Examination of the Reaction of Fully Reduced Cytochrome Oxidase with Hydrogen Peroxide by Flow-Flash Spectroscopy†

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Received July 20, 1999; Revised Manuscript Received September 30, 1999

ABSTRACT: The reaction of cytochrome *c* oxidase with hydrogen peroxide has been of great value in generating and characterizing oxygenated species of the enzyme that are identical or similar to those formed during turnover of the enzyme with dioxygen. Most previous studies have utilized relatively low peroxide concentrations (millimolar range). In the current work, these studies have been extended to the examination of the kinetics of the single turnover of the fully reduced enzyme using much higher concentrations of peroxide to avoid limitations by the bimolecular reaction. The flow-flash method is used, in which laser photolysis of the CO adduct of the fully reduced enzyme initiates the reaction following rapid mixing of the enzyme with peroxide, and the reaction is monitored by observing the absorbance changes due to the heme components of the enzyme. The following reaction sequence is deduced from the data. (1) The initial product of the reaction appears to be heme a_3 oxoferryl ($Fe^{4+}{=}O^{2-} + H_2O$). Since the conversion of ferrous to ferryl heme a_3 (Fe^{2+} to Fe^{4+}) is sufficient for this reaction, presumably Cu_B remains reduced in the product, along with Cu_A and heme *a*. (2) The second phase of the reaction is an internal rearrangement of electrons and protons in which the heme a_3 oxoferryl is reduced to ferric hydroxide ($Fe^{3+}OH^-$). In about 40% of the population, the electron comes from heme *a*, and in the remaining 60% of the population, Cu_B is oxidized. This step has a time constant of about 65 μs. (3) The third apparent phase of the reaction includes two parallel reactions. The population of the enzyme with an electron in the binuclear center reacts with a second molecule of peroxide, forming compound F. The population of the enzyme with the two electrons on heme *a* and Cu_A must first transfer an electron to the binuclear center, followed by reaction with a second molecule of peroxide, also yielding compound F. In each of these reaction pathways, the reaction time is 100−200 μs, i.e., much faster than the rate of reaction of peroxide with the fully oxidized enzyme. Thus, hydrogen peroxide is an efficient trap for a single electron in the binuclear center. (4) Compound F is then reduced by the final available electron, again from heme *a*, at the same rate as observed for the reduction of compound F formed during the reaction of the fully reduced oxidase with dioxygen. The product is the fully oxidized enzyme (heme a_3 $Fe^{3+}OH^-$), which reacts with a third molecule of hydrogen peroxide, forming compound P. The rate of this final reaction step saturates at high concentrations of peroxide (V_{max} = 250 s^{-1}, K_m = 350 mM). The data indicate a reaction mechanism for the steady-state peroxidase activity of the enzyme which, at pH 7.5, proceeds via the single-electron reduction of the binuclear center followed by reaction with peroxide to form compound F directly, without forming compound P. Peroxide is an efficient trap for the one-electron-reduced state of the binuclear center. The results also suggest that the reaction of hydrogen peroxide to the fully oxidized enzyme may be limited by the presence of hydroxide associated with the heme a_3 ferric species. The reaction of hydrogen peroxide with heme a_3 is very substantially accelerated by the availability of an electron on heme *a*, which is presumably transferred to the binuclear center concomitant with a proton that can convert the hydroxide to water, which is readily displaced.

Cytochrome *c* oxidase is the terminal enzyme in the mitochondrial respiratory chain. The enzyme reduces dioxygen to water, using reducing equivalents supplied by ferrocytochrome *c*. The free energy made available by this very favorable reaction is used to generate the protonmotive force across the mitochondrial inner membrane that is used to power ATP synthesis. For each turnover of the enzyme (O_2 reduced), four protons are pumped electrogenically across the membrane (1 H^+/e^-). Much of the interest in this enzyme is motivated by the desire to understand how the oxygen chemistry catalyzed by the enzyme is coupled to the proton pump mechanism. Attaining this goal requires a full under-

† Supported by the National Institutes of Health (HL-16101 to R.B.G.), by an NSF International Research Collaboration Award (to R.B.G.), by the American Heart Association (fellowship to D.Z.), by the Swedish Natural Science Research Council (to P.B.), by The Swedish Foundation for International Cooperation in Research and Higher Education (STINT) (to P.B.), by Grants-in-Aid for Scientific Research on Priority Areas (Molecular Science on the Specific Roles of Metal Ions in Biological Functions) (to S.Y.), and by Grants-in-Aid for Scientific Research (40068119) from the Ministry of Education and Culture of Japan (to S.Y.).

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10.1021/bi9916675 CCC: $18.00
Published on Web 11/10/1999

standing of the sequence of intermediates formed during turnover.

The dioxygen chemistry is catalyzed at the heme a_3/Cu_B binuclear center, which is buried within the enzyme (*1*−*4*). In addition to the two redox centers at the binuclear center, the enzyme also contains two additional redox centers, Cu_A and heme *a*, which can be considered as electron input centers. Electron transfer proceeds linearly from cytochrome *c* to Cu_A and then to heme *a*, and all electron input to the heme a_3/Cu_B binuclear center is via heme *a* (*5*).

The catalytic cycle of the enzyme has been examined using fast kinetics methods to define the nature of the oxygenated intermediates and their rates of formation and decay. The two most widely used approaches have been the flow-flash technique (*6*−*13*) and the use of photoactivated electron donors (*14*−*16*). In the flow-flash technique, the reactions of dioxygen with the fully or partially reduced enzyme are examined. In this approach, one "pre-loads" the enzyme with reducing equivalents (four-electron-reduced = fully reduced; two-electron-reduced = mixed valence), and the enzyme is blocked from reaction with dioxygen by forming the CO adduct. After mixing with dioxygen, the reaction is initiated by a pulse of light that expels CO from the enzyme, thus allowing dioxygen to react. This increases the time resolution by about 1000-fold over typical stopped-flow experiments, allowing monitoring of events spectrophotometrically in the microsecond time frame.

In the flow-flash experiments with fully reduced enzyme, several oxygenated intermediates are resolved optically. Dioxygen binds to reduced heme a_3, forming the Oxy complex. This is then reduced by electron transfer from heme *a*, resulting in compound P_r, in which Cu_B remains reduced. Compound P_r is then reduced to compound F by electron transfer from Cu_B to the oxygenated heme a_3 species and, at the same time, there is electron transfer from Cu_A to rereduce heme *a*, redistributing the electron between these two redox centers. This equilibration of the electron between the input centers favors reduced heme *a* by about 5:1 over reduced Cu_A. In the final step, the electron remaining in the input centers is transferred to the oxoferryl form of heme a_3 (compound F), reducing it to the ferric heme.

The second rapid kinetics approach is to use photoactivated reductants to inject electrons in different forms of the oxidase (*14*−*16*). Of particular interest is the observation that the first electron introduced into the fully oxidized enzyme is not transferred from heme *a* to the binuclear center, even in the presence of dioxygen. This implies that the two-electron-reduced oxidase species, in which both heme *a* and Cu_A are reduced, must be formed before an electron is transferred to the binuclear center. This two-electron-reduced species is, thus, an obligatory intermediate during steady-state conditions (in contrast to the single-turnover flow-flash experimental conditions). We will refer to the species where Cu_A and heme *a* are reduced as "species H" for the half-reduced form of the enzyme. Note that the redox status of the metal centers in the enzyme in the H form is just the opposite as in the well-characterized two-electron-reduced mixed-valence (MV) form of the enzyme, in which the heme a_3/Cu_A binuclear center is reduced (stabilized by CO binding to heme a_3) and the input centers (heme *a* and Cu_A) are oxidized (*5*). For further electron transfer from cytochrome *c* and for the enzyme reaction to proceed, it is necessary that electron transfer from heme *a* to the binuclear center must occur.

Although it is evident that the H form of the enzyme must participate in turnover, this species has not been examined experimentally. In principle, species H should be transiently observable by rapid two-electron reduction of the fully oxidized enzyme or by rapid two-electron oxidation of the fully reduced enzyme. However, at present the efficiency of either chemical or photochemical electron donors is insufficient to generate the H species in reasonable yield starting with the fully oxidized enzyme. In the current work, it is demonstrated that the H species can be generated in a high yield using the flow-flash technique starting with the fully reduced enzyme by reaction with hydrogen peroxide, a two-electron oxidant, in place of dioxygen. Furthermore, rapid one-electron transfer to the binuclear center from heme *a* occurs once the H species is formed, and this one-electron-reduced form of the bincuclear center is rapidly trapped by reaction with a second molecule of hydrogen peroxide.

MATERIALS AND METHODS

Cytochrome oxidase was prepared as described in ref *17*. Concentrated (1 mM) solutions of the enzyme were diluted in 100 mM HEPES[1] 1 buffer, pH 7.5, 0.1% dodecyl maltoside, reduced with 1 mM ascorbate in the presence of 1−2 μM PMS saturated with CO, and used for the flow-flash experiments. The preparation of anaerobic H_2O_2 was described in ref *18*, and the flow-flash apparatus was described in refs *9* and *19*.

RESULTS

Overview of the Reaction. Unlike dioxygen, hydrogen peroxide not only oxidizes fully reduced or partially reduced cytochrome oxidase, but also will react with the fully oxidized enzyme (*20*−*23*):

$$\text{fully reduced COX} + 2H_2O_2 \rightarrow \text{oxidized COX} \quad (1)$$

$$\text{oxidized COX} + H_2O_2 \rightarrow \text{compound } P_m \quad (2)$$

The reaction of the fully oxidized enzyme with hydrogen peroxide has been examined previously, and the initial product (at pH = 7.5) of a single turnover is compound P_m (*18*). This reaction is complete within 30 ms, and further reaction with peroxide (P→F) is not observed within this time scale. Hence, within 30 ms following mixing, the final product in the reaction of fully reduced cytochrome oxidase with hydrogen peroxide is compound P_m. The spectroscopic features of the oxygenated intermediates (compounds P and F) as well as the reduced and oxidized states of hemes *a* and a_3 are well-known. It is also clear that the reaction of the oxidase with hydrogen peroxide will involve optical changes that can be interpreted in terms of these states of the two hemes, as has been done previously in the analysis of the flow-flash reaction kinetics of the oxidase with dioxygen (*5*, *9*). Figures 1 and 2 show the time course of the reaction with hydrogen peroxide, starting with the fully reduced enzyme and ending with species P_m. In Figure 1 the reaction is monitored by the absorbance at 436 nm. This

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PMS, phenazine methosulfonate; EPR, electron paramagnetic resonance.

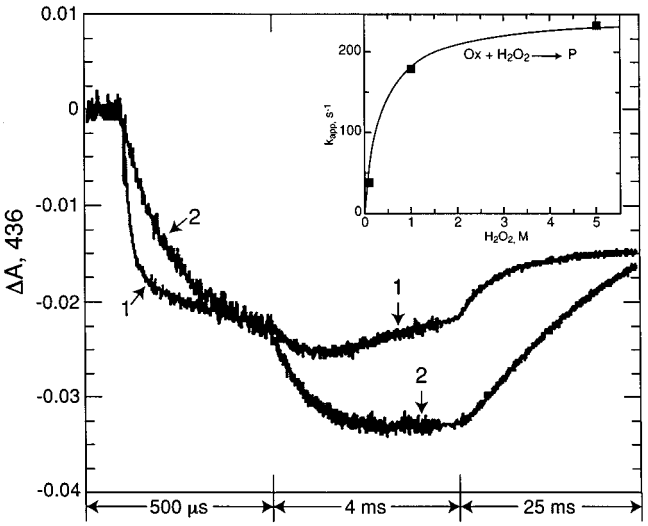

FIGURE 1: Flow-flash reaction of fully reduced oxidase with H_2O_2 monitored at 436 nm. The transients at 436 nm have almost no contribution from changes in heme *a* oxidation, and thus monitor the conversions of heme a_3. Shown are the data obtained in the reaction of the fully reduced enzyme with 0.1 and 1 M H_2O_2. The rates of the microsecond and millisecond phases of the reaction depend on the H_2O_2 concentration. Heme a_3 is oxidized and converted to compound P_m during the course of these reactions. The dependency of the latter reaction on the concentration of H_2O_2 is shown in the inset graph. The solid line represents a hyperbolic fit with $K_m = 350$ mM and $V_{max} = 250$ s^{-1}.

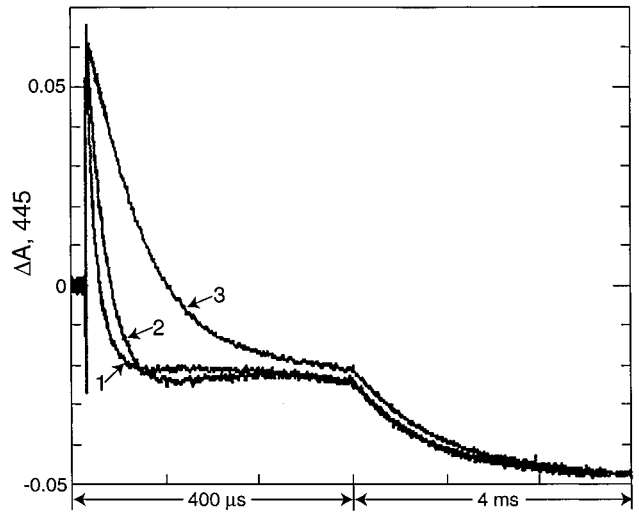

FIGURE 2: Flow-flash reaction of the fully reduced oxidase with H_2O_2 monitored at 445 nm, showing the oxidation of both hemes *a* and a_3 by H_2O_2. Shown are the transients at 445 nm in the reaction of the fully reduced enzyme with 0.1 (curve 3), 1 (curve 2), and 5 M H_2O_2 (curve 3) presented. The rate of the initial phase of the reaction has a clear dependency on the H_2O_2 concentration, while the millisecond phase and the duration of the lag phase are not dependent on H_2O_2 concentration.

is an isosbestic point for the reduced and oxidized forms of heme *a*, and monitors changes primarily in the state of heme a_3. In Figure 2, the reaction progress is monitored at 445 nm, which shows changes in the redox states of both hemes. The last step in the reaction, that is apparent at 436 nm (Figure 1), results in only small changes at 445 nm, and is not shown in Figure 2. The reaction has been examined at three different concentrations of hydrogen peroxide, 0.1, 1.0, and 5.0 M, and both Figures 1 and 2 reveal several distinct

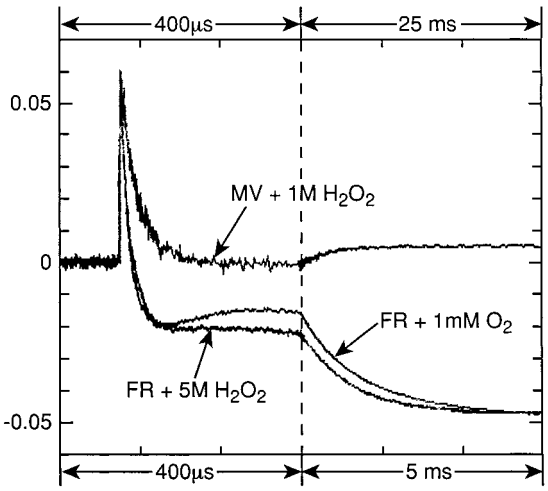

FIGURE 3: Oxidation of fully reduced (FR) cytochrome oxidase by dioxygen (1 mM) or anaerobic hydrogen peroxide (5 M). The transients of absorbance changes at 445 nm are presented. The initial parts of the reactions have approximately the same rates. When the enzyme is oxidized by dioxygen, a "bump" on the transient is present due to rereduction of heme *a* from Cu_A. This feature is almost completely gone when the enzyme reacts with H_2O_2. The transient from the reaction of mixed-valence oxidase (MV) with H_2O_2 (1 M) is given for comparison. Note that the millisecond time scale for this (MV) reaction is different (upper scale) than for the other two reactions (lower scale). The absorbance level of the "plateau" corresponds to the 40% difference between the final levels of oxidized heme *a* in the cases of mixed-valence (MV) and the fully reduced (FR) enzyme. Note that, in principle, the electron remaining on Cu_B can redistribute to heme a_3 to some extent. This can affect the precision of the estimate of the extent of heme *a* oxidation (see text).

steps in the reaction that occur in the microsecond and millisecond time spans.

Figure 3 directly compares the traces at 445 nm of the fully reduced enzyme reacting with 5 M hydrogen peroxide with the well-studied reaction of the fully reduced enzyme with 1 mM dioxygen. Since dioxygen is a four-electron oxidant, the product of the reaction with dioxygen is the fully oxidized enzyme plus $2H_2O$. It is clear from Figure 3 that the reaction with hydrogen peroxide occurs on the same time scale as does that with dioxygen, though a much higher concentration of peroxide is required to attain this rate. An important feature of the flow-flash reaction sequence with dioxygen is the "bump" in the trace observed at 445 nm (Figure 3), where the absorbance increases for a time before resuming its downward trend. This feature is due to the initial oxidation of heme *a*, followed by its rereduction by electron transfer from Cu_A and, finally, the reoxidation of heme *a*. Significantly, this "bump" is not observed in the trace of the reaction of the fully reduced enzyme with hydrogen peroxide. This will be discussed further below. It is important to note that the plateau in the absorbance transient levels at the bottom of the "bump", not at the top, as would be expected according to the model discussed below. This can be accounted for by the difference in the absorbance of the fully oxidized binuclear center and that of the oxygenated intermediates. Indeed, the binding of peroxide to the mixed-valence enzyme results in an adduct with the fully oxidized binuclear center associated with an increase in the absorbance at 445 nm which is roughly equal to the amplitude of the "bump" on the transient observed in the transient obtained with dioxygen (Figure 3).

The spectroscopic changes that are observed at 436 and 445 nm in the reaction of the fully reduced enzyme with hydrogen peroxide can be interpreted in terms of the reactions 1 and 2 (above). Two of the optical phases observed at both 436 and 445 nm (Figure 1) are dependent on peroxide concentration in the range examined (100 mM to 5 M). The rates of both the initial part of the reaction as well as the final step in the reaction increase as the peroxide concentration is raised. The initial step observed at 436 nm is the reaction of the first equivalent of peroxide with reduced heme a_3, and the last step is the much slower reaction of the third peroxide molecule with the recently formed fully oxidized form of the enzyme (reaction 2). The last step, i.e., the reaction of peroxide with ferric heme a_3 to yield compound P_m, has been examined previously (*18*) and serves as a check of the interpretation of the overall reaction sequence.

Interaction of the Fully Oxidized Cytochrome Oxidase with H_2O_2. The reaction of peroxide with the fully oxidized enzyme has been shown previously (*18, 22*) to result in an increase in the absorbance at 436 nm, corresponding to the conversion of ferric heme a_3 to compound P_m. Earlier studies (*22, 24*−*26*) have shown that the rate of this reaction increases linearly with the peroxide concentration within the millimolar range of concentrations of hydrogen peroxide. In the current work, much higher concentrations of peroxide have been used, and the rate of reaction, monitored by the final phase shown in the traces in Figure 1, is observed to saturate with an apparent K_m of 350 mM and a V_{max} of 250 s^{-1}, based on data collected with 100 mM, 1 M, and 5 M hydrogen peroxide. The V_{max}/K_m value of 700 M^{-1} s^{-1} is in good agreement with previously reported values for the second-order rate constant for the binding of H_2O_2 to the fully oxidized form of the enzyme (*22, 24*).

The rate of the reaction of peroxide with the fully oxidized enzyme to form compound P_m is considerably slower than the preceding reactions of hydrogen peroxide with the fully reduced or partially reduced forms of the enzyme, that are monitored in the traces in Figures 1 and 2. The important consequence is that in these earlier steps in the reaction of peroxide with the reduced or partially reduced forms of the enzyme, the formation of species P_m is negligible, and the reaction sequence is very unlikely to proceed via this species.

Reduction of Compound F to the Fully Oxidized Enzyme. Since peroxide is a two-electron oxidant, the reaction of two peroxide molecules with the four-electron (fully) reduced oxidase will naturally lead to the fully oxidized enzyme species, just as is the case with the reaction with one molecule of dioxygen, a four-electron oxidant. Figure 3 directly compares the flow-flash reactions of peroxide and dioxygen with the fully reduced enzyme, monitored at 445 nm. It is clear that the millisecond phases are essentially the same in the two reactions. In the case of the flow-flash reaction with dioxygen, the F→Ox transition is well characterized as the last step in the oxidation of the fully reduced enzyme, and is essentially the intramolecular transfer of an electron that is initially shared by heme *a* and Cu_A to reduce oxoferryl heme a_3 to ferric heme a_3.

In the case of the reaction of hydrogen peroxide with the fully reduced enzyme, the F→Ox transition is easily rationalized as being the penultimate step in the hypothetical reaction sequence. This millisecond phase, known to be the F→Ox step in the reaction with dioxygen, is monitored by transients observed at both 436 nm (Figure 1) and 445 nm (Figure 2). Both the rate and amplitude changes are similar for the flow-flash reactions with dioxygen and hydrogen peroxide. However, hydrogen peroxide reacts slowly with the oxidized enzyme, as described previously, resulting in the additional phase in the reaction observed at 436 nm (Figure 1), ferric heme a_3 to compound P_m.

Initial Phase of the Reaction of Fully Reduced Cytochrome Oxidase with Hydrogen Peroxide. (i) Formation of Compound F′. Whereas both the F→Ox and the Ox→P_m transitions are readily identified as the final two steps in the observed reaction sequence with hydrogen peroxide with the fully reduced enzyme, the interpretation of the initial steps of the reaction sequence is more speculative, albeit potentially more informative. Previous studies of the reaction of hydrogen peroxide with the mixed-valence (two-electron-reduced) form of the enzyme provide a basis for the interpretation of the events observed in the current experiments (*18*). The reaction of the first equivalent of hydrogen peroxide with either the fully reduced or the mixed-valence forms of the enzyme involves the same two events: (i) formation of oxoferryl heme a_3; (ii) intramolecular electron transfer, reducing oxoferry heme a_3 to ferric heme a_3. In both reactions with fully reduced and with mixed-valence forms of the enzyme, it is expected that the initial product will have oxoferryl heme a_3 and Cu_B in the reduced state. Hence, this state of the enzyme does not exactly coincide with "compound F", in which heme a_3 is oxoferryl but Cu_B is oxidized. We will refer to the state of the enzyme with heme a_3 oxoferryl and reduced Cu_B as compound F′. The oxoferryl form of heme a_3 is a powerful oxidant, and the presence of reducing equivalents on both Cu_B and heme *a* ensures that compound F′ will be short-lived in this experiment.

The reaction of hydrogen peroxide with fully reduced cytochrome oxidase is much faster than the reaction of peroxide with the fully oxidized enzyme. This is shown in the initial portions of the traces in Figures 1 and 2, which monitor the reaction immediately following the photolysis of CO. The rate of the reaction monitored at 445 nm is a function of peroxide concentration. Unlike the rate of the Ox→P_m reaction, the rate of the reaction of peroxide with fully reduced enzyme does not saturate at high peroxide concentrations (not shown), but there are insufficient data to warrant fitting this to a particular kinetic model. It is important to note that it takes molar concentrations of hydrogen peroxide to achieve rates of reaction with the fully reduced enzyme equivalent to the rates obtained with millimolar concentrations of dioxygen.

(ii) Reduction of Compound F′. In the previously examined reaction of the mixed-valence form of the oxidase with hydrogen peroxide, the formation and decay of compound F′ could be kinetically resolved at sufficiently high concentrations of hydrogen peroxide. In the case of the mixed-valence oxidase, the electron transferred to heme a_3 must come from Cu_B, and this has been measured to occur in about 80−90 μs. However, if one starts with fully reduced oxidase, the electron can also come from heme *a*. At the highest concentration of hydrogen peroxide (5 M), the initial phase of the reaction (at either 436 or 445 nm) can be deconvoluted into a 12 μs phase and a 65 μs phase. The 12 μs phase most likely corresponds to the rate of formation of compound F′, and the slower phase (65 μs) is likely the reduction of

compound F′ due to electron transfer from either heme *a* or Cu_B.

A closer examination of the data allows a rough estimate that in about 40% of the population, compound F′ is reduced to ferric heme a_3 by electron transfer from heme *a*, and in the remaining 60% of the population, the electron comes from Cu_B. An indication of this comes from the comparison of the flow-flash reaction of fully reduced enzyme with dioxygen. Figure 3 shows the optical changes at 445 nm for the reactions with dioxygen and with hydrogen peroxide. The notable feature in the trace of the dioxygen reaction is the distinct increase in the absorbance observed at 445 nm, followed by the continuing decrease. This rise or "bump" in the trace is caused by the transfer of an electron from Cu_A to reduce heme *a*, which had been oxidized at an early phase in the reaction with dioxygen (formation of compound P). In the reaction with hydrogen peroxide, if the electron used to reduce compound F′ were entirely from heme *a*, it is expected that the subsequent reduction of heme *a* would cause a similar rise in the absorbance at 445 nm. However, a clear rise or bump in the trace is not observed (Figure 3).

The alternative scenario is that the electron reducing compound F′ comes from Cu_B. This would leave the enzyme in a state where both heme *a* and Cu_A (the input centers) are reduced. In this case, the subsequent step would necessarily involve electron transfer from heme *a* to the binuclear center, which would result in a decrease in the absorbance at 445 nm or at most it would remain the same.

What is observed (Figure 1) is reasonably explained by a branched reaction sequence in which heme *a* is oxidized in about 40% of the population, yielding species E, and Cu_B is oxidized in about 60% of the population. The oxidation of Cu_B is the same reaction observed in the reaction of hydrogen peroxide with the mixed-valence enzyme (*18*). However, in the current experiment, starting with fully reduced enzyme, this leaves at least half of the enzyme in a form where both of the input redox centers are reduced and the heme−copper binuclear center is oxidized. We refer to this as the half-reduced form of the enzyme, or species H. This species H then reacts with the second equivalent of hydrogen peroxide.

Rapid Reaction of Species H with Hydrogen Peroxide and Its Implications. A rigorous kinetic analysis of the full transient in the reaction of the fully reduced enzyme is complicated by the apparent parallel mechanisms by which compound F′ is reduced. Nevertheless, it is possible to obtain a good estimate of the rate of electron transfer in the half-reduced enzyme (species H) to the fully oxidized heme a_3/Cu_B binuclear center, since this represents over half of the enzyme.

The lag phase in the reaction observed at 445 nm (Figures 2 and 3) has a duration of about 200 μs. This phase in the reaction includes electron transfer from reduced heme *a* to ferric heme a_3 in species H. The reduced heme a_3 would then be expected to be trapped by reaction with a second equivalent of hydrogen peroxide to form compound F (heme a_3 oxoferryl plus water, with oxidized Cu_B). Indeed, the transient that is observed at 580 nm during this portion of the reaction (the first 400 μs, Figure 4) is consistent with the product of the overall reaction being compound F. At 580 nm, the formation of compound F is observed more clearly than at 436 nm. The reason is that in the population of the enzyme with an electron left in the binuclear center, the electron is likely distributed between heme a_3 and Cu_B. Since oxidation of heme a_3 causes a significant absorbance decrease at 436 nm, the formation of compound F does not leave a clear signature at this wavelength.

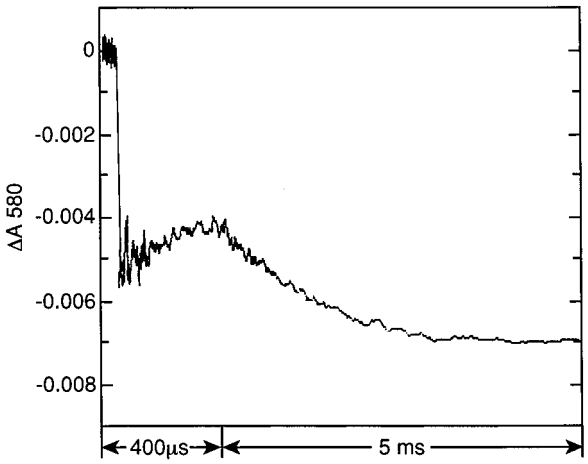

FIGURE 4: Flow-flash reaction of the fully reduced oxidase with 1 M H_2O_2 monitored at 580 nm. The increase in the absorbance at 580 nm is due to the formation of compound F, which then decays. Note that the time scale is split.

It is important to note that the reaction of hydrogen peroxide with species H (with reduced heme *a* and Cu_A) is considerably faster than with the fully oxidized enzyme. In other words, the second molecule of peroxide reacts considerably faster with the enzyme than does the third molecule of peroxide. At 436 nm, this is most evident in the trace of the reaction with 100 mM hydrogen peroxide (Figure 1). The transient observed at 580 nm (Figure 4) provides additional support for this conclusion. Indeed, compound F forms within the 100−200 μs phase as the product of the reaction with the second molecule of H_2O_2, while even at 1 M peroxide concentration, the third molecule of H_2O_2 takes about 4 ms to react. Clearly, the presence of electrons in the enzyme facilitates the reaction of hydrogen peroxide with heme a_3 to a great extent. This can be viewed as electron transfer from heme *a* to the binuclear center, forming the (unobserved) one-electron-reduced binuclear center (species E), which reacts very rapidly with hydrogen peroxide to yield compound F. This reaction is complete within about 200 μs, which puts a lower limit on the rate of electron transfer from heme *a* to heme a_3 of about 5000 s^{-1}. This is 10-fold faster than the rate V_{max} for the catalytic cycle (about 500 s^{-1} for the bovine enzyme). These data suggest that the steady-state rate of cytochrome *c* oxidation by hydrogen peroxide catalyzed by the enzyme under anaerobic conditions should be comparable to the rate of oxidase activity. Indeed, this is the case, and at 1 M hydrogen peroxide, the rate of cytochrome *c* peroxidase activity by the enzyme (measured at pH 7.5 in the absence of dioxygen) is about 1.5-fold higher than the "traditional" oxidase activity (Figure 5). The population of the enzyme with an electron in the binuclear center also forms compound F within the 200 μs phase. Thus, peroxide appears to be a very efficient trap of an electron in the binuclear center.

DISCUSSION

In the present work, the reaction of fully reduced cytochrome oxidase with hydrogen peroxide has been examined

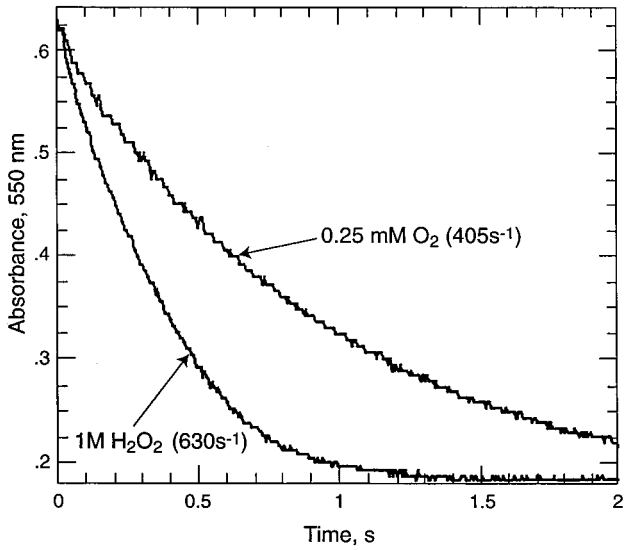

FIGURE 5: Stopped-flow reactions showing the cytochrome *c* peroxidase reaction and the cytochrome *c* oxidase reaction of the oxidase. In each case, one syringe contained both 80 μM cytochrome *c* and 40 nM cytochrome oxidase in the presence of 1 mM ascorbate. Before cytochrome oxidase was added, the solution of cytochrome *c* with ascorbate was degassed and purged with argon. For the peroxidase activity, the second syringe contained 1 M anaerobic H_2O_2, which then was substituted with air-saturated buffer without H_2O_2 for the oxidase activity measurement.

using the flow-flash technique. The primary motivation was to transiently generate the "half-reduced" form of the enzyme in which the two input centers (heme *a* and Cu_A) are reduced and the heme a_3/Cu_B binuclear center is oxidized (species H). This is an obligatory intermediate during steady-state turnover of the enzyme. Since hydrogen peroxide is a two-electron oxidant, one likely product of the initial reaction with the four-electron-reduced oxidase was expected to be species H (reaction 6 in Figure 6), and the data show that this is the case. Note that the oxidized status of the heme−copper binuclear center in species H is identical to that in the fully oxidized enzyme, but species H is capable of internal electron transfer. In the schematic shown in Figure 6, species H is united with Ox due to the common status of the binuclear center.

The interpretation of the kinetics data is greatly aided by the considerable previous experience studying the equivalent reaction with dioxygen (*5, 9, 27*) and also by the recent examination of the reaction of hydrogen peroxide with the mixed-valence form of the enzyme (*18*). It is expected that 2 equiv of peroxide will be consumed to oxidize the enzyme in two two-electron steps and that a third equivalent of peroxide then reacts with the fully oxidized enzyme to yield compound P_m. The data are consistent with this sequence of events. One deviation from the expectations is the observation that the traces at 445 nm recorded at 1 and 5 M H_2O_2 cross each other (Figure 2). However, this could be due to contamination of the 1 M H_2O_2 by a trace amount of dioxygen. Since the "bump" in the transient is very small in comparison to the corresponding trace with O_2 (Figure 3), any contamination must be quite small. If 1 M H_2O_2 is purposefully supplemented by 1 mM O_2, the transient becomes almost superimposable with the trace observed with O_2 alone in the absence of H_2O_2(not shown).

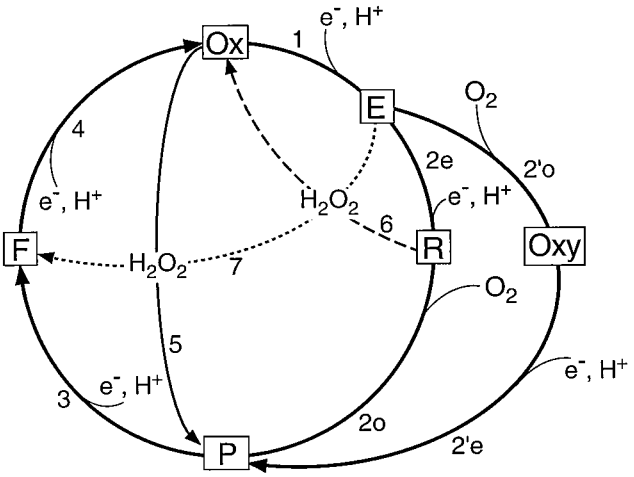

FIGURE 6: Oxidase and peroxidase cycles in cytochrome oxidase. The schematic illustrates mechanisms of O_2 and H_2O_2 reduction by cytochrome oxidase in terms of the status of the binuclear center. Both cycles start from the fully oxidized binuclear center (Ox). After species H is formed (denoted also as Ox), an electron goes into the binuclear center, forming species E. This species is H_2O_2-reactive and forms compound F in the reaction with H_2O_2 (reaction 7). At pH 7.5, this route is faster than the formation of compound P in the reaction of the fully oxidized enzyme with H_2O_2 (see text). Reduction of compound E yields species R with the fully reduced binuclear center. Species R is known to be a dioxygen-reactive species, but species E, in principle, can also react with O_2 (reaction 2′o) and then reduce into compound P. Species R reacts with O_2, yielding compound P. Species R reacts also with H_2O_2. In this reaction, the oxidized binuclear center is formed, and the mechanism includes transient formation of compound F′ (see text). Compound P, in turn, is reduced into compound F and, then, back into compound Ox. Reduction of compound F closes both oxidase and peroxidase cycles.

The model used to interpret the data must rationalize the very different rates of reaction of hydrogen peroxide with the heme a_3 Fe for each of the three forms of the enzyme with which the reaction is observed: fully oxidized, fully reduced, and the half-reduced form, species H. This can be done relatively simply based on two postulates: (i) The freshly generated, fully oxidized form of the oxidized binuclear center has hydroxide coordinated to the heme a_3 Fe; (ii) no ligand binding to ferric heme a_3 is possible without hydroxide first being displaced from this coordination site.

In flow-flash resonance Raman spectroscopy using dioxygen, it has been shown that the oxidized enzyme formed as the final product is hydroxide-coordinated to the heme a_3 iron (*13, 28*). These same resonance Raman experiments show that the hydroxide has a lifetime, measured as $^{18}O/^{16}O$ isotopic exchange with bulk water, of at least several milliseconds after its formation. It is unlikely that hydroxide coordinated to the ferric heme a_3 will dissociate on this time scale without becoming protonated and leaving the site as a water molecule. Protonation of the hydroxide ligated to the ferric heme a_3 is postulated to regulate the off-rate of the ligand on the time scale of interest, and therefore to regulate the maximal rate of reaction of peroxide with the ferric heme. In the case of the fully reduced enzyme with ferrous heme a_3, it is expected that water, rather than hydroxide, will be associated with the heme Fe, and the off-rate will not be rate-limiting for the reaction with peroxide. With these considerations in mind, the observed reaction sequence of hydrogen peroxide with the fully reduced enzyme is interpreted as the following:

1. Fully reduced enzyme rapidly reacts with hydrogen peroxide, forming compound F′, which quickly oxidizes Cu_B in about 60% of the population. This forms species H, the half-reduced enzyme, in which the heme/copper binuclear center is fully oxidized but the input centers are reduced (reaction 6 in Figure 6). This reaction of peroxide with ferrous heme a_3 is very fast since the water molecule coordinated to reduced heme a_3 readily leaves and allows ligand interaction with the heme iron.

2. In species H, formed in more than half of the enzyme population upon reduction of the first peroxide molecule, the heme/copper center is in the fully oxidized form. It is reasonable to presume that the freshly generated oxidized binuclear center has hydroxide coordinated to the heme a_3 Fe. Whereas in the fully oxidized enzyme the exchange of this hydroxide, presumably requiring protonation, takes a few milliseconds, the presence of two electrons in the input centers in species H stimulates the protonation as a result of electron transfer. Thus, at high concentrations of peroxide, the rate of ligand (peroxide) binding becomes concentration-independent and reflects the electron-transfer rate within species H from heme *a* to heme a_3 (reaction 1 in Figure 6). This process takes place within about 200 μs and includes the trapping of an electron in the heme/copper center, yielding compound F (reaction 7 in Figure 6). The last electron (F→Ox) (reaction 4 in Figure 6) is transferred at a much slower rate (about 1000 s^{-1}), in excellent agreement with previous measurements of the rate of reduction of compound F (*5*).

3. The formation of the oxidized enzyme (F→Ox) once again leaves the enzyme in a form with hydroxide coordinated to the ferric heme a_3 Fe. In the absence of electrons in the input centers, the rate of dissociation of the coordinated hydroxide limits the maximal rate of interaction of the oxidized heme a_3 with peroxide to 250 s^{-1} (reaction 5 in Figure 6).

The model described above has as its central feature that protonation of the hydroxide at the heme/copper center effectively creates the species that is capable of readily interacting with external ligands, including peroxide and, presumably, dioxygen. As an important consequence, the internal electron transfer in species H to the heme/copper center plays a dual role in the dioxygen reaction cycle. Not only does the electron transfer reduce the heme a_3 Fe, potentially capable of binding to dioxygen, but it also promotes protonation of the hydroxide associated with heme a_3, thus allowing dioxygen to bind.

Implications for Steady-State Peroxidase Activity of Cytochrome Oxidase. In the presence of hydrogen peroxide as well as a reductant such as cytochrome *c*, cytochrome oxidase exhibits peroxidase activity (*21, 29, 30*). Since peroxide can react with the binuclear center in three different states (reactions 5−7 in Figure 6), as described above, the routes by which peroxide is reduced will depend very much on the concentrations of reductant and peroxide. For example, when the rate of reduction of the binuclear center is high and the concentration of peroxide is very low, then it is likely that the fully reduced enzyme will be formed more rapidly from species H than the reaction with hydrogen peroxide. Hence, the dominant route for peroxide reaction with the enzyme will be via the fully reduced binuclear center (route 1−2e−6 in Figure 6). At the opposite extreme, when the rate of reduction of the binuclear center is very slow, then the reaction of the fully oxidized binuclear center with hydrogen peroxide to form compound P will be faster than the rate of formation of the one-electron-reduced binuclear center. Slow reduction will sequentially convert P to F and then F to Ox to complete the catalytic cycle (route 5−3−4 in Figure 6). At an intermediate level of hydrogen peroxide, the reaction with the one-electron-reduced binuclear center will predominate because the rate of formation of the fully reduced binuclear center is too slow to compete with the reaction of the one-electron-reduced species with peroxide (route 1−7−4 in Figure 6).

Implications for the Catalytic Cycle with Dioxygen. Two significant features of the reaction of cytochrome oxidase with hydrogen peroxide might also be applicable for the reaction with dioxygen: (1) peroxide can react very rapidly with the one-electron-reduced binuclear center and essentially serve to trap the electron; (2) the reaction with peroxide may be rate-limited by the need to eliminate hydroxide from the binuclear center.

It is plausible that dioxygen may also react very rapidly with the one-electron-reduced binuclear center (species E), though this has not been demonstrated experimentally. In the case of hydrogen peroxide, the product of the reaction is compound F, in which the O−O bond has been cleaved to form oxoferryl heme a_3. In the case of dioxygen, the initial product will be the Oxy complex with reduced heme a_3, which will then be converted to compound P_m (route 1−2′o−2′e−3−4 in Figure 6). By analogy with the reaction examined in the current work with peroxide, the initial reduction of heme a_3 generates the species that can readily bind O_2, and then the transfer of the second electron from the input centers (via heme *a*) will lead to compound P_m.

$$\text{species H} + H_2O_2 \rightarrow [\text{heme } a^{2+}]\text{F} \rightarrow \text{Ox}$$

$$\text{species H} + O_2 \rightarrow [\text{heme } a^{2+}]\text{Oxy} \rightarrow P_m$$

If this occurs readily, then this could plausibly be the preferred pathway in the steady-state dioxygen catalytic cycle. Usually, the oxygen-reactive species is viewed as being the two-electron-reduced binuclear center. The current work suggests the possibility that this might not be the case. There is also an early report (*31*) demonstrating the formation of a one-electron-reduced binuclear center, with CO bound to ferrous heme a_3 (Fe^{2+}−CO) with oxidized Cu_B^{2+}, showing an EPR signal from Cu_B^{2+} at $g = 2.28$. Note that this model presumes that the two-electron reduction of the fully oxidized enzyme yields a species which is dioxygen-reactive. Indeed, previous results (*14, 15*) show that in the one-electron-reduced enzyme there is no electron transfer to the binuclear center on a time scale that is consistent with the overall turnover rate.

Apparently, the oxidation of heme a_3 yields the $Fe^{3+}OH^-$ species, requiring that hydroxide must be eliminated prior to any reaction with heme a_3. Possibly, this is the rate-limiting feature in the reaction of the oxidized enzyme with hydrogen peroxide. Regardless of whether dioxygen reacts with the one-electron- or two-electron-reduced binuclear center, it is necessary for hydroxide to be displaced first. Since it is clear that reduction of the binuclear center is accompanied by protonation (one proton taken up per

electron), it is reasonable to postulate that rapid delivery of a proton to convert hydroxide to water is likely.

The current work suggests that the state of cytochrome oxidase with a single electron in the binuclear center (i) rapidly reacts with peroxide, forming compound F; (ii) can be formed from species H in 100−200 μs; and (iii) has a water molecule coordinated with heme a_3 rather than hydroxide. It appears that proton delivery to heme a_3 upon reduction not only stabilizes the reduced state capable of O_2 binding, but also facilitates the ligand exchange (H_2O for H_2O_2 and O_2) required for this reaction to proceed. The state of the enzyme [heme a_3^{2+}−$(H_2O)Cu_B^{2+}$] can be effectively trapped with peroxide in the flow-flash experiments and also during the steady-state peroxidase reaction.

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BI9916675