Proton Transfer during the Reaction between Fully Reduced Cytochrome *c* Oxidase and Dioxygen: pH and Deuterium Isotope Effects†

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ABSTRACT: The pH dependence of proton uptake and electron transfers during the reaction between fully reduced cytochrome *c* oxidase and oxygen has been studied using the flow–flash method. Proton uptake was monitored using different pH indicators. We have also investigated the effect of D_2O on the electron-transfer reactions. Proton uptake was biphasic throughout the pH range studied (6.3–9.3), and the decrease of the observed rate constants at increasing pH could be described by titration curves with pK_a values of 8–8.5. Of the four phases resolved in the redox reaction, the rate constants for the first two were independent of pH, whereas that of the third decreased at increasing pH with a pK_a of 7.9. All phases except the first were slower in D_2O than in H_2O. The values obtained for k_H/k_D were 1.0 for the first phase, 1.4 for the second and third phases, and 2.5 for the fourth phase. We suggest from these results that the fast phase of proton uptake is initiated by the second phase of the redox reaction and that this step includes a partially rate-limiting internal proton transfer. The third and fourth phases of the redox reaction are suggested to be rate limited by proton uptake from the medium. The pH dependencies of the proton uptake reactions are consistent with the participation of a titrable group in the protein in proton transfer from the medium to the oxygen-binding site.

Mitochondrial cytochrome *c* oxidase is a member of a superfamily of terminal oxidases whose function is to transfer electrons from the respiratory chain to molecular oxygen. The oxidases are integral membrane proteins, and the catalyzed redox reaction is coupled energetically to the translocation of protons across the membrane [see Saraste et al. (1991) and Babcock and Wikström (1992) for recent reviews].

Oxygen is bound and reduced at a bimetallic site composed of cytochrome a_3 and a copper ion, Cu_B. The other two redox-active metal sites, cytochrome *a* and Cu_A, are the initial acceptors of electrons from cytochrome *c*. The three-dimensional structure of the protein is not known. However, recent efforts to identify the ligands of the redox-active metals in cytochrome *c* oxidase from *Rhodopseudomonas spheroides* and the closely related cytochrome *bo* from *Escherichia coli* using site-directed mutagenesis suggest that the bimetallic site is located in the hydrophobic interior of the protein, close to the middle of the membrane-spanning part (Shapleigh et al., 1992; Lemieux et al., 1992; Minagawa et al., 1992). This location is consistent with effects of membrane potential on the redox properties of the bimetallic site observed earlier (Wikström, 1988).

The reduction of oxygen to water requires that protons can be transported from the bulk medium into the active site. It has been shown that these so-called scalar protons are taken up from the matrix side of the membrane (Wikström, 1988). Furthermore, proton translocation includes proton uptake from the medium on the matrix side, proton transfers within the protein, and release of protons to the medium on the cytosolic side. Energetic coupling of proton translocation to the redox reaction requires that proton uptake from the matrix side and proton release to the cytosolic side cannot take place in the same state of the enzyme (Tanford, 1983).

The maximal turnover number (k_{cat}) for catalysis by isolated cytochrome oxidase approaches 700 s^{-1} (Sinjorgo et al., 1986). This catalytic rate suggest the operation of specific mechanisms for the transport of protons consumed by oxygen reduction to the oxygen-binding site as well as for the uptake and relase of translocated protons. In order to study directly the uptake and release of protons we have earlier (Oliveberg et al., 1991) applied the flow–flash method (Gibson & Greenwood, 1967). This method employs flash photolysis for initiating the reaction between the fully reduced enzyme and oxygen and has been used extensively for the kinetic investigations of the redox chemistry of cytochrome oxidase. In our previous study, the pH indicator phenol red was used to monitor protolytic reactions accompanying oxygen reduction, and the faster proton uptake detected took place at about 10^4 s^{-1}. This rate is comparable to that observed for proton uptake by the bound quinone (Q_B) in the photosynthetic reaction center from *Rps. spheroides* at neutral pH (Maroti & Wright, 1989) and faster than the rate observed for proton uptake in the bacteriorhodopsin photocycle (Grzesiek & Dencher, 1986). In these cases, it is known that protonatable amino acid side chains are necessary for rapid proton transfer from the medium (Paddock et al., 1989; Otto et al., 1989), and it would seem likely that protonatable groups participate in the transfer of protons from the medium to the active site also in cytochrome oxidase.

The present work aims at illuminating the mechanism for the transfer of protons from the medium to the oxygen-binding site in cytochrome oxidase. We have used the flow–flash method to investigate the kinetics for proton uptake over a wide pH range. The reaction was biphasic at all pH values, and both rate constants showed a marked decrease at increasing pH values, with pK_a values of 8–8.5. To complement these studies, we have also reinvestigated the pH dependence of the electron transfers associated with oxygen reduction using near-infrared detection. It was found that, of the four kinetic phases resolved, the first two were independent of pH, whereas the third phase slowed down at increasing pH with a pK_a value

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of about 8. The possible rate limitation of the redox chemistry by proton transfers was further investigated by comparing the rates for electron transfers in H_2O and D_2O. In the initial phase, believed to reflect the binding of oxygen, no effect was obtained, whereas the two following phases both showed deuterium isotope effects of 1.4. The final electron transfer was the one most sensitive, with a deuterium isotope effect of 2.5.

From these results, we suggest that internal proton transfer partially limits the rate of the second phase of the redox reaction and that proton uptake from the medium limits the rates for the two last phases. The results are consistent with the participation of at least two titratable groups in proton transfers accompanying the reduction of oxygen. One of these is suggested to be located in the immediate vicinity of Cu_B in the bimetallic site, and the second is suggested to mediate proton transfer from the medium into the site.

MATERIALS AND METHODS

Materials. Bovine cytochrome *c* oxidase was isolated from mitochondrial membranes by sequential extraction with Triton X-100 followed by hydroxyapatite chromatography and exchange of Triton X-100 to dodecyl maltoside, essentially as described by Brandt et al. (1989). This preparation exhibited rapid binding of cyanide to the oxidized enzyme, an absorption maximum at 423 nm for the Soret band and very little of the $g' = 12$ EPR signal, suggesting that it is composed mostly of the "rapid" form of the enzyme (Baker et al., 1987). The pH indicators pyranine (8-hydroxy-1,3,6-pyrenetrisulfonic acid), phenol red, and cresol purple were purchased from Eastman Kodak (Rochester, NY), Merck (Darmstadt, Germany), and Sigma (St. Louis, MO), respectively. Their pK_a values under the conditions used in the present work were determined by spectrophotometric titration. The values obtained were 6.9 for pyranine, 7.8 for phenol red, and 8.6 for cresol purple. D_2O (99.9%) was from Sigma, and all other chemicals were of analytical grade.

Flow–Flash Measurements. The CO complex of the fully reduced enzyme was prepared as described earlier (Oliveberg et al., 1991). For measurements on redox reactions (without pH indicator), samples were prepared in 0.1 M Hepes[1]–KOH (pH 6.0–7.5) or 0.1 M Tris-HCl buffer (pH 8.0–9.5) at the indicated pH. For proton uptake kinetics, the enzyme stock solution (about 300 μM) was diluted 1:10 in a medium of 0.1 M KCl and 0.5% Tween 80 (also used as reaction medium) at a pH of about 7.5. To minimize the buffer capacity, the enzyme solution was then dialyzed against 200 vol of the same medium for about 15 h at 4 °C. Buffers prepared in D_2O were adjusted to the same pH meter reading (pH*) as those in H_2O. Samples were transferred anaerobically to a drive syringe of an RX-1000 rapid kinetics accessory (Applied Photophysics, Leatherhead, U.K.) in which anaerobic conditions were maintained according to the instructions of the manufacturer. The other drive syringe contained the reaction medium saturated with oxygen. A mixing ratio of 1:1 or 1:5 was used. In measurements on proton uptake, the oxygen syringe also contained a pH indicator. Concentrations of pH indicators were 40 μM of phenol red or cresol purple and 90 μM of pyranine (all concentrations given are after mixing). In these experiments, the pH after mixing was measured using a FTPH-2 microflow pH meter (LASAR Research Inc., Los Angeles, CA). Laser flash photolysis was as described earlier (Oliveberg et al., 1991), and the reaction was monitored at visible wavelengths as described by Nilsson (1992) or in the near-infrared as described by Hoganson et al. (1991). The recording system described in the latter work was also used to digitize the photomultiplier output in some of the experiments in the visible region. In proton uptake experiments, traces were recorded with the pH indicator present in both the presence and absence of buffer at the same pH. The time course for proton uptake was then obtained from the difference as described in Oliveberg et al. (1991). All experiments were performed at 22 °C.

Static Measurements of Proton Uptake. Three-milliliter samples of 5 μM enzyme in 0.1 M KCl and 0.5% Tween 80 were, after dialysis (see above), adjusted to the desired pH and then made anaerobic in a stirred Thunberg cuvette by replacing air with argon. The enzyme was reduced with 0.6 mM ascorbate using 7 nM of hexammineruthenium(III) as redox mediator. Formation of the fully reduced enzyme was complete after about 3 h as judged from the optical spectrum. Concentrations of the pH indicators were the same as those described for the flow–flash experiment. To monitor the pH change coupled to the reaction between the fully reduced enzyme and molecular oxygen, the cuvette was placed in a Shimadzu UV 3000 spectrophotometer equipped with a magnetic stirrer. Then, oxygen was admitted, and the increase in absorbance (i.e., deprotonation of the indicator dye coupled to proton uptake to the enzyme) was recorded as a distinct step and was immediately followed by additions of 10 μL of 1 mM HCl to calibrate the signal. The absorbance changes obtained on addition of acid were corrected for dilution effects. The actual pH value was determined after readjusting the indicator absorbance to the starting value by small additions of KOH. It was measured directly with a pH electrode and also calculated from the absorbance of the indicator dye. Rereduction of the enzyme by the ascorbate/hexammineruthenium(III) system was sufficiently slow as not to interfere with these procedures. Absorbance changes due to oxidation of the enzyme were determined separately in the absence of pH indicator and subtracted from those obtained with the indicator present.

Data Processing. Curve fitting was done by using algorithms provided in the Matlab software package from The Mathworks, Inc. (South Natick, MA). Proton uptake data were digitally filtered (time constant: 4 μs) using the ASYST program from Macmillan Software Co. (New York, NY).

RESULTS

pH Dependence of Proton Uptake. In order to measure the kinetics for proton uptake in a wide pH range, we have used three different indicators with different pK_a values. Time courses for proton uptake at different pH values using the indicators pyranine, phenol red, and cresol purple are shown in Figure 1. The traces shown were obtained from the difference between kinetic traces recorded in the presence and absence of buffer (0.1 M Hepes–KOH) at the same pH.

The initial lag observed earlier (Oliveberg et al., 1991) was seen in all traces. The subsequent proton uptake was biphasic at all pH values, and the rate constants obtained for the rapid and slow phases are shown as function of pH in Figure 2. These rate constants were determined by fitting a sum of two exponentials to the kinetic traces. This analysis, in particular the determination of the rate constant for the rapid phase, is complicated by the presence of the initial lag. The starting point for curve fitting was therefore systematically moved away from the flash point to minimize the influence of the lag

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

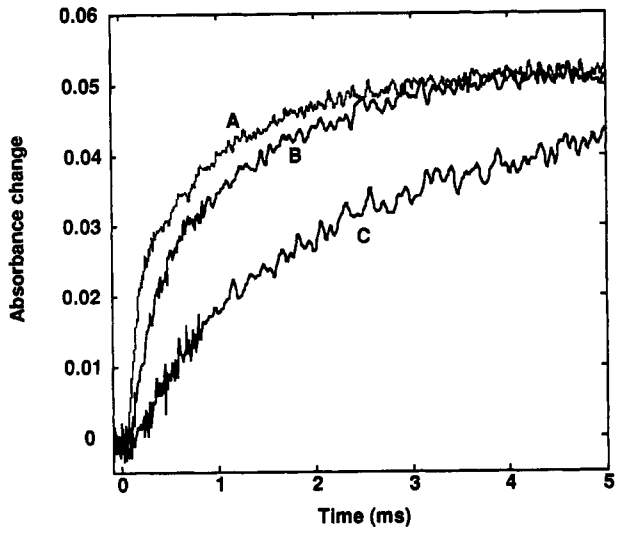

FIGURE 1: Transient absorbance changes of pH indicator dye during the reaction between fully reduced solubilized enzyme and molecular oxygen followed at different pH values. (A) 90 μM pyranine, pH 7.0; (B) 40 μM phenol red, pH 8.3; and (C) 40 μM cresol purple, pH 8.9. The pyranine signal was followed at 461 nm, and phenol red was together with cresol purple detected at 560 nm. The absorbance scale applies to trace C, and the other traces have been scaled to give the same total amplitude to facilitate comparison of the kinetics. This excludes comparisons of the stoichiometries from the data shown in this figure. The concentrations of enzyme and oxygen after mixing were 5 μM and 0.6 mM, respectively.

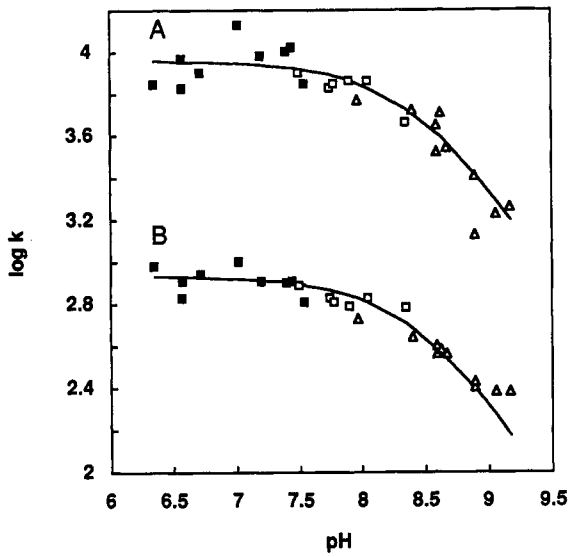

FIGURE 2: Effect of pH on the proton uptake rates. (A) Rate of the fast phase as a function of pH together with a fitted titration curve (solid line) with $pK_a = 8.5$ and a limiting rate constant at low pH of 9.2×10^3 s^{-1}. (B) pH dependence of the slow phase and a fitted titration curve with $pK_a = 8.2$ and a limiting rate constant at low pH of 870 s^{-1}. (■) pyranine, (□) phenol red, and (▲) cresol purple. For experimental conditions, see Figure 1.

on the rate constant obtained for the first phase of proton uptake. This procedure may somewhat underestimate the rate constant.

In a kinetic study of proton release and uptake by bacteriorhodopsin in the purple membrane (Grzesiek & Dencher, 1986), it was found that the addition of small amounts of phosphate buffer accelerated the observed rate for the rapid proton release, suggesting that protonation of the pH indicator and/or deprotonation of the protein was limited by proton transfer in solution. We have therefore investigated the rates for proton uptake also in the presence of 0.1 or 0.5 mM phosphate buffer. In our case, only the amplitude of the

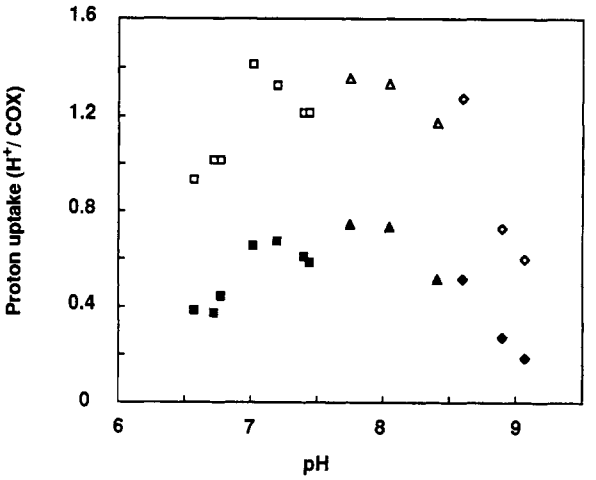

FIGURE 3: pH dependence of the net proton uptake stoichiometries obtained when fully reduced solubilized enzyme reacts with molecular oxygen in the static experiment. Open symbols: total net proton uptake. Closed symbols: proton uptake during the fast phase calculated from the fraction of the total absorbance change due to the indicator dye in the fast phase and the total number of protons taken up at the same pH. (□, ■), 90 μM pyranine; (△, ▲), 40 μM phenol red; and (◇, ◆), 40 μM cresol purple. The enzyme concentration was 5 μM.

indicator signal was affected, and no effect on the rate constants was seen (data not shown). It is therefore unlikely that the rates observed here are limited by proton transfers in solution. Also, it can be seen in Figure 2 that the rate constants obtained were independent of the indicator used, whereas in the case of rate limitation by deprotonation of the indicator the observed rate would be expected to vary with its pK_a value (Gutman & Nachliel, 1990).

The pH dependence of the net stoichiometry for proton uptake was studied by reacting the reduced enzyme with oxygen in the presence and absence of indicator in an ordinary spectrophotometer. This procedure allows for more convenient calibration of the response of the indicator. The number of protons taken up per enzyme molecule on reoxidation at different pH values is shown in Figure 3.

pH Dependence of Redox Reactions. The pH dependence of the four kinetic phases observed in the reaction between the fully reduced enzyme and oxygen has been studied earlier by Oliveberg et al. (1989). However, the complicated kinetics obtained at visible wavelengths makes it difficult to extract the rate constants from kinetic traces by curve fitting. Since accurate knowledge about the pH dependence of the accompanying redox reactions is necessary to interpret the kinetics for proton uptake, we have reinvestigated the effect of pH on the earlier phases using near-infrared (830 nm) detection. Advantages of using this technique include better resolution of the initial phases (Oliveberg & Malmström, 1992), which facilitates the kinetic analysis, and the high signal-to-noise ratio obtained with the diode laser system.

Figure 4 shows kinetic traces obtained in the pH range 6.5–9. Analysis of these traces give rate constants of 9×10^4 and 3×10^4 s^{-1} for the first two phases at all the pH values. The pH dependence of the third phase is shown in Figure 5.

Kinetic Deuterium Isotope Effects. The pH dependencies found for the third (above) and fourth phases (Oliveberg et al., 1989) suggest that these redox reactions can become rate limited by proton transfer from the medium. To investigate further the possibility of rate limitation of the redox processes by proton transfers, we have also investigated the effect of D_2O on the rates. A pH* value of 7.4, where the effect of the ionization state of the enzyme on the rates is small, was chosen

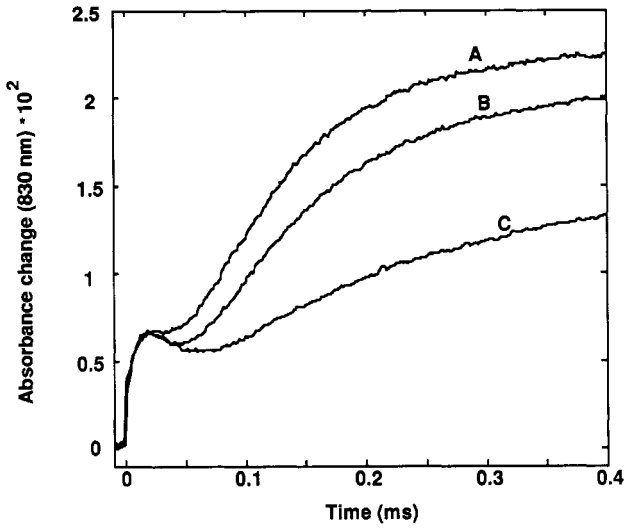

FIGURE 4: Time course of the absorbance changes at 830 nm after flash photolysis of the fully reduced carboxy cytochrome oxidase (8.5 μM) in the presence of about 1 mM oxygen and at three different pH values. (A) pH 6.5, (B) pH 7.5, and (C) pH 9.5.

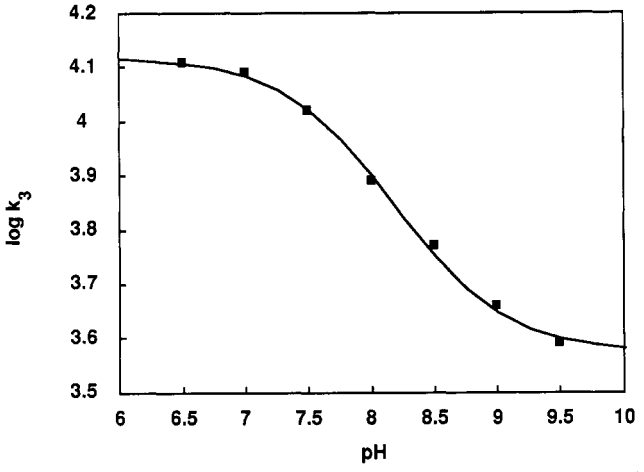

FIGURE 5: pH dependence of the rate constant obtained for the third phase (k_3) detected at 830 nm. The solid line is a fitted titration curve with pK_a = 7.9 and limiting rate constants at low and high pH of 1.4×10^4 and 3.1×10^3 s^{-1}, respectively. For experimental conditions see Figure 4.

for these experiments. At this pH*, pD becomes 7.8 due to the equilibrium isotope effect on the glass electrode (Schowen, 1978). However, equilibrium isotope effects on titratable groups in proteins are commonly of the same magnitude, but oppositely directed (Schowen, 1978). Therefore, in measurements at the same pH* value, the ionization state of the enzyme is expected to be the same in H_2O and D_2O.

Kinetic traces obtained at 605 and 445 nm in H_2O and D_2O are compared in Figure 6 (top and bottom panels). Inspection of these traces shows a clear difference in the slowest phase. From curve fitting we obtain rate constants for this phase of 930 and 350 s^{-1} in H_2O and D_2O, respectively. The appearance of the plateau region is also different, suggesting D_2O effects also on the earlier phases. Since the rate constant for these are easier to extract from kinetic traces observed in the near-infrared as discussed above, experiments were also carried out using near-infrared detection. The results are shown in the middle panel. From these traces, we obtain a rate constant of 9×10^4 s^{-1} for the first phase in both H_2O and D_2O. The rate constants for the second phase were 3×10^4 and 2.2×10^4 s^{-1} in H_2O and D_2O, respectively. Corresponding values for the third phase were 1.1×10^4 and 7.6×10^3 s^{-1}. The rate constants obtained for all phases in H_2O and D_2O and the

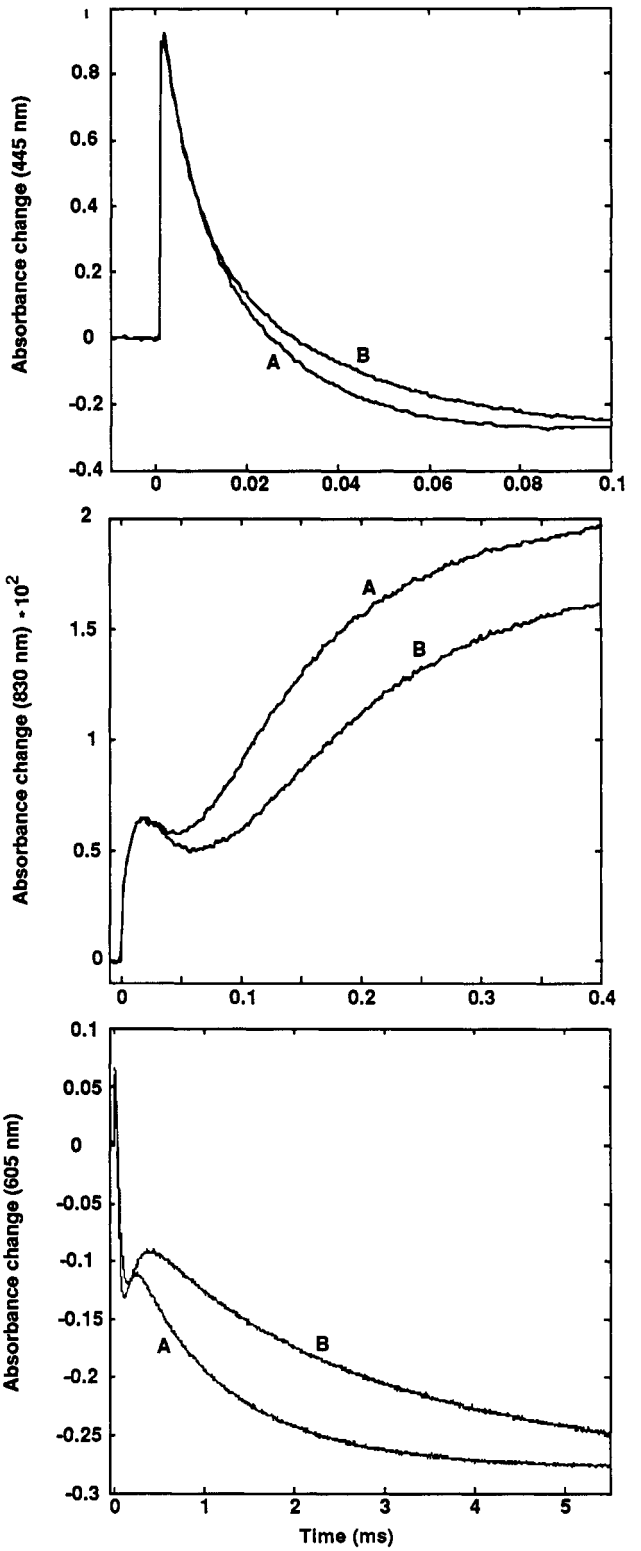

FIGURE 6: Solvent isotope (D_2O) effects on the redox reaction between the fully reduced enzyme and molecular oxygen monitored at 445, 605, and 830 nm with different time scales. (A) H_2O, (B) D_2O. The upper panel shows the effect on the two initial phases followed at 445 nm. The middle panel demonstrates the effect on the third phase detected at 830 nm, and the lower panel shows the influence on the fourth phase followed at 605 nm. Experimental conditions: pH* = 7.4; enzyme concentrations 8.5 μM in the middle panel and 11.5 μM in the upper and lower panels. The oxygen concentration was 1.0 mM.

deuterium isotope effects are summarized in Table I. Although the deuterium effects on the second and third phases are smaller than on the slowest phase, the different appearances of the kinetic traces, both in the visible and in the near-infrared,

Table I: Rate Constants for the Four Phases of Oxygen Reaction in H_2O and D_2O at pH* = 7.4

	phase			
	first	second	third	fourth
k_H, s^{-1}	9×10^4	3×10^4	1.1×10^4	930
k_D, s^{-1}	9×10^4	2.2×10^4	7.6×10^3	350
k_H/k_D	1.0	1.4	1.4	2.5

clearly show that they are significant.

The optical spectra of the oxidized and reduced enzyme in D_2O were identical to those obtained in H_2O (not shown). It is therefore unlikely that major structural perturbations of the protein by D_2O are responsible for the observed effects on the reaction rates.

DISCUSSION

This work deals primarily with proton transfers during the reaction between the reduced enzyme and oxygen. The interpretation of our results is, however, also dependent on the knowledge of the chemistry of oxygen reduction and the assignment of the four kinetic phases resolved in the oxygen reaction. In a recent review, Babcock and Wikström (1992) summarize a large body of kinetic and spectroscopic information on the reaction between fully reduced cytochrome oxidase and oxygen. The sequence of redox events in the reaction scheme proposed by these authors will be used as a starting point in discussing the present study of the acid–base chemistry accompanying the reduction of oxygen.

Kinetics of Proton Uptake. The earliest uptake of protons from the medium takes place at a rate of maximally 10^4 s^{-1}, which suggests that it is initiated by the formation of the ferric peroxide recently observed by Zhang et al. (1992; this is reflected in the second phase of the redox reaction). However, the decay of the primary oxygen adduct is accompanied by oxidation of cytochrome *a* (Hill & Greenwood, 1984; Han et al., 1990), with the consequence that the oxidation state of the oxygen-binding site cannot be that of a ferric cupric peroxide. In an earlier study (Oliveberg et al. 1991), we therefore suggested that proton uptake was initiated by the formation of a ferrous cupric peroxide intermediate (Blair et al., 1985). The recent results of Zhang et al. (1992) show, however, that cytochrome a_3 is ferric rather than ferrous at this stage. Consequently, Cu_B must be reduced.

Involvement of acid–base chemistry already at the earliest steps in the oxygen reaction is suggested by the finding that the reaction preceding proton uptake is subject to a deuterium isotope effect of about 1.4 (Table I). The reaction scheme in Figure 7 shows a possible scenario in which Cu_B is the immediate electron donor to the ferrous oxygen adduct, as suggested by Babcock and Wikström (1992). This first electron transfer (the second kinetic phase) is accompanied by a proton transfer from a group in the vicinity of Cu_B to the peroxide or another group nearby. Such proton movement can be rationalized on electrostatic grounds: on electron transfer from Cu_B to the ferrous oxygen adduct, residues nearby the former are expected to become more acidic, whereas the resulting ferric peroxide is expected to be basic. Rereduction by electron transfer from cytochrome *a* then restores the charge on Cu_B and initiates proton uptake from the medium.

The decrease in the stoichiometry of proton uptake in the fast phase at alkaline pH can be explained by titration of the peroxide. A possible explanation for the decrease in the acidic pH range is that alternative proton donors become available

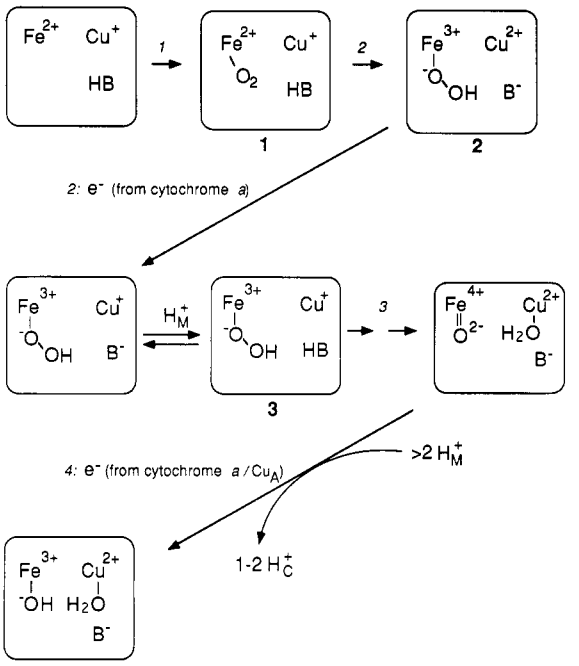

FIGURE 7: Reaction scheme illustrating the suggested proton transfers during the oxygen reaction. The italic numbers at the arrows indicate the corresponding kinetic phase (see Table I for rate constants and isotope sensitivities), and the bold numbers refer to intermediates discussed in the text. The indices C and M refer to the matrix and cytosolic sides. Determination of the number of protons taken up in the fourth kinetic phase is complicated by the release of protons that takes place on the same time scale [see Oliveberg et al. (1991)]. The protonation state of the end product should therefore be taken as tentative.

at low pH; these must then be more acidic in the final state (**3**) than in the initial state (**1**). It should be noted that when the reaction is started with the mixed-valence CO compound, there is no extra electron available and the reaction stops at the ferric cupric peroxide (**2**). In this case, no net proton uptake is predicted at neutral pH, in agreement with previous observations (Oliveberg et al., 1991).

The redox reaction suggested to initiate the rapid phase of proton uptake takes place at around 3×10^4 s^{-1}, yet the observed rate for proton uptake is maximally 10^4 s^{-1}. This suggests that it is the rate of proton transfer from the medium to the binuclear site rather than the rate of redox reaction that results in proton uptake that limits the observed rate. The rate constant for proton uptake is also distinctly pH-dependent (Figure 2A), whereas no pH dependence was found for the preceding reaction [the second phase; see also Oliveberg et al., (1989)]. We have earlier suggested the possibility that this rapid phase of proton uptake is diffusion-controlled (Oliveberg et al., 1991). However, the pH dependence of the observed rate constant is not that expected for a diffusion-controlled protonation (Gutman & Nachliel, 1990). Also, no acceleration of the uptake was obtained on the addition of buffer, which would have been expected in that case (Grzesiek & Dencher, 1986). Rather, the pH dependence of the rate for proton uptake suggests that the reaction is controlled by the protonation state of a protonatable group with a pK_a of 8–8.5. It is likely that this group is the immediate donor of protons to the oxygen-binding site. Its function would then be analogous to that of Asp-96 in bacteriorhodopsin (Otto et al., 1989).

In the reaction scheme suggested by Babcock and Wikström (1992), the subsequent redox reaction is the electron transfer from Cu_B to the ferric peroxide, resulting in the formation of the ferrous cupric peroxide observed at low temperature by

Blair et al. (1985). This reaction is reflected in the third kinetic phase, which includes oxidation of Cu_A and reduction of cytochrome *a*. Cu_B oxidation has been suggested to initiate this event by causing cytochrome *a* to go from a low-potential to a high-potential state as a result of the anticooperative redox interaction between Cu_B and cytochrome *a* (Oliveberg & Malmström, 1992). The following step is the heterolytic cleavage of the oxygen–oxygen bond with formation of the ferryl intermediate. The pH dependence of the rate of the redox reaction parallels that of proton uptake (compare Figures 2A and 5), and the reaction shows a deuterium isotope effect of 1.4 (Table I). Both these observations suggest that its rate is limited by the proton uptake. Furthermore, the similar rates of the redox reaction and proton uptake suggest that protonation at the binuclear site does not occur faster than the disappearance of the proton from the medium. This means that the rate-limiting event in proton transport from the bulk medium is the proton transfer from a position in rapid equilibrium with the medium to the residue that needs to be protonated for rapid electron transfer.

Formation of the ferryl intermediate is followed by the transfer of the fourth electron and the formation of the oxidized enzyme (the fourth kinetic phase). Since the rate of electron transfer shows a marked decrease at increasing pH (Oliveberg et al., 1989), it is likely that it must be preceded by the uptake of additional protons. The slow phase of proton uptake is thus probably initiated by the formation of ferryl intermediate. This is also consistent with the observed maximal occupancy of the ferryl state within 0.5 ms (Han et al., 1990).

The rate observed for the early part of proton uptake shows that, in that case, the mechanism for proton transfer from the bulk medium into the bimetallic site is capable of operating at a rate of 10^4 s^{-1} at the low-pH values. One would therefore expect the rate of proton uptake to be limited only by the formation of the ferryl species. On the contrary, this proton uptake is slower than the formation of the ferryl species, and its rate shows a marked pH dependence (Figure 2B). Apparently, proton transfer from the medium is slower in this case. This is consistent with the larger deuterium isotope effect observed for the transfer of the fourth electron (Table I), which suggests rate limitation by proton transfer to a greater extent than for the earlier electron transfers. Although the proton release that also takes place on this time scale (Nilsson et al., 1990) could contribute to the isotope effect, the decrease of the electron-transfer rate at high-pH values (Oliveberg et al. 1989) suggests that proton uptake rather than proton release is rate-limiting.

In the rapid phase of proton uptake, the slow step appears to be proton transfer from a donor in communication with the bulk medium to a residue in the immediate vicinity of the binuclear site. The similarity of the rate for the transfer of the fourth electron to the rate for the slow phase of proton uptake suggests that the situation is similar during the slow phase of proton uptake. Possible explanations for the 10-fold difference in rate between the two proton transfers include an increased barrier to proton transfer due to structural differences or a decreased driving force due to decreased basicity of the recipient group in the ferryl state. Both these possibilities are consistent with the larger deuterium isotope effect on the transfer of the fourth electron.

Stoichiometry of Proton Uptake. Although we have focused on investigating the pH dependence of rates, the stoichiometries for the two phases of proton uptake may also be informative for understanding the events initiating proton uptake. We can, however, measure only net stoichiometries, and these are expected to reflect formation of base during the reduction of bound oxygen, uptake or release of vectorial protons, and redox Bohr effects. We have earlier reported a net uptake of about 1 proton/enzyme molecule on oxidation by the solubilized enzyme and the release of protons to the outside of the vesicles during the oxidation of reconstituted enzyme at pH 7.5 (Oliveberg et al., 1991). From these results, an uptake of 2–3 protons at pH 7.5 from the matrix side was inferred. This is less than expected from the reduction of oxygen and proton translocation associated with conversion of the ferryl intermediate to the oxidized enzyme [Wikström (1990), but note that the suggested proton translocation associated with conversion of the peroxide intermediate to the ferryl intermediate may be by-passed when the reaction is started with the fully reduced enzyme; see Babcock and Wikström (1992) and Nilsson et al. (1990)]. We have therefore in the present work determined the stoichiometry for proton uptake in a wide pH range using a somewhat different approach that sacrifices time resolution but is more suitable for a pH titration.

The maximal stoichiometry, 1.4 protons/enzyme molecule was obtained at pH 8, and the observed stoichiometry decreased at lower and higher pH values (Figure 3). Assuming a release of two protons to the cytosolic side, this gives an maximal uptake of 3.4 protons from the matrix side. A possible explanation for this low stoichiometry is that the release of protons by redox Bohr mechanisms takes place in parallel to proton uptake induced by oxygen chemistry and to uptake and release of translocated protons during the reaction between the reduced enzyme and oxygen. Such proton release has been observed by Capitanio et al. (1990) on the oxidation of cytochrome *a* and Cu_B in the cyanide-inhibited, solubilized enzyme. Since the reduction potential of cytochrome *a* is sensitive to the pH on the matrix side (Artzatbanov et al., 1978), release of Bohr protons is expected to take place on the matrix side.

Structural Implications. In order to explain the kinetic features observed in this work, we have suggested the participation of two different groups in the acid–base chemistry accompanying oxygen reduction. One protonatable group is proposed to be in close proximity to the bimetallic site, and a second one is proposed to mediate proton transfer from the bulk medium into the site. The presence of a titratable group close to the bimetallic site has also been inferred from the pH dependence of the reduction potential of Cu_B (Moody & Rich, 1990), from pH effects on the optical spectrum of the oxidized enzyme (Papadoupolos et al., 1991), and from the pH dependence of cyanide binding to the oxidized enzyme (Konstantinov et al., 1986).

A structure for the catalytic core of the proton-translocating terminal oxidases has recently been proposed from site-directed mutagenesis of the conserved histidine residues thought to serve as ligands to the heme groups and Cu_B (Shapleigh et al., 1992; Lemieux et al., 1992; Minagawa et al., 1992). Here, these metal sites are bound via histidine side chains to the transmembrane helices II, VI, VII, and X of subunit I. It has also been suggested that helix VIII is included in the core structure (Babcock & Wikström, 1992). In this model, the only conserved amino acids with protonatable side chains in the immediate vicinity of the bimetallic site are the histidine ligands of Cu_B and, possibly, threonine redidues in helix VIII. Other possible proton donors include bound water (Kornblatt & Kornblatt, 1992) and the propionyl groups of cytochrome a_3. A conserved lysine residue in helix VIII, located approximately halfway between the bimetallic site and the surface of the membrane on the matrix side, is an attractive candidate

for a group mediating proton transfer from the solvent into the active site.

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Registry No. O_2, 7782-44-7; D_2, 7782-39-0; cytochrome *c* oxidase, 9001-16-5.