A Role for the Protein in Internal Electron Transfer to the Catalytic Center of Cytochrome *c* Oxidase†

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ABSTRACT: Internal electron transfer (ET) to heme a_3 during anaerobic reduction of oxidized bovine heart cytochrome *c* oxidase (CcO) was studied under conditions where heme *a* and Cu_A were fully reduced by excess hexaammineruthenium. The data show that ET to heme a_3 is controlled by the state of ionization of a single protolytic residue with a pK_a of 6.5 ± 0.2. On the basis of the view that ET to the catalytic site is limited by coupled proton transfer, this pK_a was attributed to Glu60 which is located at the entrance of the proton-conducting K channel on the matrix side of CcO. It is proposed that Glu60 controls proton entry into the channel. However, even with this channel open, there is the second factor that regulates ET, and this is ascribed to the rate of proton diffusion in the channel. In addition, it is concluded that proton transfer in the K channel is reversibly inhibited by the detergent Triton X-100. It is also found that the rate of ET to heme a_3 in the as-isolated resting enzyme and in CcO "activated" by reaction of fully reduced enzyme with O_2 is the same, implying that the catalytic sites of these two forms of oxidized enzyme are essentially identical.

Mitochondrial cytochrome *c* oxidase (CcO)[1] is a membrane protein that catalyzes the oxidation of ferrocytochrome *c* by molecular oxygen. The reduction of O_2 to water requires the delivery of four electrons and four protons into the catalytic center of the enzyme. Electrons enter oxidase from the cytosolic side and protons from the matrix side of the inner mitochondrial membrane. This redox reaction is one of two processes that contribute to the generation of a transmembrane proton gradient. The second process, proton pumping, is proton translocation from the mitochondrial matrix space to the cytosolic side of the membrane driven by electron transfer (ET).

Bovine heart CcO is composed of 13 subunits (*1*), but all four of the redox centers involved in ET and in the reduction of O_2 to water are located in subunits I and II (*2*). Three of these centers, heme *a*, heme a_3, and the mononuclear copper site, Cu_B, are present in subunit I, and the dinuclear copper center, Cu_A, is located in subunit II (*2*). Cu_A is close to the cytosolic surface of the protein and serves as the acceptor of electrons from the physiological reductant, cytochrome *c* (*3*−*6*). Electrons received by the oxidase are distributed between Cu_A and heme *a* on the microsecond time scale (*7*−*9*). ET then continues to the catalytic center composed of heme a_3 and Cu_B. At this binuclear center, the interaction of electrons, protons, and oxygen occurs. The three centers, heme *a*, heme a_3, and Cu_B, are buried within the protein at about the same depth from the membrane surface (*2*).

The anaerobic transfer of two electrons to the oxidized catalytic center is accompanied by the uptake of two protons, ultimately from solution (*10, 11*). These two protons are assumed to compensate for the charge of the electrons delivered to heme a_3 and Cu_B keeping the catalytic center in the electrically neutral state (*10*). The rate of heme a_3 reduction appears to be synchronous with proton uptake (*12*), and it has been concluded that the rate-limiting process for this internal ET is the stabilization of the transferred electrons by these protons (*12*).

Protons are delivered through the protein to the catalytic site by two channels, called K and D, that lead from the matrix surface of the enzyme to the binuclear center (*2*, *13*−*15*). In the X-ray structures of oxidized CcO, there is not a continuous hydrogen-bonded network in either of these two channels (*2*, *15*). It can consequently be assumed that proton diffusion through the channels involves transient structural changes together with rearrangements of internal water molecules.

The conversion of CcO from the oxidized to the reduced state is also associated with structural changes directly at the catalytic site (*16*, *17*). In fully oxidized enzyme, only a diffuse electron density is observed between heme a_3 and Cu_B, and this has yet to be convincingly interpreted. In the bovine enzyme, a peroxide anion has been proposed to bridge the two metal centers (*17*), whereas in the *Paracoccus*

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[1] Abbreviations: CcO, cytochrome *c* oxidase; CcO.DM, cytochrome *c* oxidase isolated in detergent *n*-dodecyl-*β*-D-maltoside; CcO.TX, cytochrome *c* oxidase isolated in detergent Triton X-100; **O**, oxidized cytochrome *c* oxidase as isolated; $\mathbf{O}_H$, metastable form of cytochrome *c* oxidase as the immediate product of the oxidative phase; Tris, Tris-[hydroxymethyl] aminomethane; Mes, 2[*N*-morpholino]-ethanesulfonic acid; Ches, 2[*N*-cyclohexylamino]-ethanesulfonic acid; Hepes, 3-[cyclohexylamino]-1 propanesulfonic, *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid]; TX, Triton X-100; DM, *n*-dodecyl-*β*-D-maltoside; Ru, hexaammineruthenium; DT, sodium dithionite.

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denitrificans structure, H_2O plus OH^- were consistent with the continuous electron density between Cu_B and the iron of heme a_3 (*18*). In contrast, in *Rhodobacter sphaeroides* (*19*) and in the *Thermus thermophilus* (*20*) enzymes, this density was fitted to just a single bridging oxygen (either OH^- or H_2O). No ligand is detected between Cu_B^{1+} and Fe_{a3}^{2+} in the X-ray structure of the fully reduced enzyme (*17*, *21*).

In the literature, two forms of oxidized CcO are recognized. The first is the so-called "resting" form (**O**), that is, the as-isolated enzyme. The second form is an activated oxidized CcO produced immediately following reoxidation of fully reduced enzyme with O_2 (*22*, *23*). This activated CcO ($\mathbf{O}_H$) has been postulated to be a metastable state. The energy stored in this metastable form is released by ET to the catalytic site and utilized for proton pumping. Different redox properties and coordination structures for the catalytic sites of these two forms are anticipated (*22*, 23).

The present study indicates that proton access to the catalytic site, which presumably limits the rate of ET to heme a_3 during anaerobic reduction, is under control of two factors: (i) the state of ionization of a single residue proposed to be Glu60 located at the entrance of the K channel and (ii) the rate of proton diffusion in the K channel. Moreover, our data show that there is no difference in the kinetics of ET to heme a_3 in **O** and $\mathbf{O}_H$, which in turn indicates that the catalytic site of these two forms is essentially the same.

EXPERIMENTAL PROCEDURES

Materials. Tris, Mes, Ches, and Hepes buffers were purchased from Sigma, hexaammineruthenium (III) chloride (Ru) and sodium hydrosulfite (dithionite, DT) were purchased from Aldrich; peroxide-free Triton X-100 (TX) was obtained from Roche Diagnostics and *n*-dodecyl-β-D-maltoside (DM) from Anatrace.

Enzyme Purification. Bovine heart cytochrome *c* oxidase (CcO) was isolated by the modified method of Soulimane and Buse into either TX or DM (*24*). After purification, the concentrated enzyme was stored at 77 K in 10 mM Hepes, pH 7.8, 50 mM K_2SO_4 and containing either 0.1% TX, for CcO isolated in TX (CcO.TX), or 0.05% DM, for CcO isolated in DM (CcO.DM). The enzyme concentration was determined from the absorbance of the oxidized enzyme at 424 nm using an absorption coefficient A_{424} of 156 mM^{-1} cm^{-1} (*25*).

Kinetic Measurements. The kinetics of reduction of oxidized CcO (**O**) by hexaammineruthenium (Ru) was measured under anaerobic conditions in an argon atmosphere using a stopped-flow apparatus. An anaerobic solution of **O** was mixed in a 1:1 ratio with the mixture of Ru and sodium dithionite (Ru−DT). Unless noted otherwise, all kinetics of internal ET to heme a_3 were obtained with CcO in the **O** state in the presence of 2.5 mM Ru and 5 mM DT (final concentrations). All rate constants for ET are averages of at least three measurements.

We also compared the kinetics of ET to heme a_3 of CcO in the **O** and $\mathbf{O}_H$ states at pH 9.0 and 8.0 (*23*). For the measurement of ET in the $\mathbf{O}_H$ state anaerobic, fully reduced CcO (3 μM) in the presence of 5 mM Ru and 20 mM DT was mixed in the stopped-flow with air-saturated buffers (either 200 mM Ches, pH 9.0, or 200 mM Hepes, pH 8.0, both containing 0.1% DM). In the approximately 3 ms dead time of the stopped-flow apparatus, the enzyme is oxidized, any excess oxygen present is consumed by DT, both Cu_A and heme *a* are reduced by the excess reductant, and only the re-reduction of heme a_3 is available for monitoring.

The solutions of **O** and Ru−DT were made anaerobic by repeated evacuation followed by equilibration with argon. For the Ru−DT mixture, the buffer solution containing Ru was first made anaerobic in a tonometer, and the appropriate amount of solid DT was then added from the tonometer's sidearm. Solutions of anaerobic enzyme were prepared in a similar manner with stock **O** in the sidearm of the tonometer. To remove oxygen from the stopped-flow instrument, the whole flow system was filled with a buffered solution of 5 mM DT for about 1 h prior to measurement. The driving syringes were submerged in water which was continually purged with nitrogen; the temperature was 23 °C.

We noticed that the greater the time that **O** was kept in buffers at pH 10.0 or above or at pH 6.0 or below the slower was the rate of ET to heme a_3. This decrease in the rate of ET developed slowly, on the scale of tens of minutes at 23 °C. For this reason, data were collected within 5 min after the stock **O** was dissolved in the final buffer. That this approach yielded valid data was verified by mixing enzyme diluted in a weak buffer at pH 7.8 with reductant in strong buffer at the desired final pH in the stopped-flow apparatus. The data obtained from the two procedures were essentially identical.

The acidification of buffer solutions caused by adding solid DT was compensated for by addition of concentrated KOH.

Kinetic measurements were performed on two different stopped-flow instruments: the OLIS RSM-1000 apparatus was used to collect spectra (1000/s) and an in-house instrument for data collection at single wavelength. Both instruments were equipped with 20 mm path length observation cell.

Ferrocyanide oxidation by CcO.TX was measured at pH 7.0 (50 mM MOPS−KOH buffer, 0.5 mM EDTA, and 0.1% Triton X-100) in the HP 5453 diode array spectrophotometer at 23 °C following the protocol described earlier (*26*). The reaction was initiated by the addition of 5 mM hydrogen peroxide to a solution of 1.1 μM oxidized CcO.TX that contained 0.5 mM ferrocyanide. The reaction was inhibited by the addition of 5 mM NaCN.

RESULTS

Measurement of Internal ET. The kinetics of internal ET from heme *a* to heme a_3 can be readily distinguished from the reduction of heme *a* by changing the rate of electron entry into **O** by varying the Ru concentration (*12*, *27*, *28*). For example, at pH 8.0 and with 50 μM Ru, there is no kinetic differentiation in the anaerobic reduction of heme *a* and heme a_3 in CcO.DM (Figure 1A). The reduction is characterized by a rapid phase with rate constant k_1 of 7.0 $\pm$ 0.1 s^{-1} and a relative contribution of about 92%. The second, slower component is described by a rate constant k_2 of 2.0 $\pm$ 0.6 s^{-1}. Using an absorbance coefficient of 146 mM^{-1} cm^{-1} at 445 nm for the reduced hemes *a* plus a_3 (*25*), we estimate that the reduction of 96% CcO is observed. A 2-fold increase or decrease in the DT concentration, at constant Ru and enzyme concentrations, does not affect the reduction kinetics.

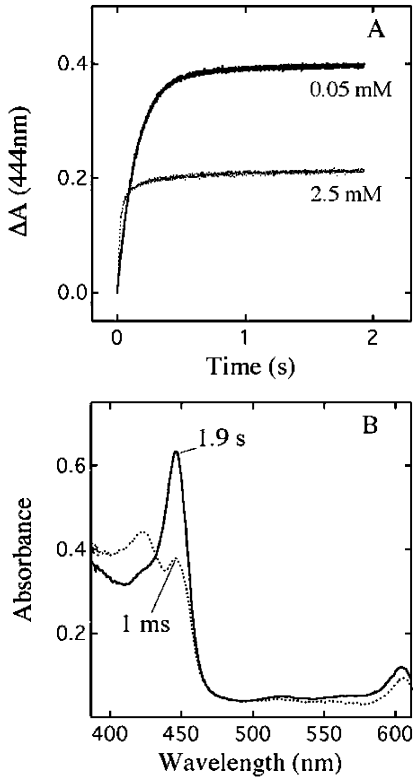

FIGURE 1: Kinetics and evolution of CcO optical spectra during anaerobic reduction. (A) Kinetics of CcO reduction by 0.05 mM and 2.5 mM Ru monitored at 444 nm. (B) Spectra of CcO collected at 1 ms and 1.9 s after mixing of CcO with 2.5 mM Ru. Measurement conditions: 2.7 μM oxidized CcO.DM was mixed in the stopped-flow with a solution of 0.1 mM or 5 mM Ru plus 10 mM dithionite. Buffer: 200 mM Hepes, pH 8.0, containing 0.05% DM, 23 °C.

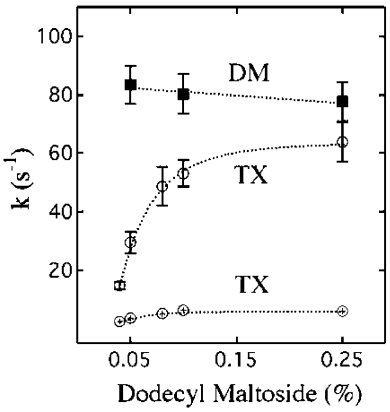

FIGURE 2: Influence of detergents on the internal electron transfer to heme a_3. Dependence of the rate constants of ET to heme a_3 for both CcO.DM and CcO.TX dissolved in buffers containing increasing concentration of DM. (■), rates for the rapid phase of ET in CcO.DM; (○), rates in the rapid and (⊕) the slow phases of ET in CcO.TX. Dashed lines: linear fit (DM) or one exponential fits used solely as guide lines (TX). Conditions: 2.4 μM CcO was reacted in the stopped-flow with 5 mM Ru plus 10 mM dithionite. Both solutions were made anaerobic as described in Experimental Procedures. Buffer: 200 mM Tris, pH 8.0, 23 °C.

Table 1: Influence of Detergents on the Rate Constant of the Rapid Phase for Heme a_3 Reduction in CcO Purified Either in Triton X-100 (CcO.TX) or in DM (CcO.DM)[a]

	k (s^{-1})	
	0.1% TX	0.05% DM
CcO.TX	1.6 ± 0.1	33.0 ± 3.7
CcO.DM	4.5 ± 0.2	83.4 ± 5.6

[a] Solutions: 200 mM Tris, pH 8.0, containing TX or DM at 23 °C.

However, heme *a* is fully reduced during the dead time of the stopped-flow apparatus, and only the reduction of heme a_3 is observed when 2.5 mM Ru is employed (Figure 1). This is demonstrated by the two spectra collected at 1 ms and 1.9 s (Figure 1B). The spectrum of CcO.DM collected at 1 ms shows maxima at 423, 446, and 604 nm, while the spectrum at 1.9 s has maxima at 446 and 604 nm and corresponds to fully reduced CcO. At 1 ms, the α-band at 604 nm is almost fully developed and the main increase in absorption, which occurs between 1 ms and 1.9 s, is in the Soret region. This spectral change is characteristic of the reduction of heme a_3 exclusively (*25*). From the observed absorption changes in the Soret region, it can be estimated that about 89% of heme a_3 has been reduced. When the same high concentration of Ru is used, Cu_A is also fully reduced within 1 ms. For these latter measurements, 20 μM CcO had to be employed because of the low absorbance of Cu_A at 830 nm (data not shown).

The kinetics of reduction of heme a_3 with 2.5 mM Ru plus 5 mM DT is a two-exponential process. At pH 8.0, the rate constants obtained from fitting to multiple experiments are 83 ± 6 s^{-1} and 10 ± 3 s^{-1} with the fast process contributing 75% to the observed absorbance changes. The amplitude of the slow component is almost independent of Ru concentration, and over concentration range 50 μM to 10 mM, varies between 10 and 25%.

Reversible Modulation of Internal ET by Detergents. It was found that ET is substantially and reversibly modulated by replacing the detergent DM with TX (Figure 2). When CcO.DM is dissolved in buffer containing various concentrations of DM, the rate of ET to heme a_3 is almost independent of detergent concentration (Figure 2). However, when stock CcO.DM is dissolved in the same buffer containing 0.1% TX in place of DM, the rate of ET to heme a_3 is decreased about 20-fold (Table 1). In this reaction, the rate constants for the rapid and slow phases of ET are 5.0 ± 0.2 s^{-1} and 0.5 ± 0.1 s^{-1}, respectively. Substituting 200 mM Tris (pH 8.0) for the Hepes buffer led to the same rates.

For CcO isolated in TX (CcO.TX) and dissolved in TX-containing buffer, the rate constants for ET to heme a_3 are 2.0 ± 0.1 s^{-1} and 0.30 ± 0.03 s^{-1} at pH 8.0. When CcO.TX is dissolved in a buffer containing increasing amounts of DM, the rate of ET increases and the rate constants for the rapid and slow phases approach limiting values of 64 ± 7 s^{-1} and 6.0 ± 0.7 s^{-1}, respectively, at ca. 0.25% DM (Figure 2). The modulation of the rate constants by TX and DM for the rapid phase of ET to heme a_3 is summarized in Table 1.

The decreased rate of ET to heme a_3 observed for CcO.TX in TX-containing buffers is reflected in a decrease in the steady-state activity. However, we would like to demonstrate that the slow turnover of CcO.TX can be stimulated by hydrogen peroxide in the similar manner as it was observed for the mutants with the impaired proton transfer in the K channel (*26*). As shown in Figure 3, CcO.TX does not oxidize ferrocyanide at any measurable rate at pH 7.0. Addition of H_2O_2 initiates the oxidation of ferrocyanide, which is fully inhibited by cyanide. The turnover rate under these conditions is 0.3 s^{-1} and is limited presumably by the

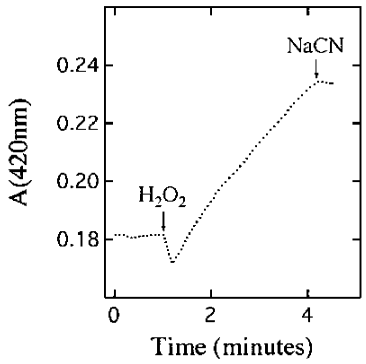

FIGURE 3: Stimulation of turnover of CcO.TX by hydrogen peroxide. The electron donor is ferrocyanide, and accumulation of ferricyanide is monitored as an absorbance increase at 420 nm. The sample contained 1.1 μM CcO.TX in 50 mM Mops−KOH buffer, 0.5 mM EDTA, 0.5 mM ferrocyanide, and 0.1% TX, pH 7.0. As indicated by arrows, 5 mM H_2O_2 and 5 mM NaCN were added to initiate or inhibit the oxidation of ferrocyanide. Temperature, 23 °C.

rate of electron transfer from the donor, ferrocyanide, to CcO (*26*).

Dependence of Internal ET on pH. The effect of detergents on internal ET for the enzyme in the **O** state is reversible. The rapid transfer observed for CcO.DM can be decreased by TX, and conversely, slow ET in CcO.TX can be accelerated by DM. This modulation of ET by detergents allowed us a more complete characterization of the possible role of protons. With increasing proton concentration, the rate of reduction of CcO increases (Figure 4A). For CcO.DM, the dependence of the logarithm of the rate constant observed in rapid phase on pH is linear with a slope of −0.85 over the pH range 8−10 (Figure 4B). The rates below pH 8.0 were too fast to measure reliably, and we consequently employed CcO.TX to obtain the rate of ET over a wider pH range. The dependence of log *k* on pH for CcO.TX is also linear above pH 7.0 with a slope of −0.94. However, below pH 7.0, the rate appears to reach a limiting value (Figure 4B). At pH 5.8, this limiting value remains the same even when higher concentrations of 5 mM Ru or 20 mM DT were used. A fit of the dependence of log *k* on pH, assuming that the deprotonation of a single protolytic group abolishes internal ET to heme a_3, yields a pK_a of 6.5 ± 0.2 (Figure 4B).

Thus far, the rates of internal ET were determined for the **O** state of both CcO.DM and CcO.TX, (Figure 4) when the enzyme, as isolated, was mixed anaerobically with excess reductant. However, we have also compared ET to heme a_3 for CcO in both the **O** and $\mathbf{O}_H$ states. The measurements at pH 8.0 and 9.0 showed that the rates and amplitudes of the fast and slow phases are almost identical for the two forms of oxidized CcO. The observed rate constants for the rapid phase of ET in the **O** and $\mathbf{O}_H$ states of CcO.DM are presented in Table 2.

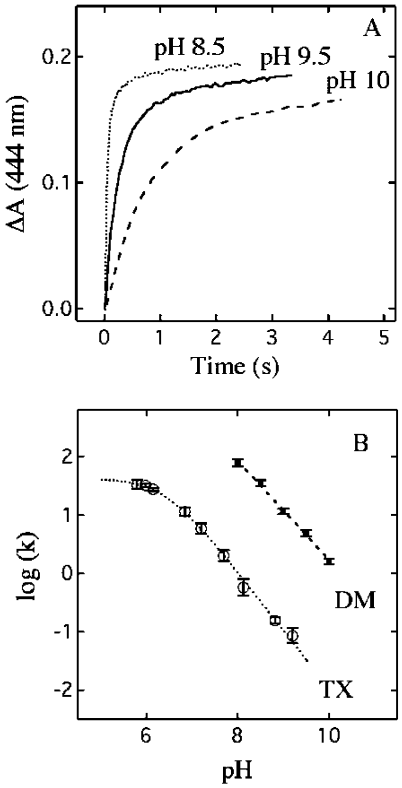

FIGURE 4: The pH dependence of kinetics and rate constants of electron transfer to heme a_3. (A) Effect of pH on kinetics of heme a_3 reduction for CcO.DM dissolved in buffers containing 0.05% DM. (B) The pH dependence of the rate constants for the rapid phase of heme a_3 reduction for CcO.DM in buffer containing 0.05% DM (DM) and CcO.TX dissolved in buffer with 0.1% TX. Broken lines: linear fit (DM) or fit to a group with pK_a = 6.5 controlling ET to heme a_3 (TX). The kinetics were measured in the anaerobic conditions described in Figure 2. Buffers (200 mM) were used for each pH: Mes between pH 5.8 and 6.7, Hepes between pH 7.0 and 8.0, Tris between pH 8.0 and 8.5, and Ches in the pH range from 9.0 to 10.0.

Table 2: The Rate Constants for Heme a_3 Reduction during the Rapid Phase for CcO.DM in the **O** and $\mathbf{O}_H$ States at Two pH Values[a]

	k (s^{-1})	
	CcO as-isolated (**O**)	CcO after reoxidation ($\mathbf{O}_H$)
pH 8.0	83.4 ± 5.6 (81%)	77.8 ± 4.9 (89%)
pH 9.0	7.1 ± 0.2 (74%)	7.0 ± 0.2 (84%)

[a] The relative contribution of the rapid phase to the observed spectral change is in parentheses.

DISCUSSION

*Kinetic Equivalence of **O** and $\mathbf{O}_H$ States.* It is recognized that the purification protocols used for isolation of CcO from bovine heart can produce enzyme with a modified catalytic center (*29*−*32*). CcO isolated by some of these methods reacts slowly with external ligands, the reduction of the catalytic site is slow, and enzyme can be transiently activated by reoxidation (*29*, *30*, *32*). However, there are now at least three methods available for the isolation of enzyme with unaltered catalytic site (*33*−*35*). Enzyme purified by these three methods reacts rapidly and homogeneously with ligands such as cyanide (*33*−*35*) and has been called the fast form (*33*). Consequently, the term "as-isolated" or "resting" CcO (**O**) does not necessarily represent one state of oxidized enzyme, as would be desirable, and is dependent on the method of purification. Our data for the fast form show that the kinetics of ET to heme a_3 in **O** state and in the recently reoxidized CcO ($\mathbf{O}_H$) are basically the same at pHs 8.0 and 9.0 (Table 2). Thus, it appears that the catalytic site in these two forms of the enzyme are in a similar, if not identical, state and that the enzyme, when isolated in the fast form, is not activated by reoxidation.

Origin of Two Phases of ET to Heme a_3. Under conditions of high concentration of Ru (e.g., 2.5 mM), heme *a* and Cu_A are fully reduced within the dead time of the stopped-flow apparatus and only internal ET to the binuclear center can be observed. With both heme *a* and Cu_A, reduced ET to heme a_3 occurs in two phases. Both phases exhibit the same response to a change of detergent and to a change of pH. Since the catalytic site consists of two redox centers, heme a_3 and Cu_B, it has been suggested that the biphasic reduction reflects the dependence of ET to heme a_3 on the redox state of Cu_B (*12*). In this situation, the fast phase (about 75%) might then represent ET to heme a_3 in that population of CcO with both heme a_3 and Cu_B oxidized (Fe_{a3}^{3+} Cu_B^{2+}). In the population of CcO with Cu_B reduced (Fe_{a3}^{3+} Cu_B^{+}), the rate of ET to heme a_3 would be less, a consequence of a postulated interaction between these two centers (*36*).

However, as previously noted and confirmed by us, the changes in the optical spectrum of heme a_3 during the reduction in both the rapid and slow phase are identical (*37*). Were the slow phase to represent population of CcO with reduced Cu_B, one would expect that the redox state of Cu_B site would influence the optical spectrum of heme a_3, which is not the case. In addition, our preliminary data on the rate of electron entry into the catalytic site, determined by the combination of the rapid freeze-quench method and EPR spectroscopy, indicate that the reduction of Cu_B does not affect the rate of reduction of heme a_3 (Fabian, M., Antalik, M., Berka, V., Jancura, D., and Palmer, G., preliminary studies). Therefore, it seems more likely that the two phases of ET simply represent two subpopulations of CcO, possibly arising during purification.

The conclusion about heterogeneity in CcO population, which is revealed by ET to the heme a_3, is in contrast to the characteristics of the fast form of CcO. Typically, about 90% of oxidized CcO, purified by the modified method of Soulimane and Buse (*24*) in our laboratory, reacts rapidly and homogeneously with ligands such as cyanide, hydrogen peroxide, and carbon monoxide. This discrepancy may indicate that these two inhomogeneities, one revealed by the ligand binding and the second one by ET, could have different origins.

Reversible Inhibition of ET by TX. The rate of ET to heme a_3 is reversibly modulated by DM and TX, and the higher rate is observed in DM-containing buffers (Figure 2, Table 1). This result is in qualitative agreement with previous studies on the influence of detergents on oxidase activity (*38*−*41*) and the individual ET steps (*40*). However, compared with published data, the magnitude of inhibition by TX is surprisingly high. In previous work, it was reported that the detergents TX and DM only slightly affect the rate of ET to heme a_3 for enzyme in the resting state (*40*). This is in contrast with our observation that replacement of TX with DM increases the rate of ET substantially, about 40-fold at pH 8.0 (Table 1). However, the effect of TX and DM on ET in the resting form of CcO observed in the present study is comparable with the influence of detergents on enzyme activated by reoxidation *(40*).

The reversible modulation of ET to heme a_3 by the detergents TX and DM has been attributed generally to an effect of the detergent environment on the conformational flexibility of the enzyme (*40*). This proposal could explain why the rate of ET to heme a_3 observed with CcO.TX dissolved in DM-buffers approaches a limiting value as the concentration of DM is increased (Figure 2). Upon increasing the DM/TX ratio, the TX originally bound to the enzyme would be displaced by DM leading to an increase in the flexibility of the enzyme. At 0.25% DM, the displacement is essentially complete and the enzyme exhibits the maximum rate of heme a_3 reduction (Figure 2).

However, our data taken together with published results provide a more specific explanation. Namely, that Triton X-100 causes a reversible inhibition of proton transfer in the K channel. This conclusion is based on a strong analogy between the behavior of TX-inhibited CcO with that of mutants of bacterial oxidases with impaired proton transfer in the K channel. Five characteristics describing the phenotype of K-channel mutants were recently summarized as follows (*42*): (1) reduced steady-state activity, (2) impaired rate of reduction of the catalytic site prior to reaction with O_2, (3) no effect on the rate of reaction of fully reduced enzyme with O_2, (4) stimulation of turnover upon substituting H_2O_2 for O_2, and finally (5) impaired proton release coupled to electron backflow from heme a_3 to heme *a* that accompanies the photodissociation of CO from mixed-valence CcO (MV.CO). In MV.CO, only heme a_3 and Cu_B are reduced and stabilized by bound CO and two protons (*8*, *9*). Photolysis of CO lowers the apparent midpoint potential of heme a_3 causing the flow of electrons from the catalytic site to oxidized heme *a* and Cu_A.

For TX-inhibited CcO, it has been already shown that steady-state activity is low and also there is no inhibition of the reoxidation of fully reduced enzyme by O_2 (*41*). The present study demonstrates that internal ET to heme a_3 is inhibited by TX (Figure 2). Moreover, we have determined that turnover of CcO.TX is stimulated by hydrogen peroxide (Figure 3), as expected for K-channel mutants (*26*). Altogether, these four characteristics of CcO.TX are equivalent to those of K-channel mutants, which suggests that the inhibition of ET by TX is a result of the modification of proton conductivity within the K channel.

That TX could modify the rate of proton access to the catalytic site was already suggested in a recent study of single-electron reduction of the ferryl form of CcO (*43*). It was observed that exposure of CcO to high concentration of TX had a very strongly influence on the rate of ET from heme *a* to oxyferryl heme a_3; this was attributed to a modulation of proton transfer by the detergent.

The most simple explanation for the inhibition of CcO by TX is that TX, presumably bound to the enzyme surface, modifies the K channel, which consequently leads to the inhibition of proton diffusion via this channel. One possible site of interaction of TX with oxidase that might be critical for proton transfer in the K channel can be inferred from the crystal structure of bovine CcO (Brookhaven Protein Data Bank number: 1v54). In the structure of the dimer, there are four cholic acid molecules bound to each monomer. One cholate molecule is located close to the matrix side of the protein surface and is bound to subunits I and II from one monomer and subunit VIa from the other monomer. Two OH groups from this cholate are hydrogen-bonded to Glu62 and Thr63 of transmembrane helix II of subunit II. These two residues are in the proximity to the entry of the K channel, and one can speculate that TX can also bind to this site and, subsequently, modulate proton diffusion within the

channel. However, this is a very simplified picture because, as it has been shown, about 180 molecules of Triton X-100 are bound per oxidase monomer (*44*).

What Controls Internal ET? Data similar to ours on the dependence of internal ET to heme a_3 on pH were reported earlier (*12*), and it was concluded that this ET reaction is limited by the rate of proton access to the catalytic site. Assuming that ET to the catalytic site is only limited by this rate of proton transfer, then the slope of the dependence of log k versus pH should be -1.0 in the pH range studied. However, for the activated $\mathbf{O}_H$ form, the slope of this dependence was found to be about -0.5 between pH 7.0 and 9.0 (*12*). The data presented in this work for the **O** state better support the theoretical expectation (Figure 4B), because between pH 8.0 and 10.0 the slope is -0.85 in the case of CcO.DM. For CcO.TX (Figure 4B), this dependence parallels that of CcO.DM and remains linear approximately until pH 7.0. At pH below 7.0, the rate asymptotes a limiting value. This dependence is unexpected and indicates that ET is under control of a single protolytic group with pK_a of 6.5 $\pm$ 0.2. It also implies that the approximately 10-fold change of rate per pH unit, observed at pH above 7.0, does not reflect a simple change of proton concentration in the solution as was assumed earlier. Instead, the rate is dependent on CcO populations having the protolytic group that undergoes a change from its protonated to deprotonated state.

A group with an essentially identical pK_a was already identified in several reactions of CcO (*11*, *45*−*47*). Thus, the rate of cyanide binding to oxidized CcO in mitochondria obeys Michaelis−Menten kinetics with respect to cyanide concentration (*45*). The pH dependence of the K_m for cyanide binding implicates a pK_a of 6.9 that was attributed to a single group interacting with heme a_3 (*45*). It has also been shown that the binding of cyanide is sensitive to pH from the matrix compartment of the mitochondrion (*48*). A pK_a of 6.6−6.9 was also established from the pH dependence of the yield of the two ferryl−oxo intermediates (**P** and **F**) at the catalytic site of CcO (*46*, *47*) that are produced by the interaction of hydrogen peroxide with oxidized CcO when incorporated into phospholipid vesicles (*46*) or present in solution containing DM (*47*). We have recently shown that the spectrum of oxidized CcO.DM in DM-containing buffers is under the influence of a single group with a pK_a of 6.6 $\pm$ 0.2 (*11*). From the extent of the spectral response to changes in pH, it was concluded that the most sensitive component is heme a_3 (*11*). Finally, a pK_a of 6.2−6.6 was also established in several earlier studies on the pH dependence of the overall catalytic activity of CcO (*49*−*52*).

Three different processes at the catalytic site, cyanide binding, the interaction with hydrogen peroxide, and the sensitivity of the optical spectrum of CcO, indicated to be under the influence of groups with nearly identical pK_a. We believe that this pK_a is the manifestation of single group and that the state of ionization of this residue controls each of the above processes. The almost identical pK_a, observed for CcO in mitochondria (*45*), for CcO isolated in different detergents (*11*, *47*, *49*, *51*), and for CcO incorporated into vesicles (*46*, *50*, *52*), shows that the pK_a of this group is practically independent of the nature of detergent environment. Moreover, from proton uptake measurements following the reduction of oxidized CcO at different pH values, we have concluded that the pK_a of this group is independent of the redox state of heme a_3−Cu_B or of the redox states of heme a or Cu_A (*11*). These data imply that the group has to be in a relative distant location from the redox centers.

A protolytic group with a basic pK_a that influences ET between heme a and a_3 when Cu_B is reduced was identified earlier (*53*, *54*). The pK_a of this group is dependent on the redox state of heme a_3, and it is assumed that this group is located in the vicinity of the catalytic site (*55*). Protonation of this residue following a pH decrease from 10.0 to 6.0 increases the rate of ET about 10-fold. This study shows that, in addition to the basic group, there is a second group that also influences ET but with distinctly different properties. This new group is acidic, its pK_a of 6.5 is not sensitive to the redox state of enzyme (*11*), and it is located in a remote location with respect to the redox centers. Nevertheless, this acidic group is responsible for an increase in the rate of ET in CcO.TX of about 3 orders of magnitude when the pH is decreased from 9.3 to 6.0 (Figure 4B).

This dramatic effect of the distant, acidic group on ET, compared to the moderate influence of the basic group located in the vicinity of the redox centers, indicates that the acidic group has to affect some process distinct from, but coupled to, ET. What is this coupled process? It is known that reduction of the catalytic site is associated with two processes: the first is proton uptake from solution (*10*, *11*) and the second is the dissociation of a native ligand(s) from the catalytic site (*17*, *21*). According to the current view, ET to heme a_3 is restricted by the rate of proton diffusion to the catalytic site (*12*, *42*, *56*). During the reductive phase, proton uptake is coincident with ET to heme a_3 (*12*) and at least the transfer of one proton occurs through the K channel (*14*). Mutations of even the distant residues in this channel profoundly influence the rate of ET to heme a_3 (*42*). These facts suggest that group with pK_a of 6.5 is a part of, or interacts with, some residue(s) in the K channel. That this residue is part of the K channel is also suggested by the study of the pH dependence of the reaction of oxidized *Rhodobacter* CcO with hydrogen peroxide (*57*), where it was found that in a mutant with impaired proton transfer in the K channel the pH dependence of formation of **P** and **F** was eliminated. On the basis of these facts, we suggest that the acidic group under consideration modulates proton transfer in the K channel.

What is the identity of this residue controlling proton transfer in the K channel? The pertinent residue has to be distant to the catalytic site because its pK_a is not affected by the redox state of the enzyme (*11*). Identification of this group as Lys319, the key residue in the K channel, can be excluded because this residue appears to be in the protonated state at pH 7−8 in oxidized enzyme (*58*). Since the pK_a of the group is 6.5, the most obvious candidate for the residue would be His256 of subunit I. His256 is located close to the entry of the proposed K channel in bovine CcO (*2*). However, mutation of the corresponding histidine in bacterial oxidases showed that this residue is not required for proton delivery to the binuclear center (*42*). Surprisingly, it was found that a glutamic acid located in subunit II and at the surface of *R. sphaeroides* oxidase (Glu101, Glu60 in bovine enzyme) is critical for proton diffusion within the K channel (*42*). Similarly, the mutation of the equivalent residue, Glu89, in cytochrome bo_3 from *Escherichia coli* resulted in inhibition of catalytic activity (*59*).

A different conclusion concerning the entrance of the K channel was drawn in a recent study of *Paracoccus denitrificans* oxidase (*37*). It was found that mutation of the corresponding glutamate residue (Glu78 in *Paracoccus denitrificans* oxidase) does not have a substantial impact on ET to heme a_3 or on the steady-state activity (*37*). This discrepancy is not yet well-understood.

Given the prevailing observations in favor of Glu60 as the entry point of the K channel, we propose that this residue is also responsible for the pH dependence of ET to heme a_3. This group could gate proton entry into the K channel and consequently to the binuclear center. When Glu60 is protonated, the gate to the K pathway is open and protons can enter the channel. Conversely, when the group is deprotonated, the gate is closed and access of protons into the channel is blocked. This assignment requires that the pK_a of Glu60 be a little more than 2 pH units higher than is the pK_a of free glutamate. As the glutamate in question is surrounded by a large number of nonpolar residues, such an increase in pK_a is not unreasonable.

Our data also show that the state of ionization of Glu60 cannot be the only factor that controls proton access to the catalytic site. At acidic pH, when Glu60 is protonated and the gate is open, an increase in proton concentration in solution does not increase the rate of ET (Figure 4B). Thus, even with an open channel, there is some additional limitation to proton transfer. We suggest that the rate of proton diffusion through the K channel is the second limiting factor. This in turn regulates ET to the catalytic site, which is faster in CcO.DM than in CcO.TX.

The idea that the group with pK_a of 6.5 controls proton diffusion in the K channel provides an explanation for the pH dependence of catalytic activity, the yield of the two ferryl−oxo intermediates, **P** and **F**, in the reaction of oxidized CcO with hydrogen peroxide, and for the cyanide binding to the catalytic site of the enzyme. On the basis of the equivalent pK_a values, determined in this study, and from the dependence of catalytic turnover on pH (*49*−*52*), we suggest that the rate-limiting step in the overall turnover of oxidase at acidic pH values is ET to heme a_3 during the reductive phase. It then follows that under these conditions proton access to the binuclear center via the K channel controls the activity of the enzyme.

An explanation for the pH-dependent yield of the **P** and **F** forms was already presented in our recent study (*11*). The critical point for this explanation is the pH dependence of the rate of decay of **F** ⇒ **O**, which is very similar to that described here for the rate of heme a_3 reduction and also indicates that this transition is under control of a single group with a pK_a of 6.8 ± 0.2 (Fabian, M., and Palmer, G. preliminary results).

Another process that suggests the involvement of the K channel is cyanide binding to oxidized CcO in mitochondria (*45*). The Michaelis−Menten kinetics of cyanide binding implies the formation of the initial transient complex, undetected by optical spectroscopy (*45*). This intermediate is converted in the following step into the spectrally distinct form with cyanide bound to heme a_3. We have suggested that, in this initial complex, cyanide is coordinated to Cu_B (*60*). Since it is the electroneutral HCN which probably enters the catalytic site (*61*, *62*) while the bound form is the CN^- anion (*63*), the reaction requires proton dissociation. However, no pH changes in the solution following cyanide binding to oxidized oxidase were observed (*64*). The following scheme takes these observations into account:

$$\underset{\mathbf{O}}{Fe_{a3}^{3+}-OH_2\ {}^{-}HO-Cu_B} + HCN \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \underset{\mathbf{I.CN}}{Fe_{a3}^{3+}-OH_2\ Cu_B-CN^-} + H_2O \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \underset{\mathbf{O.CN}}{Fe_{a3}^{3+}-CN^-\ Cu_B} + H_2O$$

where **O** represents oxidized enzyme with a water molecule ligated to heme a_3 and hydroxide anion to Cu_B, **I.CN** is a spectrally undetectable intermediate, **O.CN** is the final product of cyanide binding, and k_i and k_{-i} are the rate constants for the particular forward and reverse reactions, respectively. We suggest that formation of **I.CN** is associated with the transfer of proton, released from HCN, as a proton or water from the catalytic site through the K channel into the medium. This transfer is again under control of the residue with a pK_a of 6.5. However, to explain the dependence of K_m on pH, we also have to postulate that reverse proton diffusion from the bulk solution to the catalytic site, when cyanide is bound to Cu_B, is very weakly dependent on pH; that is, k_{-1} is insensitive to pH. This proposal is justified by two observations: (i) the K channel is selectively open and closed during the catalytic cycle (*57*, *65*), and this transition is probably triggered by the redox or ligation state of the metal centers of the binuclear center (*57*, *65*) and (ii) we have recently shown that the pH sensitivity of heme a_3 is lost in the complex of oxidized CcO with cyanide (*11*). With these two suggestions and knowing that k_{+2} is only weakly pH-dependent (*45*), the effect of pH on the Michaelis−Menten constant, $K_m = (k_{-1} + k_2)/k_1$, is reduced to the variation of k_1. Thus, the pH dependence of K_m is inversely proportional to k_1, which increases in a manner similar to that observed for the rate of ET to heme a_3 (Figure 4B).

Another process that might be a rate-limiting step for the internal ET is the displacement of ligand(s) localized between Fe_{a3} and Cu_B in oxidized CcO (*16*, *18*). We believe that this is less likely as is suggested by the reaction of oxidized CcO with an excess of hydrogen peroxide. In this reaction, not only is the bridging ligand displaced but the catalytic site is structurally modified since Fe_{a3}^{3+} is converted to the ferryl−oxo state (*66*). If the displacement of ligand were rate-limiting then, at any given pH and temperature, the rate of ligand displacement should not have exceeded the rate of ET. However, our observations contradict this expectation. In the reaction of CcO.TX (in TX buffer) and CcO.DM (in DM buffer) with 25 mM H_2O_2 (pH 8.0, 23 °C), the rate constants, obtained from single-exponential fits, were 14 ± 2 s^{-1} and 14 ± 1 s^{-1}, respectively. Since both rates of ligand displacements are the same and the rate of reaction of CcO.TX with peroxide is about 10-fold faster than the rate of heme a_3 reduction (Table 1), it would appear that ligand displacement is not a limiting step for ET.

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