

MATERIALS AND METHODS

Cytochrome oxidase was purified from bovine hearts essentially by the method of Brandt et al. (1989), which yields

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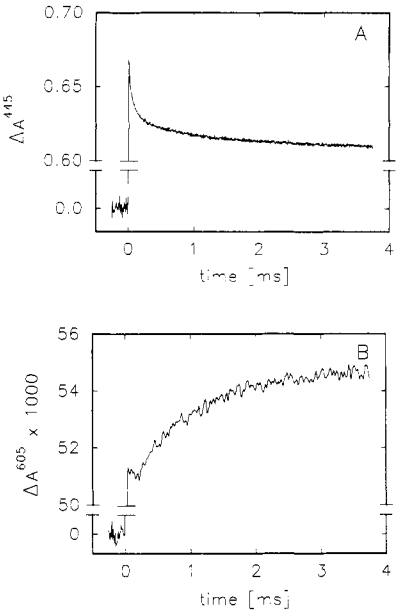

FIGURE 1: Time course of the absorbance changes at pH 7.5 after photodissociation of CO from mixed-valence cytochrome oxidase followed at 445 nm (A) and 605 nm (B) with a band-pass of 5 nm. At 445 nm, there is a significant contribution from cytochrome *a* oxidation which occurs in the 10^4 s^{-1} phase. The concentrations of enzyme and CO were 10 and 15 μM, respectively, and the temperature was 23 °C.

"fast" enzyme as defined by Baker et al. (1987). Mixed-valence oxidase was prepared as described by Brzezinski and Malmström (1985). The following buffers in 0.10 M concentration, all containing 0.1% (w/v) dodecyl maltoside, were adjusted to the desired pH with HCl or KOH: 2-(*N*-morpholino)ethanesulfonic acid (Mes)[1] at pH 6.0 and 6.5; Hepes at pH 7.0 and 7.5; Tris at pH 8.0, 8.5, and 9.0; glycine at pH 10.3. For the pH studies, concentrated enzyme was diluted into the buffers. At pH 7.4, experiments were also made with 0.50 mM buffer, in which case the change in ionic strength was compensated by the addition of K_2SO_4. For the D_2O experiments, the enzyme stock solution was diluted with 2 volumes of D_2O (99.9%) buffer and reconcentrated; this procedure was repeated 3 times, which required 24 h. The pD of the solution was estimated by adding 0.4 to the pH meter reading (Schowen, 1978).

The excitation laser and observation equipment were modified from an earlier design (Brzezinski & Malmström, 1987; Oliveberg & Malmström, 1991), as described in detail by Hallén and Brzezinski (1993). The rate constants were determined from the kinetic traces by a Nelder–Mead simplex algorithm in the Matlab software (Math Works) or by a program based on the Levenberg–Marquardt algorithm, written by Dr. Örjan Hansson in this department.

RESULTS

Figure 1 shows the absorbance changes at 445 and 605 nm following flash photolysis of CO from mixed-valence cytochrome oxidase. At both wavelengths, four kinetic phases can be resolved, the last one representing CO recombination (Oliveberg & Malmström, 1991). The first three phases have

[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

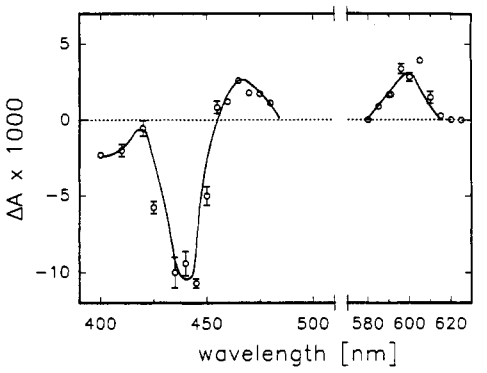

FIGURE 2: Kinetic difference spectrum of the slow phase recorded under the same conditions as in Figure 1.

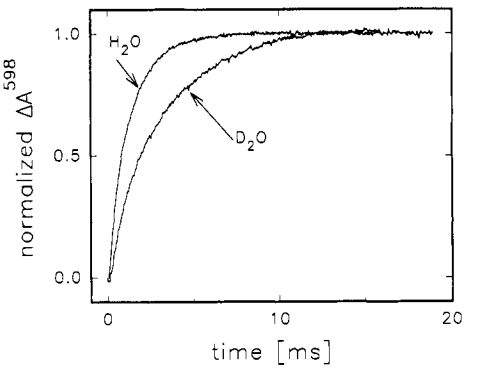

FIGURE 3: Effect of solvent exchange from H_2O to D_2O on the slow phase monitored at pH-meter reading 7.5. The small contributions to the absorbance changes at 598 nm from the CO recombination reaction have been subtracted from the traces presented. The concentrations of the enzyme and CO were 10 μM and 1 mM, respectively, and the temperature was 23 °C.

rate constants of approximately 10^5, 10^4, and 10^3 s^{-1}, as found previously (Oliveberg & Malmström, 1991), except that the third phase was not observed at 445 nm in the earlier study. To resolve the slowest reaction better from the CO recombination, and thus obtain a better estimate of its rate constant, we have used a low CO concentration. Any absorbance changes at 830 nm were too small to be detected.

The kinetic difference spectrum for the slow phase is given in Figure 2. There is a decrease in absorbance in the Soret region and an increase in the α region, the ratio in the changes at 445 and 605 nm being -3.3 ± 0.5. Figure 3 demonstrates that this reaction at pH 7.5 is slower in D_2O compared to H_2O. The two kinetic traces have been recorded at the same pH meter reading, which means that the pD value is 0.4 unit higher. The reason for this is the fact that the relevant comparison should be made at the same degree of ionization of the acid–base groups in the enzyme in H_2O and D_2O, and it has been found that for almost all acids with pK_a values between 3 and 10 the pK_a is increased by about 0.4 unit in D_2O (Schowen, 1978). In this manner, it can be estimated that k_H/k_D is 2–3. There was no isotope effect on the rate of the two faster phases. The effect of D_2O was also measured at pH 9.2 and 10.3. It was small but still measurable at the intermediate pH but was not seen at all at the highest pH (data not shown). We have shown that the isotope effect is reversible by diluting the D_2O enzyme into H_2O. The optical spectrum of the enzyme is the same with the two isotopes (Hallén & Nilsson, 1992).

The pH dependence of the slow reaction is shown in Figure 4. The other two phases do not depend on pH, in agreement with earlier results (Brzezinski & Malmström, 1987; Oliveberg

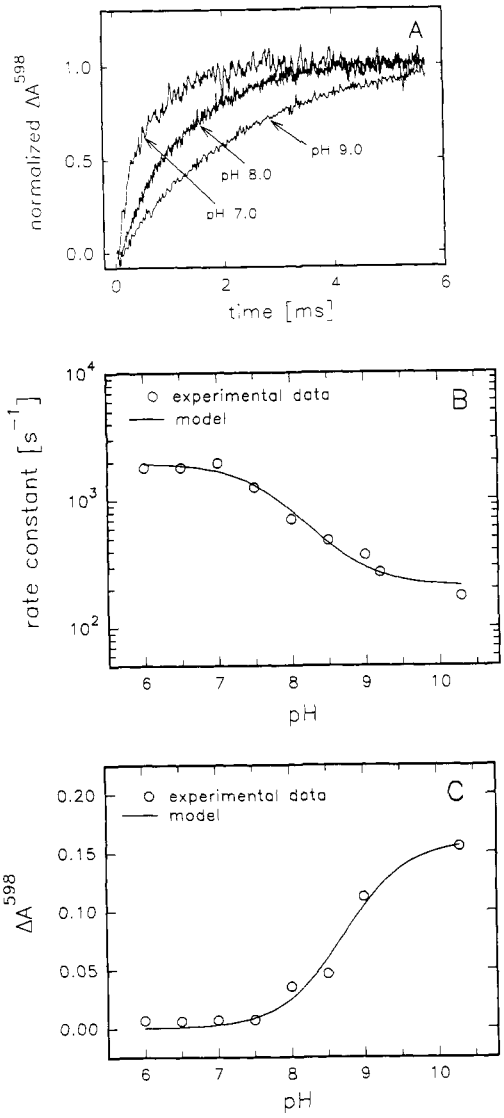

FIGURE 4: pH dependence of the slow phase monitored at 598 nm. In (A), the reaction at pH 7.0, 8.0, and 9.5 is shown, with the traces normalized to the same amplitude [cf. (C)] to highlight the changes in rates. Experimental conditions were the same as in Figure 3. In (B), the experimentally observed rate constants are plotted against pH, the curve being calculated from eq 1 and eq 2, derived on the basis of the model in Figure 6. The effect of pH on the amplitude of the slow phase is shown in (C), with the curve calculated from eq 3.

& Malmström, 1991). It can be seen that the rate of the slow phase decreases with increasing pH, whereas the amplitude of the absorbance changes increases. The curve for the rate in Figure 4B is calculated from a model (see Discussion) and gives a pK_a value of 7.7 ± 0.2. The amplitude data (Figure 4C) can be described with a pK_a of 8.7 ± 0.3. The rates measured at pH 7.4 were the same in 0.50 mM as in 0.10 M buffer (data not shown).

The possible effect of azide on the kinetics of the reaction, by facilitating proton transfer, was also tested. In 50 μM concentration, it had no effect, whereas at 10 mM concentration it slowed down the CO recombination but did not affect the other phases.

Figure 5 illustrates the temperature dependence of the rate of the slow phase, which corresponds to an Arrhenius activation energy of 42 ± 4 kJ·mol^{-1}. The same buffer solution was used at all temperatures, since the pK_a of Hepes has a very small temperature dependence (0.01/°C). Thus, no pH adjustment with changing temperature was necessary.

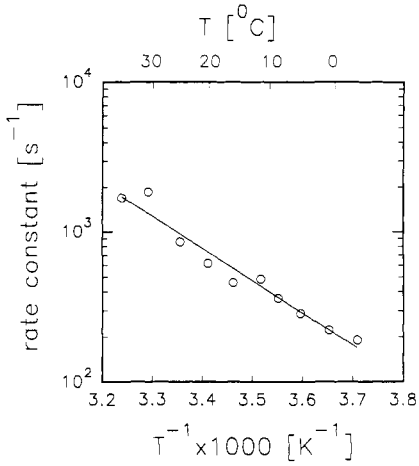

FIGURE 5: Arrhenius plot of the slow phase at pH 7.5, recorded at 598 nm. The buffer contained 30% glycerol in this experiment.

DISCUSSION

Figure 1 shows that the slow reaction phase following CO dissociation from mixed-valence cytochrome oxidase can be detected also at 445 nm, if a low CO concentration is used to slow down the recombination reaction. Earlier it had only been seen in the α region (Brzezinski & Malmström, 1987), and this was one reason for suggesting that it represents a structrual change rather than electron transfer (Oliveberg & Malmström, 1991). This phase is easier to observe in the α region than in the Soret region, because its relative amplitude compared to the recombination reaction is larger in the α region. One can also make measurements at 598 nm, which is an isosbestic point for the recombination reaction. There is an isosbestic point at 435 nm in the Soret region as well, but for some reason this has not been used earlier.

The fact that we now observe absorbance changes of opposite sign connected with the slow phase suggests that this, like the first, most rapid phase (Oliveberg & Malmström, 1991), involves electron transfer from cytochrome a_3 to cytochrome a. This is strongly supported by the difference spectrum in Figure 2, which gives a ratio in the absorbance changes at 445 and 605 nm of approximately −3.3. Attempts have earlier been made to estimate the absorbance changes associated with redox changes in the two cytochromes (Vanneste, 1966; Blair et al., 1982), and values of the absorbance ratio in the range of 2.9–3.7 have been found, depending on which ligand was used to prevent reduction of cytochrome a_3.

The kinetic isotope effect demonstrated in Figure 3 suggests that the electron transfer in the slow phase is also linked to a proton-transfer reaction. It has been shown that the resonance Raman spectrum of cytochrome a is changed in D_2O (Argade et al., 1986), but the isotope effect cannot be due to a shift in its redox properties, since the other two phases are not affected. The effect disappears at high pH, however, which may mean that there are also isotope effects on the ionization of the reactive form of the enzyme or a conformational change (Jencks, 1969). We will, however, later argue that there is a change in the rate-limiting step as the pH is raised, which may also explain the change in the isotope effect. With *lac* permease, it has earlier been proposed that the kinetic isotope effect disappears at high pH because of a change in the rate-limiting step (Viitanen et al., 1983).

The pH dependence of the reaction rate (Figure 4B) shows that this is controlled by the degree of protonation of a group with a pK_a of 7.7, as estimated by curve-fitting to equations derived for a model described later. This value is close to that

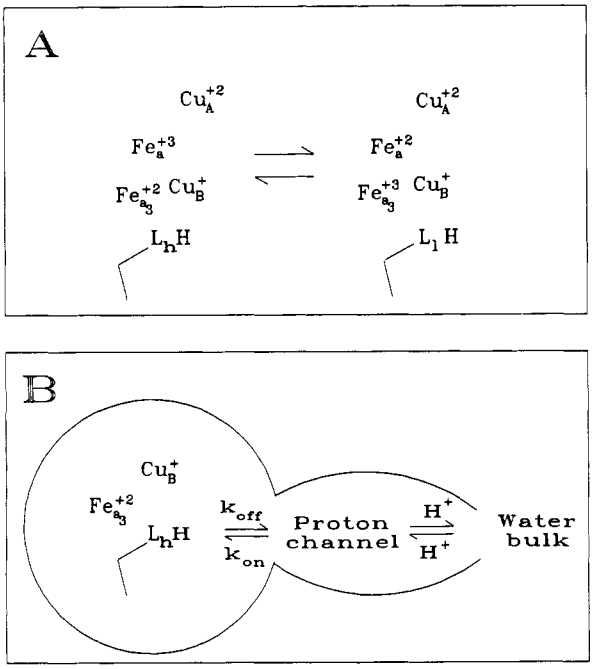

FIGURE 6: Model illustrating the results presented as a series of coupled electron and proton equilibria. (A) Fast electron equilibrium between cytochrome a_3 and cytochrome a after the flash and dissociation of CO out of the bimetallic center. L represents a protonatable group with its pK_a dictated by the redox state of cytochrome a_3 (the subscript h means a high pK_a and l a lower one). As cytochrome a_3 becomes oxidized in the first rapid electron-transfer reaction, the pK_a of LH is lowered, and a shift in the proton equilibria in the pocket is induced. The observed rate of proton dissociation from LH to the medium is a function of the internal proton activity in a proton channel (B), as described in the text.

which has earlier been observed for a proton donor in the vicinity of the binuclear site (Hallén & Nilsson, 1992). That the proton exchange involves cytochrome a_3 and not cytochrome a is, in addition, supported by the amplitude changes in Figure 4C. A proton bound in the vicinity of a cytochrome will stabilize the reduced state electrostatically. Thus, if the proton were associated with cytochrome a, the equilibrium would be shifted toward oxidized cytochrome a as the pH was increased, and the amplitude would drop, but the opposite is actually observed. The amplitude data give a pK_a of 8.7.

The activation energy of the reaction (42 kJ·mol^{-1}) is slightly higher than that found for the fast phase (Oliveberg & Malmström, 1991), which may be because the electron transfer is limited by the protonic equilibria. It may be noted that a similar activation energy has been found for proton transfer to a surface-bound indicator in bacteriorhodopsin (Heberle & Dencher, 1992).

We have tested the effect of azide on the kinetics of the reaction, because in two other proton-translocating systems, bacteriorhodopsin and a bacterial photosynthetic reaction center, it has been found that azide can stimulate proton transfer in mutant forms in which possible proton-transfer groups have been removed (Otto et al., 1989; Takahishi & Wraight, 1991). We found no effect of azide addition on the reactions studied, except on CO recombination, which is perhaps not surprising, since the proton-translocating machinery is intact. An effect of azide on the relaxation rate of the redox equilibrium between cytochrome a_3 and Cu_B has, however, been observed (Hallén & Brzezinski, 1993).

In Figure 6, we present a model which can account for our results, as shown by the calculated curves in Figure 4B–C. In this model, there is an acid–base group LH associated with cytochrome a_3. This is assumed to have a high pK_a value when the cytochrome is reduced, but it drops to 8.7 on oxidation because of repulsion of the proton by the extra positive charge. When CO is flashed off, there is an initial very rapid electron transfer to cytochrome a from cytochrome a_3, which thus becomes oxidized, whereas Cu_B remains reduced (Figure 6A) (cf. Verkhofsky et al. (1992)]. This electron-transfer reaction then causes a drop in the pK_a, which leads to a dissociation of the proton from LH. The proton loss, in turn, results in a further shift in the redox equilibrium between the two cytochromes, giving rise to the slow reaction.

In the model in Figure 6, it is assumed that LH is in proton equilibrium with the solvent via a specific proton pathway. This is suggested to be a proton channel with protonatable groups facilitating proton diffusion (Figure 6B), but other possibilities would be a water wheel or a hydrogen-bonded chain (Copeland & Chan, 1989). There are several reasons for introducing a specific proton pathway. First, if the group could equilibrate directly with the solvent, one would expect the rate to increase continuously with decreasing pH, whereas a limiting rate of ca. 2000 s^{-1} is actually observed (Figure 4B). A rate increase would also be anticipated at high pH, where OH^- becomes the proton acceptor (Crooks, 1975), but again a limiting rate of 300 s^{-1} is instead found. Third, in buffered solutions, the base of the buffer rather than H_2O or OH^- would be the proton acceptor [see Lindskog and Coleman (1973)], but if this were the case, we cannot account for the pH dependence of the observed rate. Our choice of buffers has the consequence that an appreciable fraction is in the basic form at all pH values. One would then expect the proton exchange between LH and the solvent always to be rapid, so that the low rate at high pH could not be explained. With carbonic anhydrase, for example, proton transfer between the buffer and the active site has been found to have a second-order rate constant of 10^8 M^{-1} s^{-1}, and proton equilibrium becomes rate-limiting only at low buffer concentrations (Jonsson et al., 1976). In addition, we found no change in rate at pH 7.4, when the buffer concentration was decreased from 100 to 0.50 mM. This may, however, be because our rate is relatively low compared to carbonic anhydrase. If the rate constant for the reaction with the buffer base were as high as with carbonic anhydrase, we would not be able to detect any change in rate on lowering the buffer concentration.

A proton channel leading to cytochrome a_3 has been suggested earlier (Konstantinov et al., 1986; Wikström, 1988) and is supported by the distribution of conserved amino acids in subunit I (Hosler et al., 1993). Helix VIII has a highly conserved hydrophilic face, which may form part of the lining of a proton channel. There is also a highly conserved loop between helix II and helix III, located on the inside of the membrane, in cytochrome c oxidase as well as in the homologous subunit I of cytochrome *bo*. When Asp135 in the latter is changed to Asn, the pump slips, whereas it still functions in Glu135 (Thomas et al., 1993). Thus, the negative charge of this residue may be a necessary entry point to a channel. Evidence that acid–base groups associated with cytochrome a_3 are accessible via a proton channel has also been provided by optical measurements (Fabian & Malmström, 1989). In another proton pump, bacteriorhodopsin, there is direct structural evidence for the presence of a proton channel [see Lanyi (1992)]. Thus, our model in Figure 6B appears reasonable.

According to our model, the observed relaxation rate should be described by

$$k_{obs} = \alpha k_{on} + k_{off} \quad (1)$$

where α is the degree of saturation of the proton channel:

$$\alpha = \frac{1}{1 + 10^{pH-pK_a}} \quad (2)$$

The amplitude is given by the equation:

$$\frac{[L^-]}{[L^-] + [LH]} = \frac{1}{1 + 10^{pK_a-pH}} \quad (3)$$

It should be noted that the pK_a values in eq 2 and eq 3 are not the same. When $pH \ll pK_a$, α will be close to 1, so that the rate will be maximal. The amplitude at low pH, on the other hand, will be small (Figure 4C), because the degree of dissociation of LH will be minimal. At $pH \gg pK_a$, α approaches 0, and the rate will be low, whereas the amplitude will be large, in agreement with the data in Figure 4. From the fit of the data to curves calculated from eqs 1–3, it was estimated that $k_{off} = 210 \pm 80\ s^{-1}$ and $k_{on} = 1740 \pm 120\ s^{-1}$, and $pK_a = 7.7$ in eq 2. The on-rate is comparable to the rate of the second phase of proton uptake in the oxygen reaction (Hallén & Nilsson, 1992) and to the rate of proton uptake by a quinone in a photosynthetic reaction center [see Okamura and Feher (1992)].

The pK_a determined from the rate data (Figure 4B) must according to this analysis represent an average value for the groups in the proton channel. The value for the amplitude, on the other hand, refers to LH, since thermodynamics require that the equilibrium with the solvent is independent of any intermediates. The estimated pK_a in eq 3 is 8.7. It is a kinetic advantage that the two values are not too far from each other.

We can only speculate on the nature of the proton binding group. One possibility is that LH is H_2O, and L^- is then OH^-. Lanne et al. (1979) have provided EPR evidence for the coordination of OH^- to cytochrome a_3 in the partially reduced enzyme at pH 8.4. This conclusion has been questioned on the basis of magnetic circular dichroism data by Papadopoulos et al. (1991). Their experiments were performed with the fully oxidized enzyme, however, and the partially reduced form studied here may well be in a different conformation. This is evidenced by the results of Fabian and Malmström (1989), who found that azide affects the pH-induced optical changes in the oxidized enzyme, whereas we found no effect of azide. Coordination of OH^- either to cytochrome a_3 or to Cu_B has been suggested by Mitchell et al. (1992), but Cu_B is excluded in our case, since it remains reduced. A metal-coordinated H_2O would fit nicely with a pK_a close to 8 (Lanne et al., 1979), but another identity of LH is by no means excluded.

It is tempting to suggest that the proton transfer reactions observed here form part of the proton-translocating machinery of cytochrome oxidase. In normal turnover, the reactions would go in opposite direction from that in Figure 6, and there would be proton uptake. The scalar protons, i.e., those involved in water formation when dioxygen is reduced, must react at the bimetallic site, but there is increasing evidence that proton pumping involves this site as well [see Babcock and Wikström (1992)]. It may be noted that OH^- coordinated to a metal ion in the bimetallic site has been suggested to be involved in proton pumping by Mitchell (1987). According to Wikström (1989), the normal direction of the reactions in Figure 6 is not involved in proton pumping. The loading of the pump site could, however, well occur in steps that do not result in overall proton translocation. The pK_a values may seem too high, but it should be noted that the matrix pH is probably coniderably higher than 7 (Blair et al., 1986). In addition, in the normal direction of the reaction, the cytochrome is reduced, and the pK_a is even higher according to our model, which would favor proton uptake. Thus, the proton-transfer reactions reported here could be involved in the uptake of either scalar or vectorial protons. In fact, Mitchell et al. (1992) have reported the uptake of two protons on reduction of the bimetallic site.

Woodruff et al. (1991) have provided evidence from CO recombination experiments that a ligand in the bimetallic site can replace the proximal histidine on cytochrome a_3. Furthermore, a switching ligand in this site has been suggested to play an important role in the mechanism of proton pumping (Rousseau et al., 1993; Woodruff, 1993; Hallén & Brzezinski, 1993). Our group (Hallén et al., 1993; Hallén & Brzezinski, 1993) has demonstrated that light-induced structural changes involving cytochrome a_3 can occur also in the absence of external ligands. Thus, it is possible that the switching ligand, rather than OH^-, is involved in the proton equilibria reported here. Hopefully, the availability of mutants with structural changes in the bimetallic site (Hosler et al., 1993) should make it possible to test these ideas experimentally, and studies on mutant oxidases are in progress in our laboratory.

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