Proton Involvement in the Transition from the "Peroxy" to the Ferryl Intermediate of Cytochrome *c* Oxidase†

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ABSTRACT: In the absence of any external electron donor, the "peroxy" intermediate of cytochrome *c* oxidase (CcO-607) is converted to the ferryl form (CcO-580) and subsequently to oxidized enzyme. The rate of conversion of CcO-607 to the CcO-580 form is pH dependent between pH 3.0 and pH 7.6. A plot of the logarithm of the rate constant for this conversion is a linear function of pH with a slope of −0.92, implying the involvement of a single proton in the transition. Upon rapidly lowering the pH from 8.1 to 5.8, the uptake of one proton was observed by direct pH measurement, and the kinetics of proton uptake coincide with the spectral conversion of CcO-607 to CcO-580. We interpret the slow endogenous decay of CcO-607 to CcO-580 to be the result of proton transfer to a deprotonated group generated in the binuclear cavity during CcO-607 formation. This group is not freely accessible to protons from the medium, and its pK_a is probably higher than 9.0.

The membrane-bound terminal respiratory oxidases catalyze the reduction of molecular oxygen to water associated with the generation of a transmembrane proton gradient. Mitochondrial cytochrome *c* oxidase (CcO)[1] belongs to the so-called super-family of heme−copper oxidases (*1*−*3*). Members of this family have a unique bimetallic center composed of heme and a copper ion where oxygen is reduced and high-affinity ligands are bound. Despite differences in reactivity among the individual oxidases, the members of this family show substantial structural and functional homology.

Four redox centers are involved in the electron-transfer reactions. Cu_A and cytochrome *a* serve to deliver electrons from reduced cytochrome *c* to the binuclear center composed of cytochrome a_3 and Cu_B. It is at this binuclear center that dioxygen is reduced to water and most external ligands are bound.

Complete reduction of dioxygen to water requires four electrons, and this reaction is coupled to the uptake of protons from the mitochondrial matrix. Four protons are used for the formation of water (substrate protons), and four more are translocated ("pumped") from the mitochondrial matrix to the cytosolic side of the mitochondrial inner membrane. On average, each electron transferred to dioxygen by cytochrome oxidase is associated with the transmembrane transfer of two elementary positive charges. However, examination of the intermediates of the catalytic cycle shows that not each electron participates equally in generating the transmembrane electrochemical potential (*4*). Two of these intermediates, namely, the "peroxy" and "ferryl" forms, are of particular interest because they appear to be of particular relevance to the process of proton pumping (*5*).

Because the product of the reaction of two-electron-reduced enzyme with dioxygen is formally at the level of a "peroxy" intermediate, it was initially expected to have an intact O−O bond (e.g., $Fe_a^{3+}Cu_A^{2+}Fe_{a3}^{3+}$−O−O−$Cu_B^{2+}$). The "peroxy" intermediate would require two electrons to complete the conversion of dioxygen to water and to regenerate oxidized enzyme. One-electron reduction of this "peroxy" form produces the ferryl intermediate ($Fe_a^{3+}Cu_A^{2+}$-Fe_{a3}^{4+}═O HO−Cu_B^{2+}), and the fourth electron completes the conversion of the ferryl form to oxidized enzyme (*6*−*20*). It is generally accepted in the ferryl form that the dioxygen bond is broken and the iron of heme a_3 is in the oxo−ferryl state (Fe_{a3}^{IV}═O) with hydroxide/water possibly coordinated to Cu_B^{2+} (*7*−*20*). However, accumulated spectroscopic and chemical data on the "peroxy" intermediate make it clear that the dioxygen bond is already broken and that the iron of heme a_3 is also in the oxo−ferryl state (*21*−*25*). This conclusion is supported by our observation that close to one electron is sufficient for conversion of the "peroxy" form to the oxidized enzyme, which also indicates that both forms of the enzyme are essentially in the same redox state (*26*).

Originally the "peroxy" and ferryl intermediates of cytochrome *c* oxidase were observed in mitochondria under conditions of reversed electron flow (*27*−*29*). The "peroxy" intermediate was characterized by a maximum at 607 nm and a red-shifted Soret band in the optical difference

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[1] Abbreviations: CcO, cytochrome *c* oxidase; CcO-O, oxidized cytochrome *c* oxidase; CcO•CN, complex of oxidized CcO with cyanide; MV•CO ($a^{3+}Cu_A^{2+}a_3^{2+}$CO−Cu_B^{+}), mixed valence carbon monoxide complex in which cytochrome *a* and Cu_A are oxidized and cytochrome a_3 and Cu_B are reduced and ligated by CO; P (CcO-607), "peroxy" intermediate; F (CcO-580), ferryl intermediate; apNADH, reduced 3-acetylpyridine adenine dinucleotide; PMS, oxidized phenazine methosulfate; DM, *n*-dodecyl-*β*-D-maltoside; Tris, tris(hydoxymethyl)aminomethane; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; Hepes, *N*-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Ches, 2-(cyclohexylamino)ethanesulfonic acid.

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spectrum referenced to oxidized enzyme (*27*−*29*). Identical optical difference spectra were observed for: (i) an intermediate, designated as P_M, created in the reaction of two-electron-reduced enzyme with dioxygen (*30*−*34*); (ii) an intermediate (P_H) prepared by the addition of a stoichiometric quantity or a small excess of H_2O_2 to oxidized enzyme at basic pH (*35*−*40*); and (iii) a transient intermediate (P_R) observed during the reaction of fully (four-electron) reduced oxidase with dioxygen (*17*, *18*). Moreover, besides the identical optical characteristics, all three intermediates (P_M, P_H, and P_R) have the same oxygen-sensitive Raman bands (*14* , *21*−*24*, *41*), an indication that there might be but a single species.

The ferryl form is characterized by an absorbance maximum around 580 nm and a red-shifted Soret band in the optical difference spectrum recorded with respect to oxidized enzyme (*13*, *18*, *27*−*29*). Similar if not identical species are prepared by the interaction of oxidized enzyme with stoichiometric or low amounts of H_2O_2 at acidic pH (*35*−*40*) or by the addition of excess of hydrogen peroxide to oxidized enzyme (*35*−*40*). For the remainder of this report, we will use the designation CcO-607 for the "peroxy" form and CcO-580 for the ferryl form.

It thus appears that there are two ferryl forms of oxidase, CcO-607 and CcO-580, but these two forms have markedly different optical characteristics. Recently it was shown that lowering the pH of CcO-607 induces the conversion to CcO-580 (*40*). We have studied this conversion in more detail and found that this transition to CcO-580 is controlled by the uptake of one proton from the medium and that this conversion does not require any external electron donor. Our data suggest that the group involved in the proton uptake has a pK_a that is higher than 9.0 and that it is probably located in vicinity of the binuclear center when the enzyme is in the CcO-607 state.

MATERIALS AND METHODS

Tris, Bicine, Hepes, catalase, reduced 3-acetylpyridine adenine dinucleotide (apNADH), and phenazine methosulfate (PMS) were purchased from Sigma, Mes and Ches from Aldrich, dodecyl maltoside (DM) from Anatrace, and Triton X-100 (peroxide-free) from Boehringer.

Cytochrome *c* oxidase (CcO) was isolated using Triton X-100 by the method of Soulimane and Buse (*42*) with minor modifications (*43*). Enzyme concentration was determined at pH 8.0 from the absorbance of oxidized enzyme at 424 nm by using $A = 158\ mM^{-1}\ cm^{-1}$.

CcO-580 was prepared at pH 5.9 (10 mM Mes, 0.1% DM, 100 mM K_2SO_4) by the addition of 20 μM H_2O_2 to 5.0 μM CcO-O. The optical difference spectrum of CcO-580 *minus* CcO-O is pH dependent, and at acidic pH the maximum is at 575 nm compared to 578−580 nm at pH 8.0 (*36*). The conversion of CcO-O to CcO-580 was followed in a spectrophotometer by monitoring the absorption increase at 574 *minus* 630 nm. The maximum yield of CcO-580 was observed in about 6 min, and the concentration was determined from the optical difference spectrum of peroxide-treated CcO minus CcO-O using $\Delta A_{575-630} = 5.3\ mM^{-1}\ cm^{-1}$ (*29*).

The CcO-607 form of CcO was prepared from the oxidized enzyme (CcO-O) dissolved in 10 mM Tris buffer, pH 8.0, containing 0.1% DM and 100 mM K_2SO_4 in the presence of 150 units of catalase; this enzyme solution was flushed with a gentle flow of carbon monoxide (CO) for about 10−30 s at room temperature in an open spectrophotometer cuvette. The conversion of the enzyme to CcO-607 was followed via the increase in the differential absorbance between 607 and 630 nm referenced to oxidized enzyme. The maximum conversion to CcO-607 (92%) was reached in about 4 min. The sample was then outgassed several times on ice to replace residual CO with argon because the continued presence of CO accelerates the decay of CcO-607. The concentration of CcO-607 was determined from the difference spectrum of CcO-607 minus CcO-O using $\Delta A_{607-630} = 11\ mM^{-1}\ cm^{-1}$ (*29*).

To measure the rate of endogenous conversion of CcO-607 to CcO-58, we have used two methods. At pH values above pH 5.0, it was sufficient to use the standard optical spectrophotometer, but at pH values below pH 5.0, the conversion was rapid and it was necessary to use a stopped-flow instrument. The conversion was measured as the difference spectral change ΔA(606−630 nm) in the manual spectrometer and as a absorption change at a single wavelength ΔA(607 nm) when using the stopped-flow instrument.

The conversion of CcO-607 to CcO-580 at different pH values was measured as follows. CcO-607 was prepared in a weak buffer at pH 8.0 (10 mM Tris, pH 8.0, containing 100 mM K_2SO_4 and 0.1% DM); this pH was chosen because it provides both the largest yield and the highest stability of CcO-607. The reaction was started by mixing equal volumes of CcO-607 and a strong buffer of the desired final pH (200 mM buffer, containing 100 mM K_2SO_4, 0.1% DM, and 150 units of catalase); the pH of the sample was also verified at the end of reaction. To cover the pH range between pH 3 and 9.0, the following buffers were used: Ches (above pH 8.5), Bicine or Tris (pH 8−8.5), Hepes (pH 7−7.7), Mes (pH 5.6−6.6), and citric acid−NaOH (below pH 5.5). All spectral measurements were performed at 23 °C.

Below pH 5.0, oxidized cytochrome oxidase is not stable and is denatured at a rate that increases with decreasing pH with an associated spectral change at 607 nm. However, this contribution can be subtracted from the acid-induced decay of the CcO-607 form because (i) the absorption changes of CcO-O at 607 nm can be fit by a single exponential; and (ii) the contribution of CcO-O to the spectral change at 607 nm at acidic pH is always less than 35% of the change observed with CcO-607. Because the rate of spectral change of CcO-O at pH 3.0 is the same as the rate of decay of CcO-607, we limited our experiments to pH values of 3.0 and greater.

The relaxation of CcO-607 to CcO-580 is followed by the slower conversion of CcO-580 to a form that has an optical spectrum that corresponds to the oxidized enzyme. To test if this final product and the initial oxidized enzyme are in the same redox state, we have determined the number of electron equivalents required for the complete reduction of each form. For this measurement, the initial 8 μM CcO-O at pH 8.0 was divided into two aliquots. The first aliquot was converted to CcO-607 and then mixed with a strong buffer at pH 7.0 as was described above for the kinetic measurement. The CcO-607 sample was then incubated at room temperature in the dark for about 4 h, at which time the optical spectrum was that of oxidized enzyme. The

second aliquot was incubated at pH 7.0 for the same time and under the same conditions as the sample of CcO-607. At the end of the incubation, each sample was made anaerobic in a Thunberg-style optical cell by the gas-exchange technique (*26*) and placed under argon. The anaerobic samples were reduced with a 5-fold molar excess of apNADH in the presence of a small amount of PMS. First apNADH was added from one sidearm of the optical cell followed by the addition of PMS from the second sidearm. The kinetics of oxidation of apNADH and reduction of enzymes were followed simultaneously by optical spectrophotometry. At the end of reaction, the quantity of reduced enzyme and residual apNADH was calculated from the difference optical spectrum using $\Delta A_{606} = 23.2\ mM^{-1}\ cm^{-1}$ for the reduced minus oxidized absorbance change due to the enzyme (*44*) and $A = 9.1\ mM^{-1}\ cm^{-1}$ for the residual apNADH at 364 nm (*45*); the latter had to be corrected for the absorbance change due to reduction of the enzyme using the absorption coefficient $\Delta A_{364} = -4.5\ mM^{-1}\ cm^{-1}$.

Stopped-flow measurements were performed using a Kinetic Instrument apparatus with 2 cm path length observation cell. The kinetic data were analyzed using OLIS stopped-flow software. Optical spectra and kinetics were measured in a Hewlett-Packard Diode Array 8452A spectrophotometer in a thermostated cell holder.

To demonstrate that the acid-induced conversion of CcO-607 to the CcO-580 form is coupled to proton uptake from the medium, we have directly measured the pH changes in the sample using a glass electrode (response time less than 10 s). The most favorable pH for these proton uptake measurements is at pH 5.8 because (i) enzyme is stable at this pH; and (ii) the conversion of CcO-607 to CcO-580 is about 10 times faster than the subsequent conversion of CcO-580 to CcO-O. As the CcO-607 to CcO-580 transition is complete in about 4 min, it is possible to minimize any drift in pH.

The experiment was carried out as follows: CcO-607 was prepared from 53 μM oxidized enzyme at pH 8.1 in 5 mM Tris, 100 mM K_2SO_4, 0.1% DM with catalase as was described above. Then 0.2 mL of this enzyme solution was added to 1.8 mL of weakly buffered medium (0.5 mM Tris, 0.1 mM Mes, pH 8.1, 100 mM K_2SO_4, 0.1% DM), thermostated at 20 °C, with constant stirring under a flow of ultrahigh purity argon. The pH of the enzyme solution was then decreased to approximately 5.8 by one addition of 23 μL of 50 mM HCl. At the end of each measurement, a defined amount of NaOH and HCl (final concentration 5 μM) was added to the sample to calibrate the observed pH changes at pH 5.8.

Identical pH jump measurements were made for oxidized enzyme and its cyanide complex (CcO·CN). From the three dependencies of pH versus time for CcO-607, CcO-O, and CcO·CN, we have calculated the difference pH kinetics of CcO-607 *minus* CcO-O and CcO·CN *minus* CcO-O. Time zero was taken as the moment when 50 mM HCl was added and the pH was rapidly lowered. The data are presented 30 s after HCl addition because in the time interval from 0 to 30 s the calculated data were very noisy (Figure 2).

The total enzyme concentration in the pH measuring cell was identical for all samples and equal to 5.3 μM. In the CcO-607 sample, the initial concentration was 4.7 μM CcO-607 and 0.6 μM CcO-O. For pH measurements, all solutions and samples were thoroughly degassed to remove carbon dioxide. The cyanide complex was prepared by incubation of stock solution of CcO-O (287 μM) with 300 μM cyanide for 3 h at room temperature. With this protocol, there was almost quantitative formation of CcO-CN, and there is no interference from any free cyanide in the solution. As judged by optical spectra, the concentration of CcO·CN is not changed by acidification of the medium.

The pH changes were recorded using a Denver Instruments model 225 pH meter interfaced to a Macintosh laptop computer, and the software capabilities of IgorPro (Wavemetrics, Lake Oswego, OR) were used to collect data at 1 s intervals.

RESULTS

In the absence of an external electron donor, the CcO-607 intermediate of cytochrome *c* oxidase is not stable but slowly decays back to a species with an optical spectrum that corresponds to the initial oxidized enzyme. These two oxidized forms of CcO are in the same redox state because the number of equivalents of apNADH required for the full reduction of CcO-O or relaxed CcO-607 at pH 7.0 were found to be 1.74 ± 0.17 and 1.83 ± 0.06 (SD), respectively. Because apNADH is a two-electron donor, it follows that both forms of the enzyme require approximately four electrons for full reduction. It does appear that there is a slight loss of enzyme following the endogenous conversion of CcO-607 to oxidized enzyme because an optical difference spectrum between the two species reveals a slight decrease of absorption at 418−420 nm in relaxed CcO-607; the extent of this decrease is about 3% of the Soret band intensity.

When CcO-607 is prepared in a weakly buffered medium at pH 8.0 and subsequently acidified to pH 5.8, the decay of CcO-607 to CcO-O is biphasic (Figure 1A,B), and these two phases are kinetically well separated. Fifteen seconds after the pH is lowered, the CcO-607 form is still the major contributor to the optical difference spectrum (Figure 1A) with the characteristic sharp maximum at 607 nm. Four minutes later, the peak at 607 nm is almost completely lost, and the major form is CcO-580 with an absorption maximum at 574 nm (Figure 1A). The clear demonstration of the conversion of CcO-607 to CcO-580 at acidic pH is a consequence of the faster rate of CcO-580 formation compared to its slower decay (see below) to CcO-O. In the Soret region, the positions of the extrema in the difference spectrum are almost the same for both forms, as is well documented at neutral pH values (*35*−*40*).

The rate of conversion of CcO-607 to CcO-580 at acidic pH is not influenced by the presence of 10 mM ferricyanide, by omitting K_2SO_4, by the substitution of Hepes for Tris, or by the concentration of CcO-607. For example, we have observed at pH 6.0 for 3.95 and 39.3 μM CcO-607 conversion rates of $6.4 \times 10^{-3}\ s^{-1}$ and $6.9 \times 10^{-3}\ s^{-1}$, respectively.

The kinetics of the decay of CcO-607 and the formation of CcO-580 at pH 5.8 were followed by measuring the changes in the absorption differences at 606 *minus* 630 nm and at 574 *minus* 630 nm, respectively (Figure 1B). At this pH, the decay of CcO-607 can be fit with a single exponential using a rate constant of $1.1 \times 10^{-2}\ s^{-1}$. The kinetic behavior of CcO-580 is, however, biphasic with an initial increase

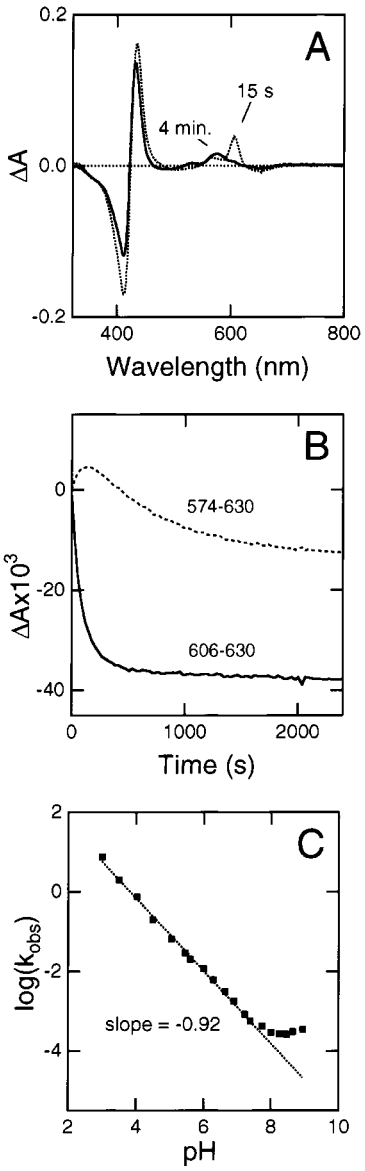


FIGURE 1: Effect of pH on the optical spectrum and kinetics of endogenous decay of the "peroxy" intermediate. 5 μM CcO-607 in weakly buffered medium at pH 8.0 was acidified to pH 5.8 as described under Materials and Methods. (A) The difference spectra recorded 15 s (- - -) and 4 min (—) after lowering the pH. The reference spectrum is oxidized enzyme obtained from the CcO-607 after 40 min incubation at pH 5.8. (B) The kinetics of absorption changes at 606−630 nm (—) and at 574−630 nm (- - -) reflecting changes in the concentration of CcO-607 and CcO-580, respectively. (C) Dependence of the logarithm of the rate of endogenous decay of CcO-607 on pH: (■) experimental values; (- - -) best linear fit between pH 3.0 and pH 7.4; the slope is −0.92.

followed by a slower decrease, and the data are very well fit using two opposed exponentials. The initial increase, corresponding to CcO-580 formation, has a rate constant of 1.1×10^{-2} s^{-1}, precisely the same as that for the decay of CcO-607; the slower decrease corresponds to the decay of CcO-580 to CcO-O and could be fit using a rate constant of 1.2×10^{-3} s^{-1}.

The conversion of CcO-607 to CcO-580 accelerated by acidification of medium cannot be reversed by restoring the initial pH. For example, CcO-580 prepared at pH 5.9 by addition of hydrogen peroxide followed by the addition of catalase to remove the residual peroxide was shifted to pH 8.0 by addition of concentrated Tris buffer. There was no detectable conversion of CcO-580 back to CcO-607; rather CcO-580 reverted to oxidized enzyme slowly with time.

The rates of decay of CcO-607 were measured at a number of pH values in the range from pH 3.0 to 9.0 and the data plotted as log k_{obs} versus pH (Figure 1C). Below pH 7.6, the plot is linear with a slope of −0.92. At pH values greater than pH 7.6, the data deviate from linearity, reaching an apparent minimum at about pH 8.3 with a rate constant of 2.7×10^{-4} s^{-1} and possibly increasing at higher pH.

We have noticed, however, that at alkaline pH values the enzyme undergoes a slow autoreduction and the rate of this autoreduction increases with increasing pH. For example, when the oxidized enzyme is incubated anaerobically at 23 °C for about 2 h at pH 8.0, the product was a mixture of CcO-580 and partially reduced enzyme; the presence of the former species is attributed to a slow leak of oxygen into the anaerobic cell and reacting with partially reduced oxidase produced during the long incubation. Using the absorbance difference ΔA(416 − 382 nm), we have determined the overall rate of conversion of oxidized cytochrome a_3 to the mixture of reduced and ferryl states to be approximately 1.9×10^{-4} s^{-1}. This value provides a minimum estimate of the true rate of reduction because at least three electrons are required to make CcO-580. However, even with this uncertainty, it is possible to conclude that the rates of decay of CcO-607 and autoreduction of CcO-O are very similar at pH 8, and we thus interpret the deviation in linearity above pH 7.6 (Figure 1C) as reflecting autoreduction of the enzyme which promotes the decay of CcO-607. At pH values higher than 8.0, the rate of autoreduction slowly increases and is approximately equal to the observed slow increase in the rate of decay of CcO-607.

The dependence of the decay rate on pH implies that one proton from the medium is consumed during the conversion of CcO-607 to CcO-580. Consequently we have examined whether the conversion of CcO-607 to CcO-580 at pH 5.8 is associated with the uptake of a proton as gauged by direct measurement of pH. As controls, identical experiments were also conducted with CcO-O and CcO·CN. The data are presented as the changes in pH observed with CcO-607 *minus* those recorded with CcO-O and CcO-CN *minus* CcO-O (Figure 2). The pH changes observed with CcO·CN *minus* CcO-O show a slight pH decrease with time that we attribute to a drift in the system. However, the time dependence of the pH changes between CcO-607 *minus* CcO-O shows a pH increase, implying the uptake of a proton. The presented pH kinetics take about 8 min, and during this time, CcO-607 is converted to CcO-580 together with some decay of CcO-580 to CcO-O. Superimposed on the pH data is a theoretical curve consisting of the sum of two exponentials generated using rate constants obtained from fitting parallel optical measurements. If we assume that the conversion of CcO-580 to CcO-O consumes one proton, then the average proton consumption for the endogenous decay of CcO-607 to CcO-580 calculated from the three measurements is 1.04 ± 0.36 (SD).

The data above show that the conversion of CcO-607 to CcO-580 at acidic pH is accompanied by proton uptake. However, the CcO-580 form can also be prepared at acidic pH by the addition of a stoichiometric amount of hydrogen peroxide to oxidized enzyme (*36*). Therefore, we have examined whether a proton is consumed from the medium

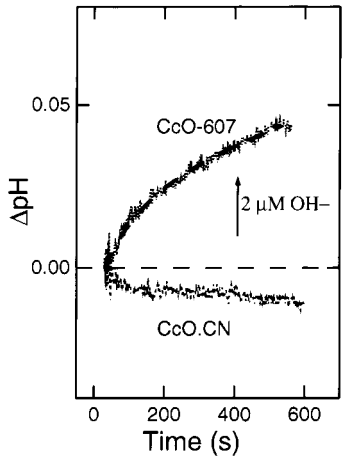


FIGURE 2: Kinetics of the change in pH of CcO-607 *minus* CcO-O and of CcO·CN *minus* CcO-O observed after lowering the pH from 8.1 to 5.8. At time zero, a small volume of dilute hydrochloric acid was added to CcO-607 prepared at pH 8.1; the final pH was 5.8. CcO-607, gray dotted line: Time dependence of the difference in the change in pH of CcO-607 with respect to CcO-O. Overlaid black dashed line: A two-exponential fit using the rate constants obtained from fitting the absorbance changes in a parallel optical measurement of the conversion of CcO-607 to CcO-580 (1.1×10^{-2} s^{-1}) and CcO-580 to CcO-O (1.1×10^{-3} s^{-1}). CcO·CN, gray dotted line: Time dependence of difference in the change in pH of CcO·CN with respect to CcO-O. The pH changes are presented relative to pH 5.8 (- - -). In all samples, enzyme (5.3 μM) was dissolved in a medium of 0.5 mM Tris, 0.1 mM Mes, 100 mM K_2SO_4, 150 units of catalase plus 0.1% DM.

during this reaction. Our measurements showed that there is no pH change in the medium when 5.4 μM CcO-O reacts with 5 μM H_2O_2 at pH 5.4 with the formation of 2.4 μM CcO-580.

DISCUSSION

The "peroxy" intermediate, CcO-607, the reaction product of two-electron-reduced oxidase with molecular oxygen, appears to decay to oxidized enzyme in a biphasic manner even in the absence of an external electron donor. These two phases are kinetically very well resolved at acidic pH (Figure 1A,B). As the same rates are observed at pH 5.8 for both the decay of CcO-607 and the formation of CcO-580, it would appear that the first phase comprises the conversion of CcO-607 to the CcO-580 form. In the second phase, CcO-580 is converted to oxidized enzyme with a rate that is an order of magnitude slower. This sequential conversion, CcO-607 → CcO-580 → CcO-O, can be discriminated at pH values below pH 7.6; above this pH, the rate of conversion of CcO-580 → CcO-O is presumably faster than that of CcO-607 → CcO-580. However, we assume that, at all pH values, the measured decay rate of CcO-607 measured at 607 nm is also the rate of conversion of CcO-607 → CcO-580.

The rate constant for the endogenous decay of the CcO-607 form is pH dependent (Figure 1C) with the rate increasing as the pH is lowered. The dependence of rate constant on pH between pH 3.0 and 7.6 is linear, and the slope of −0.92 implies the involvement of one proton in the transition of CcO-607 to CcO-580. At pH values higher than 7.6, the data deviate from linearity and give the impression that there is a group with a pK_a of around 7.6 that controls the decay rate. However, we discount this explanation for two reasons. First, we found that above neutral pH the enzyme undergoes autoreduction with a rate similar to that of the decay of CcO-607 and the rate of autoreduction increases slightly as the pH is raised; the autoreduction would accelerate the decay of CcO-607, resulting in the deviation from what would otherwise be a linear dependence of log(k_{obs}) on pH. Second, if CcO-607 were in equilibrium with some protonated species and this latter species is responsible for the decomposition, then the shape of the graph would differ dramatically from that observed by asymptoting to a limiting maximum value at acidic pH rather than a minimum value at alkaline pH as is observed. We thus conclude that the dependence of log(k_{obs}) on pH would be intrinsically linear with a slope close to −1 over the whole pH region between pH 3.0 and 9.0 were there no autoreduction of the enzyme.

It might be argued that the endogenous decay of CcO-607 to CcO-580 could have its origin in some intermolecular redox exchange reaction between partially reduced enzyme molecules. However, we believe that this is not the case because the decay rate is not dependent on the initial concentration of CcO-607 nor is it affected by the presence of ferricyanide at acidic pH.

The CcO-607 intermediate was prepared using carbon monoxide which selectively reduces the binuclear center with two electrons. The intermediate is a product of the reaction between this partially reduced enzyme and dioxygen, and at this stage, the dioxygen bond is already cleaved (*21*−*24*, *41*) by a reaction that requires the availability of four electrons. Three electrons can be provided by the two metal ions present at the binuclear center by oxidizing the iron of heme a_3 and Cu_B to the ferryl and cupric states, respectively. A plausible candidate for the source of the fourth electron is Tyr 244 (*46*, *47*) which is oxidized to the radical form. It follows that the initial redox state of CcO-607 is 2 electron-equiv above oxidized enzyme. However, this redox state is unstable even in the absence of external electron donors. We have found that the putative radical is reduced faster than is the oxo−ferryl state of heme a_3 with no observable changes in the optical spectrum of CcO-607. Thus, several minutes after CcO-607 formation at pH 8.0, we have observed that approximately 70% of CcO-607 is in a redox state that is only 1 electron-equiv above oxidized enzyme (*26*).

We have previously proposed that the instability of the tyrosine radical present at the binuclear center is the result of a redox reaction with some group within the enzyme, presumably as a result of a reaction with one-or-more amino acid side chains (*26*). That the enzyme can function as an electron donor to its own redox centers is supported by our present observations on the instability of the enzyme at basic pH. Thus, oxidized enzyme is slowly reduced in the absence of any external electron donor(s), and this process is likely to be more rapid when the enzyme is in a higher oxidation state. Further evidence for the oxidation of enzyme groups is the observation that enzyme that has undergone the sequence CcO-O → CcO-607 → CcO-580 → CcO-O has the same redox state as the starting material but appears to have suffered some irreversible autoxidation as judged by the loss of a small amount of absorbance in the Soret band.

Thus, we suspect that the rapid loss of tyrosine radical and the somewhat slower loss of ferryl heme occur by the same mechanism though the specifics of this mechanism need

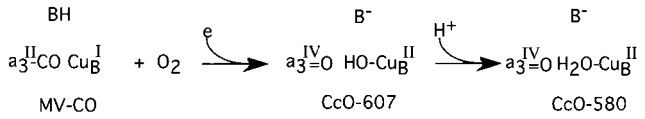
to be determined. The enhanced instability of radical is similar to the radicals generated by the treatment of myoglobin with H_2O_2 which disappear faster than the ferryl form (*48*−*51*). The mechanism for this phenomenon is not clear; one proposal is that the radical migrates to the surface of the protein and is quneched by the reaction with the second protein molecule (*52, 53*).

In this experiment, the CcO-607 form was prepared at basic pH 8.0 and used after a delay of about 10 min. Under these conditions, approximately 70% of CcO-607 should be in the ferryl state with a reduced radical content. Then the electronic state of the binuclear center of this form of CcO-607 is equivalent to the "peroxy" form (P_R) that is observed transiently during the reaction of fully reduced oxidase with dioxygen. This P_R intermediate, with its optical spectrum identical to CcO-607 (*17, 18*), is produced with the concomitant oxidation of reduced cytochrome *a*, cytochrome a_3 (*54*−*56*), and presumably Cu_B. In this case, all four electrons required for cleavage of the dioxygen bond are supplied by the metal redox centers. This P_R intermediate is converted later to ferryl form with the uptake of one proton but without any additional electron transfer to the binuclear center (*56*). Proton transfer to the binuclear center appears to be the rate-limiting step in the conversion of P_R to the ferryl form (*55, 56*).

If it is indeed the case that CcO-607 and P_R are isoelectronic, then the changes in the optical spectrum of heme a_3 during the conversion of CcO-607 → CcO-580 are a consequence only of a protonation event occurring in the vicinity of the heme a_3−Cu_B center. Experimentally we have found that the rate of the spontaneous CcO-607 to CcO-580 transition is the same as the rate of proton uptake (Figure 2). It indicates that the proton transfer to the binuclear center is like in the P_R intermediate the rate-limiting process. This similarity with the identity of optical and Raman data observed between P_R and CcO-607 (*24, 41*) provides further support that both forms of the enzyme are identical. Because one proton is consumed in the conversion both of CcO-607 to CcO-580 and of P_R to the ferryl form, we assume that CcO-580 and the transient ferryl form are also identical, at least to the extent of having same electronic states for the binuclear center.

The binuclear center is buried within the enzyme and insulated from the outer medium by a hydrophobic barrier (*57, 58*). To cross this barrier, protons need a pathway, and several potential pathways were identified from both mutagenesis studies (*59*−*62*) and the crystal structures of the bovine (*57*) and *Paracoccus denitrificans* enzymes (*58*). Three proton pathways were suggested to connect the catalytic center to the exterior. Two of these pathways, called K and D, connect the binuclear center with the matrix, and the third connects the binuclear center with the cytosol; this latter channel might serve for the exit of pumped protons or of water (*57, 58*). All three potential proton pathways are, however, interrupted by cavities, and there is no continuously spanning hydrogen-bond network between the outer medium and the catalytic center of oxidized enzyme. For the function of cytochrome oxidase as a proton pump, it is anticipated that the proton pathways must be selectively controlled by the redox and/or ligation state of the protein. We assume that when the enzyme is in the CcO-607 state, the binuclear center is not readily accessible to protons from the medium,

Scheme 1



and the acid-promoted decay of CcO-607 is due to protons that slowly leak into the closed binuclear cavity from the medium.

The closed proton conducting pathway proposed for CcO-607 would appear to be in conflict with the fast proton transfer observed in P_R that occurs within milliseconds (*55, 56*). There are two possible reasons for this difference. First, CcO-607 may be in a metastable state analogous to the oxidized state of the enzyme immediately following reoxidation (*4*). This state (O∼) is proposed to be a form of the oxidized enzyme in which approximately half of the energy for proton translocation is conserved. O∼ decays in less that 20 s (*4*), implying that the proton conduction pathways are also closed within the same time. However, if O∼ is rereduced before this decay, then proton translocation can be completed, indicating that the proton conducting pathways have not yet closed. We have earlier expressed a similar explanation for the conversion of CcO-607 to CcO-580 (*26*) and proposed that the reduction of cytochrome *a* can be the trigger for the opening of a proton channel in CcO-607 which in turn should increase the rate of relaxation of CcO-607 to CcO-580.

The competing explanation requires that the production of CcO-607 is associated with the oxidation of some group in the enzyme that is part of the pathway conducting protons into the binuclear center. However, the long lifetime of CcO-607 requires that this oxidized group is relatively stable and specific. The oxidation of a specific group is based on the rate of CcO-607 decay that is a single exponential at all pH values tested and indicates homogeneity of the CcO-607 population. However, the oxidation of the binuclear center with hydrogen peroxide produces the free radicals with multiple different origins (*36, 46, 63, 64*), suggesting that the radical is free to migrate within in the protein. The fast and quantitative conversion of CcO-607 to oxidized enzyme provides an additional argument against the oxidation of a specific group that blocks proton transfer. When CcO-607 was reduced at pH 8.0 by one electron under anaerobic conditions, the conversion to oxidized enzyme was completed in the time for manual mixing, about 20 s (*26*). Obviously the conversion is associated with proton uptake which, on this time scale, was not inhibited. Thus, it seems that the initial explanation of a metastable state is more consistent with our observations.

Our proposal that the binuclear center of CcO-607 is associated with closed proton pathways explains why the conversion rate is slow. However, it does not explain why the conversion, if it is simply an acid−base reaction, is not reversible. Our explanation for this apparent irreversibility is based on the presence of one deprotonated group with a relatively high pK_a generated in the binuclear cavity during the formation of CcO-607. We illustrate this point with a reaction scheme (Scheme 1); this scheme does not take into account the possible movement of pumped protons because we are only interested in the net protonation changes of solubilized enzyme.

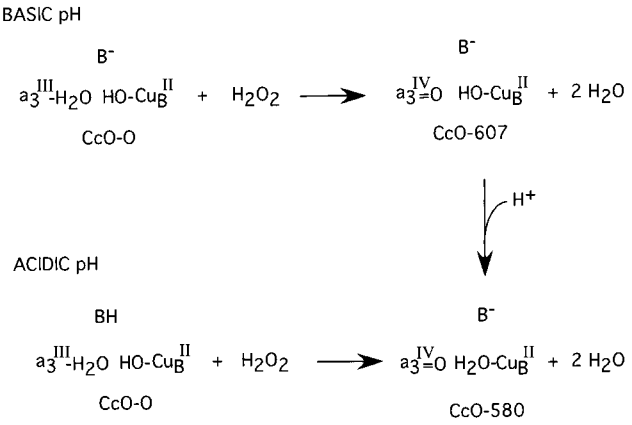
Scheme 1 starts with the MV•CO complex where cytochrome a_3 and Cu_B are reduced and stabilized by bound CO. The reduction of the binuclear center at basic pH is associated with the uptake of approximately two protons from the medium (*65*−*69*). Electrostatic calculations suggest that one proton can be accepted by hydroxide, coordinated to Cu_B^{II}, and the second is shared by the cluster of residues, designated as B in Scheme 1; this cluster interacts strongly with the binuclear center (*70*, *71*). The water molecule generated from hydroxide and coordinated to Cu_B is in all probability displaced from the binuclear cavity by CO binding as is indicated by the crystal structure of bovine enzyme with bound CO (*72*). Thus, only the single proton which resides on cluster B can be used as a substrate proton.

When MV•CO reacts with oxygen to form CcO-607, there are no observable pH changes in the medium (*65*, *67*). At this stage, the dioxygen bond is cleaved, and Tyr 244 (YH) (*46*, *47*), if oxidized to the neutral radical, also makes a second substrate proton available to the binuclear cavity. In the absence of an external electron donor, this neutral tyrosine radical is probably quenched by the oxidation of one or more amino acid residues within the enzyme with the production of tyrosinate. Because the radical quenching reactions are presently undefined, the electron donor to the tyrosine radical, that is unlikely to be located at the binuclear center, is omitted from Scheme 1. It is expected that the pK_a of H_2O/OH^- bound to Cu_B is probably lower than that of YH/ Y^- (*70*, *71*), and so we present the stable CcO-607 intermediate as a_3^{IV}═O HO−Cu_B^{II}. The next step is the irreversible conversion of CcO-607 to CcO-580. According to this scheme, the irreversible conversion is a consequence of the protonation of hydroxide at Cu_B which should also be the rate-limiting step. The sufficient requirement for the irreversibility of the conversion of CcO-607 to CcO-580 by pH change is that the pK_a of H_2O/OH^- bound to Cu_B should be higher than 9.0.

Both CcO-607 and CcO-580 can be prepared in the stoichiometric reaction between hydrogen peroxide and oxidized enzyme (*36*). At basic pH (pH 8.0), the main product is CcO-607, and at acidic pH (pH 5.8), it is CcO-580 (*36*, *38*, *40*). The dependence of the yield of CcO-607 on pH is expected to be associated with the protonation state of two groups with identical pK_a values that lie in the range 6.7−7.3 (*38*, *40*). We have observed that the yields of both CcO-607 and CcO-580 exhibit an inverse pH dependence. The reaction product of oxidized enzyme with peroxide at any pH is a mixture of CcO-607 and CcO-580 with the yield of CcO-607 decreasing with a decrease in pH and a corresponding increase in the yield of CcO-580; the pK_a of this transition is close to 7.0 (M. Fabian and G. Palmer, unpublished results). As hydrogen peroxide is completely protonated in the pH range 5−8.5, it can only be the protonation state of the enzyme group(s) that controls the reaction product.

From the present measurements, we know that the conversion of CcO-607 to CcO-580 required one proton, but the production of CcO-580 at acidic pH with stoichiometric H_2O_2 is not accompanied by proton uptake from the medium. To explain these observations, it is sufficient to assume that at acidic pH there is a proton already within the enzyme, and this proton is available for reaction in the binuclear cavity. Thus, we postulate the existence of a group with a pK_a around

Scheme 2



7.0 in the vicinity of binuclear center of oxidized enzyme. When the group is deprotonated, the product of the reaction of CcO-O with peroxide is CcO-607, and when the group is protonated, the reaction product is CcO-580 (Scheme 2). In Scheme 2, which is again a simplified version of the reaction, it is assumed but omitted that at both pHs the reaction of a stoichiometric amount of H_2O_2 with CcO-O generates a neutral tyrosine radical, Y•, which is later endogenously quenched on the time scale of minutes. B cluster represents the group with a pK_a of 7.0 of oxidized enzyme which is protonated at acidic pH and can donate the substrate proton to the binuclear center in the course of the reaction. There is kinetic evidence that this group is the conserved Glu 242 (*73*) which is believed to be part of a proton conducting pathway.

The reaction mechanism presented above is based on the suggestion that hydroxide ion is coordinated to Cu_B in oxidized enzyme (*74*). If this is not the case, calculations show that Tyr 244 will have pK_a of 7.6 in the oxidized enzyme (*70*) and that the proton distribution in the catalytic center of intermediates will also be different. Despite this uncertainty in the chemical nature of the protonatable group involved, our data indicate that the difference between the stable intermediates CcO-607 and CcO-580 is in the protonation state of the catalytic center and that uptake of one proton converts CcO-607 to CcO-580.

REFERENCES

1. Saraste, M., Holm, L., Lübben, M., and van der Oost, J. (1991) *Biochem. Soc. Trans. 19*, 608−622.
2. Calhoun, M. W., Thomas, J. W., and Gennis, R. B. (1994) *Trends Biochem. Sci. 19*, 325−330.
3. Trumpower, B. L., and Gennis, R. B. (1994) *Annu. Rev. Biochem. 63*, 675−716.
4. Verkhovsky, M., Jasaitis, A., Verkhovskaya, M., Morgan, J. E., and Wikström, M. (1999) *Nature 400*, 480−483.
5. Wikström, M. (1989) *Nature 338*, 776−778.
6. Hill, B. C., and Greenwood, C. (1984) *Biochem. J. 218*, 913−921.
7. Witt, N. S., Blair, D. F., and Chan, S. I. (1986) *J. Biol. Chem. 261*, 8104−8107.
8. Witt, N. S., and Chan, S. I. (1987) *J. Biol. Chem. 262*, 1446−1448.
9. Varotsis, C., and Babcock, G. (1990) *Biochemistry 29*, 7357−7366.
10. Han, S., Ching, Y. C., and Rousseau, D. L. (1990) *Nature 348*, 89−90.
11. Han, S., Ching, Y. C., and Rousseau, D. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8408−8412.

12. Ogura, T., Takahashi, S., Shinzawa-Itoh, K., Yoshikawa, S., and Kitagawa, T. (1990) *J. Biol. Chem. 265*, 14721−14723.
13. Babcock, G. T., and Wikström, M. (1992) *Nature 356*, 301−309.
14. Ogura, T., Takahashi, S., Hirota, S., Shinzawa-Itoh, K., Yoshikawa, S., Appelman, E. H., and Kitagawa, T. (1993) *J. Am. Chem. Soc. 115*, 8527−8536.
15. Varotsis, C., Zhang, Y., Appelman, E. H., and Babcock, G. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 237−241.
16. Verkhovsky, M., Morgan, J. E., and Wikström, M. (1994) *Biochemistry 33*, 3079−3086.
17. Morgan, J. E., Verkhovsky, M., and Wikström, M. (1996) *Biochemistry 35*, 12253−12240.
18. Sucheta, A., Georgiadis, K. E., and Einarsdóttir, Ó. (1997) *Biochemistry 36*, 554−565.
19. Sucheta, A., Szundi, I., and Einarsdóttir, Ó. (1998) *Biochemistry 37*, 17905−17914.
20. Han, S., Takahashi, S., and Rousseau, D. L. (2000) *J. Biol. Chem. 275*, 1910−1919.
21. Proshlyakov, D. A., Ogura, T., Shinzava-Itoh, K., Yoshikawa, S., Appelman, E. H., and Kitagawa, T. (1994) *J. Biol. Chem. 269*, 29385−29388.
22. Proshlyakov, D. A., Ogura, T., Shinzava-Itoh, K., Yoshikawa, S., and Kitagawa, T. (1996) *Biochemistry 35*, 76−82.
23. Proshlyakov, D. A., Ogura, T., Shinzava-Itoh, K., Yoshikawa, S., and Kitagawa, T. (1996) *Biochemistry 35*, 8580−8586.
24. Kitagawa, T., and Ogura, T. (1997) *Prog. Inorg. Chem. 45*, 431−479.
25. Fabian, M., Wong, W. W., Gennis, R. B., and Palmer, G. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 13114−13117.
26. Fabian, M., and Palmer, G. (1999) *Biochemistry 38*, 6270−6275.
27. Wikström, M. (1981*) Proc. Natl. Acad. Sci. U.S.A. 78*, 4051−4054.
28. Wikström, M. (1988) *Chem. Scr. 28A*, 71−74.
29. Wikström, M., and Morgan, J. (1992) *J. Biol. Chem. 267*, 10266−10273.
30. Chance, B., Saronio, C., and Leigh, J. S., Jr. (1975) *J. Biol. Chem. 250*, 9226−9232.
31. Chance, B., Saronio, C., and Leigh, J. S., Jr. (1979) *Biochem. J. 177*, 931−941.
32. Greenwood, C., Wilson, M. T., and Brunori, M. (1974) *Biochem. J. 137*, 205−215.
33. Hill, B. C., and Greenwood, C. (1983) *Biochem. J. 215*, 659−667.
34. Nicholls, P., and Chanady, G. A. (1981) *Biochim. Biophys. Acta 634*, 256−265.
35. Bickar, D., Bonaventura, C., and Bonaventura, J. (1984) *J. Biol. Chem. 259*, 10777−10783.
36. Fabian, M., and Palmer, G. (1995) *Biochemistry 34*, 13802−13810.
37. Wrigglesworth, J. M. (1984) *Biochem. J. 217*, 715−719.
38. Vygodina, T., and Konstantinov, A. (1989) *Biochim. Biophys. Acta 973*, 390−398.
39. Weng, L., and Baker, G. M. (1991) *Biochemistry 30*, 5727−5733.
40. Jünemann, S., Heathcote, P., and Rich, P. R. (2000) *Biochim. Biophys. Acta 1456*, 56−66.
41. Proshlyakov, D. A., Pressler, M. A., and Babcock, G. T. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 8020−8025.
42. Soulimane, T., and Buse, G. (1995) *Eur. J. Biochem. 227*, 588−595.
43. Fabian, M., and Palmer, G. (1998) *FEBS Lett. 422*, 1−4.
44. Liao, G.-L., and Palmer, G. (1996) *Biochim. Biophys. Acta 1274*, 109−111.
45. Kaplan, N. O. (1960) in *The Enzymes 3* (Boyer, P. D., Lardy, H., and Myrback, K., Eds.) pp 105−169, Academic Press, New York.
46. MacMillan, F., Kannt, A., Behr, J., Prisner, T., and Michel, H. (1999) *Biochemistry 38*, 9179−9184.
47. Blomberg, M. R. A., Siegbahn, P. E. M., Babcock, G. T., and Wikström, M. (2000) *J. Inorg. Chem. 80*, 261−269.
48. King, N. K., and Winfield, M. E. (1963) *J. Biol. Chem. 238*, 1520−1528.
49. Yonetani, T., and Schleyer, H. (1967) *J. Biol. Chem. 242*, 1974−1979.
50. Davies, M. J. (1990) *Free Radical Res. Commun. 10*, 361−370.
51. Cooper, E. C., Green, E. S. R., Rice-Evans, C. A., Davies, M., and Wrigglesworth, J. M. (1994) *Free Radical Res. Commun. 20*, 219−227.
52. King, N. K., Looney, F. D., and Winfield, M. E. (1967) *Biochim. Biophys. Acta 133*, 65−82.
53. Tew, D., and Montellano, P. R. O. (1988) *J. Biol. Chem. 263*, 17880−17886.
54. Takahashi, S., Ching, Y.-C., Wang, J., and Rousseau, D. L. (1995) *J. Biol. Chem. 270*, 8405−8407.
55. Brzezinski, P., and Ädelroth, P. (1998) *J. Bioenerg. Biomembr. 30*, 99−107.
56. Karpefors, M., Ädelroth, P., Zhen, Y., Ferguson-Miller, S., and Brzezinski, P. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 13606−13611.
57. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1996) *Science 272*, 1136−1144.
58. Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) *Nature 376*, 660−669.
59. Thomas, J. W., Puustinen, A., Alben, J. O., Gennis, M., and Wikström, M. (1993) *Biochemistry 32*, 10923−10928.
60. Fetter, J. R., Qian, J., Shapleigh, J., Thomas, J. W., Garcia-Horsman, A., Schmidt, E., Hosler, J., Babcock, G. T., Gennis, R. B., and Ferguson-Miller, S. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1604−1608.
61. Garcia-Horsman, A., Puustinen, A., Gennis, R. B., and Wikström, M. (1995) *Biochemistry 34*, 4428−4433.
62. Gennis, R. B. (1998) *Biochim. Biophys. Acta 1365*, 241−248.
63. Chen, Y.-R., Gunther, M. R., and Mason, R. P. (1999) *J. Biol. Chem. 274*, 3308−3314.
64. Rigby, S. E. J., Jünemann, S., Rich, P., and Heathcote, P. (2000) *Biochemistry 38*, 5921−5928.
65. Oliveberg, M., Hallen, S., and Nilsson, T. (1991) *Biochemistry 30*, 436−440.
66. Hallen, S., and Nilsson, T. (1992) *Biochemistry 31*, 11853−11859.
67. Mitchell, R., and Rich, P. (1994) *Biochim. Biophys. Acta 1186*, 19−26.
68. Capitanio, N., Vygodina, T. V., Capitanio, G., Konstantinov, A. A., Nicholls, P., and Papa, S. (1997) *Biochim. Biophys. Acta 1318*, 255−265.
69. Paula, S., Sucheta, A., Szundi, I., and Einarsdóttir, Ó. (1999) *Biochemistry 38*, 3025−3033.
70. Kannt, A., Lancaster, R. D., and Michel, H. (1998) *Biophys. J. 74*, 708−721.
71. Kannt, A., Lancaster, R. D., and Michel, H. (1998) *J. Bioenerg. Biomembr. 30*, 81−87.
72. Yoshikawa, S., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Yamashita, E., Inoue, N., Yao, M., Fei, M. J., Libeu, C. P., Mizushima, T., Yamaguchi, H., Tomizaki, T., and Tsukihara, T. (1998) *Science 280*, 1723−1729.
73. Ådelroth, P., Karpefors, M., Gilderson, G., Tomson, F. L., Gennis, R. B., and Brzezinski, P. (2000) *Biochim. Biophys. Acta 1459*, 533−539.
74. Ostermeier, Ch., Harrenga, A., Ermler, U., and Michel, H. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 10547−10553.

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