# Observation of a Novel Transient Ferryl Complex with Reduced $Cu_B$ in Cytochrome *c* Oxidase†

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ABSTRACT: The reaction between mixed-valence (MV) cytochrome *c* oxidase from beef heart with $H_2O_2$ was investigated using the flow-flash technique with a high concentration of $H_2O_2$ (1 M) to ensure a fast bimolecular interaction with the enzyme. Under anaerobic conditions the reaction exhibits 3 apparent phases. The first phase ($\tau \cong 25$ $\mu$s) results from the binding of one molecule of $H_2O_2$ to reduced heme $a_3$ and the formation of an intermediate which is heme $a_3$ oxoferryl ($Fe^{4+}{=}O^{2-}$) with reduced $Cu_B$ (plus water). During the second phase ($\tau \cong 90$ $\mu$s), the electron transfer from $Cu_B^+$ to the heme oxoferryl takes place, yielding the oxidized form of cytochrome oxidase (heme $a_3$ $Fe^{3+}$ and $Cu_B^{2+}$, plus hydroxide). During the third phase ($\tau \cong 4$ ms), an additional molecule of $H_2O_2$ binds to the oxidized form of the enzyme and forms compound P, similar to the product observed upon the reaction of the mixed-valence (i.e., two-electron reduced) form of the enzyme with dioxygen. Thus, within about 30 ms the reaction of the mixed-valence form of the enzyme with $H_2O_2$ yields the same compound P as does the reaction with dioxygen, as indicated by the final absorbance at 436 nm, which is the same in both cases. This experimental approach allows the investigation of the form of cytochrome *c* oxidase which has the heme $a_3$ oxoferryl intermediate but with reduced $Cu_B$. This state of the enzyme cannot be obtained from the reaction with dioxygen and is potentially useful to address questions concerning the role of the redox state in $Cu_B$ in the proton pumping mechanism.

Cytochrome *c* oxidase is the terminal enzyme in respiratory chains of mitochondria and of many bacteria (*1*−*3*). The enzyme provides all four reducing equivalents required to reduce dioxygen to two waters. The mechanism of dioxygen reduction by cytochrome oxidase has long been a topic of intensive study.

In addition to the reduction of dioxygen, cytochrome oxidase is a critical component in the energy conservation process (*4*−*6*). Free energy is conserved by two different processes in the enzyme (*1, 7*−*9*). First, since the binding site for cytochrome *c* is located at the opposite side of the membrane to the side from which the protons used for dioxygen reduction are taken up, the chemical reaction itself results in a net translocation of one electric charge per electron transferred. Second, cytochrome oxidase is a proton pump which translocates an additional four protons per dioxygen molecule across the membrane. The steps of the catalytic cycle which are coupled to proton translocation and the mechanism by which this occurs are of great interest (*9*−*12*).

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The steps involved in the reduction of dioxygen are very fast and require specific kinetic approaches for investigation (*1, 10, 11, 13*). Since the first work of Gibson and Greenwood (*14*) the flow-flash technique has been widely used to investigate the mechanism of dioxygen reduction by the enzyme (*1*). In this approach, the CO adduct of either the fully reduced or two-electron reduced (mixed-valence) forms of the enzyme is first made. Then, after being rapidly mixed with dioxygen, the CO is rapidly photodissociated by a short laser pulse to initiate the reaction of the enzyme with dioxygen. Several distinct steps in the interaction of the fully or partially reduced forms of the enzyme with dioxygen can be resolved using this approach (*1, 13, 15*−*18*).

In the flow-flash experiments, the initial form of the cytochrome *c* oxidase may be fully reduced (four-electron reduced) or two-electron reduced, the so-called mixed-valence (MV) form in which only the two metals in the dioxygen-reactive heme/copper center are reduced. Figure 1 briefly summarizes the intermediates obtained in the reactions of these two forms of the enzyme with dioxygen. If all four redox centers of cytochrome oxidase are reduced before the reaction with dioxygen, then dioxygen undergoes complete reduction to two water molecules, yielding also the fully oxidized form of the enzyme (*1, 19*). This reaction proceeds via intermediates Oxy (or A), $P_r$, and F and is pictured as the pathway on the right side of the scheme in Figure 1 (FR → Oxy → $P_r$ → F → Ox). After the initial binding of dioxygen to reduced heme $a_3$ (compound A), there

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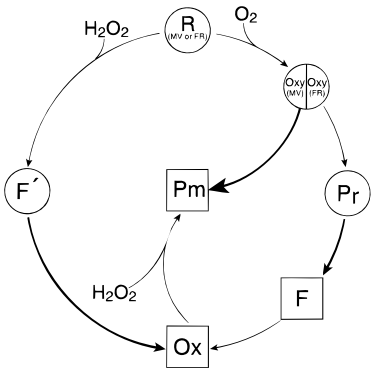


FIGURE 1: Schematic diagram showing the various reactions discussed in the text. The species indicate the state of the binuclear center. Two forms of the enzyme are depicted which have a reduced binuclear center. These forms are the fully reduced (FR, four-electron reduced) enzyme and the mixed-valence form of the enzyme (MV, two-electron reduced). In either case, oxygen binds to form the initial Oxy complex. With the fully reduced enzyme, the reaction proceeds along the outside branch, forming $P_r$, then F, and finally the fully oxidized enzyme, Ox. If the starting point is the mixed-valence form of the enzyme, the product after Oxy is species $P_m$. The reaction of the mixed-valence form of the enzyme with hydrogen peroxide is shown on the left-hand branch of the scheme. In this case, the initial product is F′, which converts to Ox and then reacts with a second molecule of hydrogen peroxide to form product $P_m$. Species in circles are presumed to have reduced $Cu_B$, and those in squares have oxidized $Cu_B$. The three bold arrows all represent reactions that involve electron transfer from $Cu_B$ to an oxygenated form of heme $a_3$ (Oxy $\rightarrow$ $P_m$, $P_r \rightarrow$ F, and F′ $\rightarrow$ Ox), and it is noted that all have similar rates ($\tau = 100$ $\mu$s).

is a rapid electron transfer from heme *a* to the heme $a_3/Cu_B$ binuclear center. This transiently forms compound $P_r$, which is defined by an increased absorbance at 607 nm (relative to the oxidized binuclear center), and formally contains three reducing equivalents at the heme/copper center (from heme a, heme $a_3$, and $Cu_B$) (*16*). In the next step of the reaction, compound $P_r$ is converted to compound F (oxoferryl heme $a_3$) while, simultaneously, the fourth electron is transferred from $Cu_A$ to heme *a*. Finally, in the last step, the last electron is transferred from heme a to the binuclear center, reducing the oxoferryl heme $a_3$ ($Fe^{4+}{=}O^{2-}$) to the ferric heme ($Fe^{3+}$ $HO^-$) (*20*).

In contrast, when MV cytochrome oxidase (two-electron reduced form) reacts with dioxygen, the reaction stops at the peroxy intermediate, because only two reducing equivalents are available in the enzyme (*15*). This is also shown in Figure 1 (MV $\rightarrow$ Oxy $\rightarrow$ $P_m$). However, although by optical spectroscopy the product appears to be the same as compound $P_r$, there are only two reducing equivalents present at the heme/copper center. This is called compound $P_m$. It is generally believed that the oxygenated state of heme $a_3$ is the same in both compounds $P_r$ and $P_m$, but that in $P_r$ $Cu_B$ is reduced whereas in $P_m$, $Cu_B$ is oxidized (*16*). The actual structure of compound P (either $P_r$ or $P_m$) is still unresolved.

In steady-state turnover of the enzyme, when the rate limitation is the delivery of electrons to the enzyme (*21*), it seems very likely that the intermediate formed will correspond to compound $P_m$ though this has not yet been demonstrated directly. Presuming this to be the case, then the conversion of $P_m$ to compound F is coupled to proton pumping. Attempts to demonstrate proton pumping during the $P_r$ to F transition using the flow-flash approach require further clarification. No proton release from reconstituted vesicles with cytochrome oxidase is observed (*22*, *23*), but substantial charge translocation, measured by the development of a membrane potential, appears associated with this step (*10*). By comparison, both proton release from reconstituted vesicles and charge translocation are clearly associated with the F-to-Ox transition (*10*−*12*, *22*).

Proposed models of proton pumping, such as the histdine cycle (*24*, *25*), invoke changes in the redox status of $Cu_B$ as a key element in the mechanism of coupling. Hence, one might expect differences in the coupling to proton pumping for the $P_r$-to-F and $P_m$-to-F transitions. Similarly, the efficiency of proton pumping in the F-to-Ox transition might also depend critically on the redox state of $Cu_B$ in the oxoferryl form of the cytochrome oxidase. Examining and comparing these redox transitions and states of the enzyme could help clarify the mechanism of the proton pump. The aim of the current work is to demonstrate a method to generate the state of cytochrome oxidase with the oxoferryl form of heme $a_3$ in which $Cu_B$ is reduced.

The oxoferryl form of the cytochrome *c* oxidase with reduced $Cu_B$ does not form in any of the reactions with dioxygen, so a different approach has to be used to prepare this compound (here referred to as F′). The approach undertaken in this work is based on the fact that cytochrome oxidase can utilize not only dioxygen as an oxidant but also hydrogen peroxide (*26*−*28*). Since $H_2O_2$ is the two-electron reduced form of $O_2$, it can be expected to react with forms of cytochrome oxidase with reduced heme $a_3$ to transiently form an oxoferryl complex. Starting with the two-electron reduced form of the enzyme, one expects the initial product to be oxoferryl with reduced $Cu_B$ (Figure 1).

In the present work, the reaction of MV (two-electron reduced) cytochrome *c* oxidase with $H_2O_2$ is examined. It is demonstrated that compound F′ is an intermediate in this reaction and that both the formation and the decay of F′ can be kinetically resolved. Compound F′ decays into the fully oxidized form of the enzyme which, in excess $H_2O_2$, reacts further to form compound $P_m$, which is the same form of the enzyme resulting from the reaction of MV cytochrome *c* oxidase plus dioxygen.

## MATERIALS AND METHODS

Beef heart cytochrome oxidase used in this work was kindly donated by Dr. Shinya Yoshikawa and represents a preparation of redissolved crystals.

The reagents used, potassium ferricyanide (Sigma), potassium ferrocyanide (Sigma), hydrogen peroxide, HEPES (Sigma), glucose, and glucose oxidase, were of analytical grade.

*Preparation of MV*. A 10 $\mu$M solution of cytochrome oxidase (100 mM HEPES, pH 7.4, in the presence of 50 mM ferricyanide) in a Thunberg cuvette was degassed and reequilibrated with nitrogen several times by means of a gas line. The nitrogen was exchanged for carbon monoxide, and 50 mM ferrocyanide was added anaerobically. The mixture was incubated until the full development of the 592 nm spectroscopic band in the difference optical spectrum that is diagnostic of MV cytochrome *c* oxidase.

*Apparatus*. The flow-flash investigation was performed using the same apparatus as described in ref *29* with some modifications. The metal syringe needles were substituted

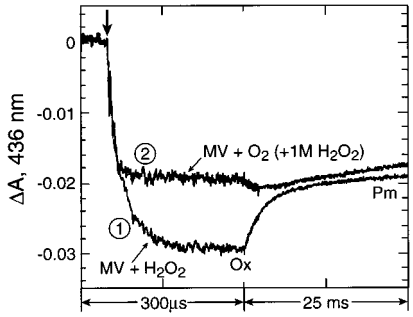


FIGURE 2: Reaction of the mixed-valence cytochrome oxidase with dioxygen and hydrogen peroxide. The transients at 436 nm show the time course of the reactions between the mixed-valence cytochrome oxidase and dioxygen (trace 2) or hydrogen peroxide (trace 1). In the reaction with $H_2O_2$ the oxidized form of the enzyme is formed within 100 $\mu$s. This is followed by reaction with an additional $H_2O_2$ molecule on the millisecond time scale, yielding compound $P_m$. In the reaction with dioxygen the final product is formed in the initial reaction with dioxygen within the microsecond time scale, so that no additional slower phase is present. The conditions of the reaction are described in the text. The reactions were initiated by a laser flash after mixing the cytochrome oxidase solution with either aerobic or anaerobic solutions of $H_2O_2$. The final solutions contained about 2 $\mu$M oxidase and 1 M $H_2O_2$ in 100 mM HEPES buffer (pH 7.5) and 0.1% lauryl maltoside. In the case of the aerobic $H_2O_2$ solution, the final solution also contained 1 mM dioxygen.

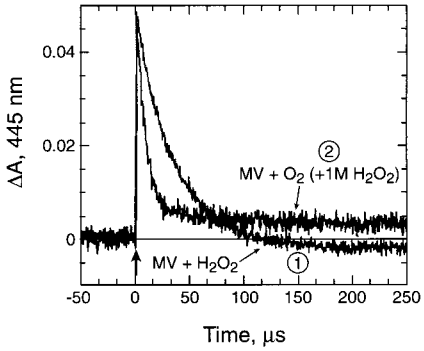


FIGURE 3: Oxidation of the mixed-valence cytochrome oxidase by $O_2$ or $H_2O_2$. The transients at 445 nm show the time course of conversion of the enzyme into either Ox or $P_m$ caused by the reactions with either $H_2O_2$ or $O_2$, respectively. The conditions are the same as described in Figure 2.

by Teflon tubing to avoid decomposition of $H_2O_2$ into oxygen catalyzed by metal surfaces. All the surfaces were tested to ensure that they did not cause $H_2O_2$ decomposition. The stopped-flow line for $H_2O_2$ was first washed using a solution of 100 mM glucose in the presence of glucose oxidase to ensure consumption of any dioxygen adsorbed on the surfaces. The syringe and tubing used for the enzyme were washed with dithionite and then with anaerobic water.

After the transients of the reaction with anaerobic $H_2O_2$ solution were recorded, the peroxide solution was saturated with $O_2$ and the reaction with the same sample of MV cytochrome *c* oxidase was studied. Thus, exactly the same concentration of the enzyme was assured in each case.

## RESULTS

*Reaction of MV Enzyme with $H_2O_2$.* Flash-induced absorbance changes were monitored at 436 nm because this is essentially an isosbestic point both for CO dissociation and for redox shifts in heme a while, at the same time, 436 nm is close to the maximum of the P-minus-Ox difference spectrum (*30*).

Figure 2 shows the transients obtained by following the reaction between MV cytochrome *c* oxidase with either anaerobic (trace 1) or aerobic (1 mM $O_2$; trace 2) solutions of hydrogen peroxide. In the absence of $O_2$, there is a larger initial absorbance change in the microsecond time scale compared to the transient obtained in the presence of 1 mM $O_2$, and under anaerobic conditions there is an additional phase of the reaction which is completed within 25 ms ($\tau = 4$ ms). The microsecond phase of the absorbance change in the case of anaerobic $H_2O_2$ is about 1.5-fold larger than in the case of the aerobic solution, because the product formed after 100 microsecond in the anaerobic reaction of MV with $H_2O_2$ is Ox, which absorbs less at 436 nm than does P, the product in the reaction with $O_2$. The millisecond phase of the reaction (half-time about 4 ms), observed only under anaerobic conditions, is also expected due to the reaction of the 100 microsecond product (Ox) with a second molecule of $H_2O_2$ to form compound P.

Note that the millisecond phase of the reaction is essentially abolished by the presence of 1 mM dioxygen. The initial rate of reaction of 1 mM dioxygen with the MV cytochrome *c* oxidase is about 3-fold faster than observed with 1 M $H_2O_2$ (under anaerobic conditions), as shown in Figure 3. Hence, it is clearly necessary to remove $O_2$ from the solution as well as to use a very high concentration of $H_2O_2$ (1 M) to ensure that the observed reaction is actually with peroxide. In the presence of 1 M $H_2O_2$, trace amounts of $O_2$ will have little effect, since a relatively high concentration of $O_2$ is necessary to effectively compete with the peroxide reaction. Under aerobic conditions, the reaction of MV cytochrome *c* oxidase with $O_2$ predominates and quickly yields compound P. Under anaerobic conditions, two sequential reactions with peroxide are required to form the same product, compound P.

Figure 3 shows the transient absorbance changes at 445 nm for the initial reaction (microsecond phase). Again, it is seen that the reaction with 1 M $H_2O_2$ is slower than with 1 mM $O_2$. The characteristic time constant of the primary interaction is $\sim$25$-$30 $\mu$s with peroxide vs 10 $\mu$s in the reaction with $O_2$. When both oxidants are present at these concentrations, the reaction proceeds predominantly with $O_2$ and not with hydrogen peroxide. The magnitude of the optical change at 445 nm is slightly larger in the case of $H_2O_2$ compared to the reaction with $O_2$. This reflects the difference in absorbance of the two products, between $P_m$ (from dioxygen) and Ox (from peroxide), though this difference in amplitude is much smaller at 445 nm than at 436 nm.

*Observation of Compound F′.* During the initial reaction of MV cytochrome *c* oxidase with the first molecule of $H_2O_2$, the transient formation of an oxoferryl intermediate with an electron on $Cu_B$ (F′) is expected (Figure 1). Then F′ is expected to convert into compound Ox, analagous to the conversion of compound $P_r$ into F during reaction of fully reduced cytochrome oxidase with $O_2$ (i.e., electron transfer from $Cu_B$ to the heme/oxygen adduct). To address the question of whether the formation and subsequent decay of compound F′ can be observed, the transients at 580 and 428 nm upon the reaction of MV cytochrome *c* oxidase with $H_2O_2$ were monitored (Figure 4). An isobestic point in the F-minus-Ox difference spectrum is 428 nm (*30*), and it is presumably

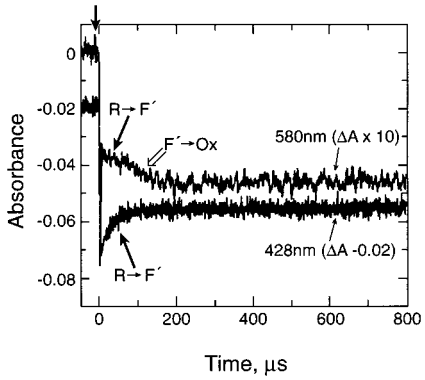


FIGURE 4: Transient observation of compound F′. The transient at 428 nm shows the rate of formation of compound F′ in the initial reaction between mixed-valence cytochrome oxidase and $H_2O_2$. The transient at 580 nm displays a lag phase after initial CO dissociation, which coincides with the rate of compound F′ formation. The lag phase is followed by the absorbance decrease as expected for the F′ → Ox conversion. The conditions are the same as described in Figures 2 and 3.

also the isobestic point for the F′-minus-Ox difference spectrum. The maximum absorbance changes expected in the F′-minus-Ox difference spectrum are expected to be at 580 nm (*30, 31*) so this wavelength was selected to monitor the decay of F′ to Ox.

The transient at 428 nm appears monophasic and has the characteristic time of ~30 $\mu$s, similar to the transient at 445 nm (Figure 3). These data are too noisy to attempt deconvolution into component parts with any confidence. However, the transient at 580 nm is clearly consistent with the formation and subsequent decay of compound F′. After the initial absorbance change due to CO dissociation, the absorbance decreases (Figure 4). The 30 $\mu$s phase, seen in the transients at 445 and 436 nm, appears as a lag phase when monitored at 580 nm. The lag phase is followed by a further absorbance decrease, indicating that the events in the microsecond time scale involve two kinetically distinguishable processes. The absorbance decrease at 580 nm is consistent with the F′-to-Ox conversion and has a characteristic time constant of about 80−90 $\mu$s.

Thus, the results demonstrate that, at 1 M $H_2O_2$, the initial interaction with reduced heme $a_3$ is fast enough to resolve the internal electron transfer during the F′-to-Ox conversion following the initial reaction with $H_2O_2$.

## DISCUSSION

The reaction of MV cytochrome *c* oxidase with hydrogen peroxide does not proceed via intermediates that are common with those obtained during reaction of the fully or partly reduced cytochrome oxidase with $O_2$ (Figure 1) and presumably also during steady-state turnover with $O_2$. In the reaction with peroxide, compound F′ is formed, which has an additional electron in the binuclear center on $Cu_B$, analogous to compound $P_r$. Thus, the reaction of $H_2O_2$ with partly or fully reduced cytochrome oxidase is of interest to explore the effects of the redox state of $Cu_B$ on the proton pumping mechanism and the properties of the oxygenated transients formed during the reaction.

*Formation of F′.* The formation of compound F′ is the initial step in the reaction between MV cytochrome *c* oxidase and $H_2O_2$. At 1 M $H_2O_2$, the reaction proceeds fast enough ($\tau \cong 25-30$ $\mu$s) to be resolved from the subsequent electron transfer from $Cu_B$ resulting in Ox ($\tau \cong 80-90$ $\mu$s).

The reaction of MV cytochrome *c* oxidase with peroxide is potentially complicated by the phenomenon of electron back-flow (*13, 32, 33*). Photolysis of CO changes the redox midpoint potential of heme $a_3$, resulting in electron redistribution to heme *a* and $Cu_A$. The rate of compound F′ formation (25 $\mu$s) is much slower than the rate of the electron equilibration from heme $a_3$ to heme *a* (3 $\mu$s). Thus, there will be equilibration of the distribution of the electron between the hemes prior to $H_2O_2$ binding. The electron also redistributes to $Cu_A$, but with a characteristic time of about 50 $\mu$s (*13*), i.e., comparable to the rate of compound F′ formation upon $H_2O_2$ binding under these conditions. Hence, a reaction of peroxide with oxidized heme $a_3$ is possible. However, this is unlikely to be a significant process. Only a relatively small fraction (10−20%) of the enzyme population has oxidized heme $a_3$ following photolysis. It is very likely that water (55 M) will rapidly associate with the heme $a_3$ iron after photolysis and that dissociation of this water molecule must precede the reaction with $H_2O_2$. Due to the stronger interaction of the water dipole, the rate of dissociation of water from ferric heme $a_3$ will be substantially slower than from ferrous heme $a_3$. This again argues that any population of oxidized heme $a_3$ that is present due to electron back-flow will not contribute significantly to the observed reaction with peroxide. At pH 7.4 a small fraction of the enzyme also becomes deprotonated following CO photolysis, but on a time scale of about 1 ms (*13*). This proton is presumably still associated with the enzyme during the more rapid reaction with peroxide.

*The F′-to-Ox Conversion and Proton Distribution.* This reaction has a characteristic time of about 80−90 $\mu$s. The F′-to-Ox transition observed in this work is substantially faster than the corresponding F-to-Ox transition, starting with an electron on heme *a* instead of on $Cu_B$ (*1*). The rate of the F-to-Ox transtion has been measured both in the reaction of the fully reduced enzyme with dioxygen (flow-flash) (*1*) and by photoinduced electron injection to preformed compound F (*11, 12, 34, 35*). In both cases, the electron transfer from heme *a* to the oxoferryl form of the binuclear center takes about 1 ms. Reduced $Cu_B$ does not accumulate during this reaction (*35*), so if it forms at all as an intermediate, its rate of oxidation must be substantially more rapid than its rate of formation. This is consistent with the current measurement showing that the halftime for the electron transfer from $Cu_B$ to heme $a_3$ oxoferryl is about 100 $\mu$s. It is worth noting that other reactions that involve electron transfer from $Cu_B$ to oxygenated heme $a_3$ species also have half-times of about 100 $\mu$s: $P_r$ to F (*36*) and Oxy to $P_m$ (*37*) (Figure 1).

The difference in the rates of the F′-to-Ox and F-to-Ox transitions is likely to be at least in part due to different proton distributions in the enzyme in the two types of experiments (*38*). In compound F′ not only is $Cu_B$ reduced but there is also an additional proton bound to the enzyme (*39*), presumably at the heme/copper center. Hence, the (intramolecular) proton-transfer reactions that accompany the electron transfer from $Cu_B$ to the heme oxoferryl (F′-to-Ox) will be different from the proton movements (i.e., proton uptake from the bulk solution) accompanying the electron transfer from heme *a* (F-to-Ox). The differences in proton distribution are likely to be critical for determining the

efficiency of coupling to proton pumping, as well as the rates of the chemical reactions occurring at the active site. It is clear that a knowledge of both the proton and electron distributions in the enzyme during turnover is essential in order to understand the mechanism of the enzyme.

*Hydrogen Peroxide Binding to the Fully Oxidized Binuclear Center*. In the reaction of MV cytochrome *c* oxidase with peroxide (<1 M), the reaction of the first peroxide molecule (MV-to-F′) is about 100-fold faster than the reaction of the second peroxide, which reacts with the fully oxidized form of the enzyme (Ox-to-$P_m$). The product of the reaction with the first peroxide with MV cytochrome *c* oxidase (i.e., after ∼100 $\mu$s) should be a form of the fully oxidized enzyme with hydroxyl, at least initially, bound to ferric heme $a_3$ (*1*). The protonation of this hydroxyl could form water directly at the ferric heme iron, and dissociation of this water is necessary prior to the binding of the second peroxide. This could limit the rate of the reaction with the second peroxide. It is of interest that the resonance Raman signal (450 $cm^{-1}$) that is assigned to the heme $a_3$ iron−hydroxide and that is observed during the last step in the reaction of the fully reduced enzyme with dioxygen decays on the millisecond time scale (*20*, *40*). This is consistent with the idea that the protonation and dissociation of this hydroxide are perhaps rate limiting for the reaction of the second peroxide with MV cytochrome *c* oxidase under the experimental conditions examined.

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