Formation of the "Peroxy" Intermediate in Cytochrome *c* Oxidase Is Associated with Internal Proton/Hydrogen Transfer

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ABSTRACT: When dioxygen is reduced to water by cytochrome *c* oxidase a sequence of oxygen intermediates are formed at the reaction site. One of these intermediates is called the "peroxy" (P) intermediate. It can be formed by reacting the two-electron reduced (mixed-valence) cytochrome *c* oxidase with dioxygen (called P_m), but it is also formed transiently during the reaction of the fully reduced enzyme with oxygen (called P_r). In recent years, evidence has accumulated to suggest that the O−O bond is cleaved in the P intermediate and that the heme a_3 iron is in the oxo-ferryl state. In this study, we have investigated the kinetic and thermodynamic parameters for formation of P_m and P_r, respectively, in the *Rhodobacter sphaeroides* enzyme. The rate constants and activation energies for the formation of the P_r and P_m intermediates were 1.4×10^4 s^{-1} (∼20 kJ/mol) and 3×10^3 s^{-1} (∼24 kJ/mol), respectively. The formation rates of both P intermediates were independent of pH in the range 6.5−9, and there was no proton uptake from solution during P formation. Nevertheless, formation of both P_m and P_r were slowed by a factor of 1.4−1.9 in D_2O, which suggests that transfer of an internal proton or hydrogen atom is involved in the rate-limiting step of P formation. We discuss the origin of the difference in the formation rates of the P_m and P_r intermediates, the formation mechanisms of P_m/P_r, and the involvement of these intermediates in proton pumping.

Cytochrome *c* oxidase catalyzes the one-electron oxidation of four cytochrome *c* molecules and the four-electron reduction of dioxygen to water (for review see refs *1* and *2*). Initially, electrons are transferred from cytochrome *c* to the primary electron acceptor, Cu_A. Reduction of Cu_A is followed by intramolecular electron transfer to heme *a* and then to a binuclear center consisting of heme a_3 and Cu_B, which is the binding site for dioxygen. During reaction of the reduced enzyme with O_2 different oxygen intermediates build up to detectable concentrations. The dynamics of formation and decay of these intermediates can be followed using the flow-flash technique (*3*). The anaerobic, CO-bound, reduced enzyme is mixed rapidly with a buffer containing dioxygen. The reaction of the enzyme with oxygen is limited by the rate of CO dissociation, which is slow under normal experimental conditions, but increases dramatically upon illumination. Therefore, pulsed illumination of the CO-bound enzyme in the presence of oxygen results in instantaneous dissociation of the CO ligand and initiation of the reaction of the reduced enzyme with oxygen.

Binding of dioxygen to the reduced binuclear center results in formation of the oxo-ferrous intermediate (A) as first described by Chance and colleagues (*4*). Chance et al. (*4*) also found that the reaction of the two-electron reduced (mixed-valence) enzyme with O_2 results in formation of a stable intermediate at the "peroxy-oxidation" level (intermediate P_m, referred to by Chance et al. as intermediate C).[1] Formation of the P_m intermediate can be followed in time using the flow-flash technique starting with the mixed-valence enzyme. For the bovine enzyme, the rate constant of its formation was found to be $\sim 6 \times 10^3$ s^{-1} (*5*, *6*). On the other hand, when the fully reduced bovine enzyme reacts with O_2, a transient peroxy intermediate (P_r) is formed with a rate of 3×10^4 s^{-1} (*6*−*9*), concomitant with electron transfer from heme *a* to the binuclear center. Even though the P_r intermediate has an additional electron at the binuclear center (as compared to intermediate P_m), the optical absorption spectra of intermediates P_r and P_m are the same (*10*, *11*), indicating that their molecular structures are similar. During reaction of the fully reduced enzyme with oxygen, the decay of the P_r intermediate is accompanied by formation of the oxo-ferryl intermediate (F) without additional electron transfer to the binuclear center. The transfer of the fourth electron from the Cu_A/heme *a* equilibrium to the binuclear

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[1] Abbreviations: DIE, deuterium isotope effect, defined as the ratio of the rate constants (k_H/k_D) measured in H_2O and D_2O, respectively; τ, time constant ($1/k$); R, fully reduced enzyme; A, ferrous-oxy intermediate; P_m and P_r, the peroxy intermediate formed upon reaction of the mixed-valence and fully reduced enzyme, respectively, with oxygen; F, oxo-ferryl intermediate; O, fully oxidized enzyme; amino acid residue and mutant enzyme nomenclature: M(II-263) denotes methionine-263 of subunit II; ML(II-263) denotes a replacement of methionine-263 of subunit II by leucine. Amino acid residues are numbered according to the *R. sphaeroides* sequence.

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center results in decay of the F intermediate and formation of the fully oxidized enzyme (O).

For a long time it was assumed that in the P intermediate the O−O bond was intact (for review, see refs *12* and *13*), i.e., the structure of this intermediate was $Fe_{a3}^{3+}-O^{-}-O^{-}(H^{+})$. However, abundant spectroscopic data have been accumulated indicating that in the P state the intermediate at the binuclear center does not have a peroxy structure, but rather the O−O bond is broken and an oxo-ferryl state ($Fe_{a3}^{4+}{=}O^{=}$) is formed (*14*−*20*). Proshlyakov et al. (*21*) used resonance-Raman spectroscopy to show that during reaction of the mixed-valence bovine cytochrome *c* oxidase with O_2, the band corresponding to the $Fe_{a3}^{4+}{=}O^{=}$ stretching motion appears with the same kinetics ($\tau \cong 200\ \mu s$) as the (kinetic) optical absorption spectrum of the P_m intermediate. In another recent study, using mass spectrometry, Fabian et al. (*22*) showed that upon reaction of the mixed-valence enzyme with an O_2 isotope (forming P_m), one of the oxygen atoms of the O_2 molecule is found in a water molecule that equilibrates with the bulk solution.

Collectively, the above-discussed results show that in the P_m state the O−O bond is cleaved, and an oxo-ferryl structure is found at heme a_3, a scenario that requires a transfer of a total of four electrons from the enzyme to dioxygen:

$$[Fe_{a3}^{2+}-O_2] + Cu_B^{+} + e^{-} + H^{+} \rightarrow [Fe_{a3}^{4+}{=}O^{2-}] + [Cu_B^{2+}-OH^{-}] \quad (1)$$

i.e., two electrons are taken from heme a_3 ($Fe_{a3}^{2+} \rightarrow Fe_{a3}^{4+}$) and one electron from Cu_B ($Cu_B^{+} \rightarrow Cu_B^{2+}$) and one more from another source. During reaction of the fully reduced enzyme with O_2, this electron is transferred rapidly from heme *a* ($Fe_a^{2+} \rightarrow Fe_a^{3+}$) to the binuclear center upon formation of P_r. In the reaction of the mixed-valence enzyme with O_2, different candidates have been suggested as the source of this electron. Because it is located close to the binuclear center, the most plausible candidate is tyrosine (I-288) (*13*, *23*−*25*), which presumably forms a tyrosyl radical. A radical has been found during reaction of cytochrome *c* oxidase with hydrogen peroxide (*26*, *27*) (which results in formation of the P intermediate at the binuclear center), although the location of this radical in the protein has not been identified. In the fully reduced enzyme, the radical species would presumably not be built up because of the rapid electron transfer from heme *a*.

Yoshikawa et al. (*28*) found that in the bovine enzyme Y(I-288) is covalently bound to H(I-284), one of the ligands of Cu_B. The same configuration was found in the *Paracoccus denitrificans* enzyme (*29*). The cross-linking between the tyrosine and the histidine was also confirmed from results using chemical methods (*30*). The structure is unusual and may hint a specific role for Y(I-288) in oxygen reduction and/or proton pumping.

From the above summarized results, questions emerge concerning the differences of the energetics of formation of intermediates P_m and P_r, whether both intermediates are formed during the catalytic cycle of cytochrome *c* oxidase (coupled to proton pumping) and the specific involvement of Y(I-288). The experiments presented in this work were designed toward addressing the similarities and differences and the mechanisms of formation of intermediates P_m and P_r, respectively.

MATERIALS AND METHODS

Mutagenesis, Growth of Bacteria, and Enzyme Purification. The *Rhodobacter sphaeroides* bacteria were grown in Sistrom's medium as described (*31*). Ni^{2+}-NTA affinity chromatography was used for purification of the wild-type and the ML(II-263) mutant enzymes (*32*). The bovine enzyme was prepared using the method of Brandt et al. (*33*). Mutagenesis was performed as described previously (*34*). Enzyme stock solutions were kept under liquid nitrogen until use.

Preparation of the Mixed-Valence and Fully Reduced CO-Bound Enzyme. To exchange H_2O for D_2O, the enzyme was repetitively diluted with 0.1 M Hepes buffer at pH 7.5, 0.05% dodecyl-β-D-maltoside in D_2O and reconcentrated using centricon-50 tubes (Amicon Inc.) (<3% H_2O remained). To prepare the fully reduced enzyme, the sample was transferred to an anaerobic cuvette in which the atmosphere was exchanged for nitrogen and was then fully reduced by adding 5 mM sodium ascorbate. An oxygen-saturated buffer solution was added that allowed for about 10 turnovers. Finally, after rereduction of the enzyme, the nitrogen atmosphere in the cuvette was exchanged for carbon monoxide. The final concentration of the enzyme was 10−15 μM.

Prior to formation of the mixed-valence enzyme, the oxidized enzyme was washed repetitively in a D_2O solution by repetitive dilution and reconcentration, after which it was turned over by adding a small amount of sodium ascorbate to the oxidized enzyme. After reoxidation of the enzyme, air was removed and the enzyme was incubated in CO. To prepare the mixed-valence CO complex of the enzyme, a redox buffer consisting of 0.5 mM ferri/ferrocyanide was added anaerobically to the sample. If necessary, the enzyme solution was titrated with ferricyanide. The mixed-valence CO complex was identified by its optical absorption spectrum.

The D_2O sample of the bacterial mutant enzyme was prepared by gel filtration on a column PD-10 (Amersham Pharmacia Biotech), preequilibrated with 0.1 M Hepes buffer at pH 7.5, 0.05% dodecyl-β-D-maltoside in D_2O. The fully reduced CO-bound enzyme was then prepared as described above, except that this enzyme was not turned over.

Flow-Flash Measurements. Measurements of absorbance changes associated with the reaction of the mixed-valence and fully reduced enzymes with O_2 were done as described previously (*35*, *36*).

Measurement of pH. Experiments in D_2O were done at the same pH meter reading (pH_{obs}) as in H_2O. This means that the pD in the D_2O sample is about 0.4 units higher than the pH (*37*). However, the exchange of H_2O for D_2O is usually associated with a change in the pK_as of titratable groups of about the same magnitude, which means that at pH_{obs}, in both H_2O and D_2O the protonation states of the titratable groups in the enzyme are the same (*37*). In addition, the reaction investigated in this study is pH-independent, which means that small deviations in pH/pD do not have any effect on the reaction characteristics.

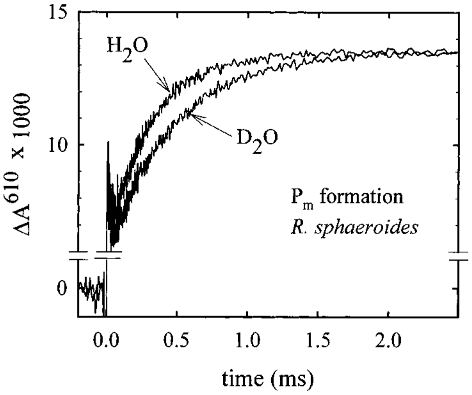

FIGURE 1: Absorbance changes at 610 nm following flash photolysis of CO from the *R. sphaeroides* mixed-valence enzyme in the presence of O_2 in H_2O and D_2O, respectively. The flash-induced CO-dissociation reaction is seen as a transient absorbance increase at time = 0. It is followed by a decrease in absorbance due to O_2 binding, followed by an increase in absorbance upon formation of P_m with a time constant of 300 and 420 μs in H_2O and D_2O, respectively. Experimental conditions: 1 μM reacting enzyme, 0.1 M Hepes, pH_{obs} 7.5, 0.05% dodecyl-β-D-maltoside, 22 °C, and 1 mM O_2.

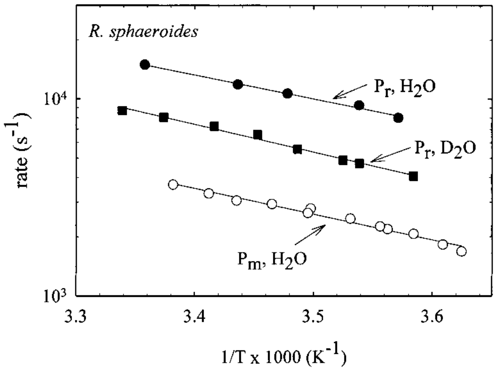

FIGURE 2: Temperature dependencies of the rate of P_m and P_r formation in the *R. sphaeroides* enzyme. The rate of P_m formation was determined from a one-exponential fit to the data recorded at 610 nm (open circles) (cf. Figure 1). The rate of P_r formation in H_2O (filled circles) and D_2O (filled squares) was determined from a two-exponential fit to the data recorded at 445 nm (see Figure 3). To avoid interference from heme *a* re-reduction during the P → F transition in the fully reduced enzyme, the P_r formation was studied in the ML(II-263) mutant enzyme of *R. sphaeroides*. Experimental conditions were the same as in Figure 1 (P_m) and Figure 3 (P_r).

Table 1: Kinetic and Thermodynamic Parameters for P_m/P_r Formation in the *R. sphaeroides* and the Bovine Enzymes

	R. sphaeroides		bovine[a]	
	$A \rightarrow P_m$	$A \rightarrow P_r$	$A \rightarrow P_m$	$A \rightarrow P_r$
τ in H_2O (μs)	300	70	200	∼30
τ in D_2O (μs)	420	130	380	∼45
DIE	1.4 ± 0.1	1.8 ± 0.2	1.9 ± 0.4	1.4
E_a in H_2O (kJ/mol)[b]	24 ± 1	20 ± 3	27 ± 4	∼28
E_a in D_2O (kJ/mol)[b]		25 ± 1	29 ± 2	
$\Delta S^\#$ in H_2O (J/mol·K)	−92 ± 5	−86 ± 6		
$\Delta S^\#$ in D_2O (J/mol·K)		−80 ± 5		

[a] The data are from refs *6*, *39*, and *41*, except for the activation energies for P_m formation (this work). [b] The activation energy, E_a, is $\Delta H^\# + RT$, where $\Delta H^\#$ is the activation enthalpy.

RESULTS

Kinetics of P_m Formation. The CO-bound mixed-valence *R. sphaeroides* cytochrome *c* oxidase was mixed rapidly with a solution of an O_2-containing buffer, after which the CO ligand was flashed off using a 10-ns laser flash. Figure 1 shows absorbance changes at 610 nm after flash photolysis of CO from the mixed-valence enzyme at pH_{obs} = 7.5. This wavelength is close to the absorbance maximum (at 607 nm) of the absorbance-difference spectrum of the peroxy intermediate minus the oxidized enzyme. After the initial increase in absorbance due to CO dissociation is a rapid decrease in absorbance, associated with formation of the ferrous-oxy (A) intermediate with a time constant of ∼20 μs, i.e., slightly slower than with the fully reduced enzyme (∼8 μs, see Discussion). This change is followed by an increase in absorbance with a time constant of about 300 μs in H_2O (see also ref *38*), associated with formation of the P_m intermediate. The kinetics of formation of P_m did not display any dependence on pH in the range 6−9.5 (data not shown). When the same experiment was done in a D_2O solution, the time constant increased by a factor of 1.4 ± 0.1 to ∼420 μs, i.e., the deuterium isotope effect was similar to that observed previously with the bovine enzyme (*39*). No proton uptake was associated with formation of the P_m intermediate (data not shown).

The formation rate of P_m was measured for different temperatures in the range ∼275−300 K. Figure 2 shows the temperature dependence of the P_m formation kinetics in H_2O for the *R. sphaeroides* enzyme. The activation energy, E_a, and entropy, $\Delta S^\#$, were found to be 24 ± 1 kJ/mol and −92 ± 5 J/mol K, respectively (see also Table 1).

Kinetics of P_r Formation. In *R. sphaeroides* cytochrome *c* oxidase, the P_r intermediate has previously been found to be formed with a time constant of 55 μs (*35*), i.e., about 5 times faster than formation of the P_m intermediate. In studies of the reaction of the fully reduced enzyme with O_2 the decay of the P_r intermediate and formation of the ferryl intermediate (F) with a time constant of about 140 μs (*35*), is associated with transfer of the 4th electron from Cu_A to heme *a*. To avoid interference from absorbance changes associated with the electron-transfer reaction, we investigated the P_r formation kinetics in a mutant enzyme [ML(II-263)] in which the redox potential of Cu_A is increased and therefore the electron transfer from Cu_A to heme *a* slowed by a factor of ∼200 (*40*). Alternatively, formation of P_r can be followed at ∼590 nm (*10*), which is close to an isosbestic point for reduction/oxidation of heme *a*. Therefore, at this wavelength, formation of P_r can be observed without interference from absorbance changes associated with changes in the reduction level of heme *a*. In addition, the absorbance change associated with the R → A transition has an opposite sign as compared to that associated with the A → P_r transition, which simplifies resolution of the latter (*10*).

Figure 3 shows absorbance changes at 445 nm (ML(II-263) mutant enzyme) and at 590 nm (wild-type enzyme) after flash photolysis of CO from the fully reduced enzyme in the presence of O_2. The traces were fitted with a sum of two exponential functions, associated with oxygen binding (R → A, $\tau \cong 8$ μs) and P_r formation (A → P_r, $\tau \cong 70$ μs), respectively. The same formation rates of P_r were obtained with the ML(II-263) as with the wild-type enzyme, which indicates that the mutation at the Cu_A site does not perturb the binuclear center [as also found in more extensive studies of the ML(II-263) mutant enzyme (*34*, *40*)].

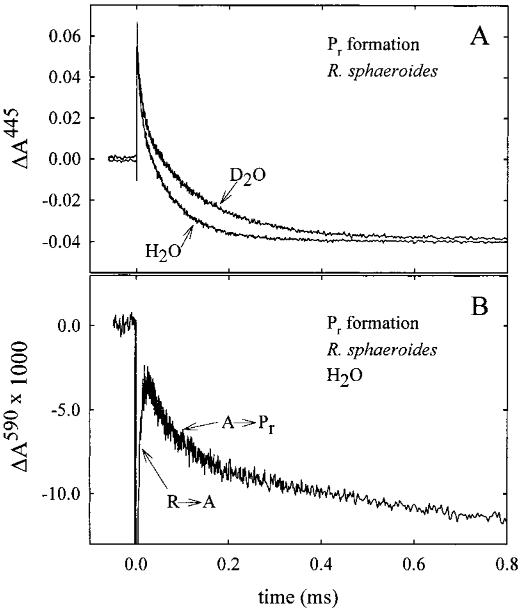

FIGURE 3: (A) Absorbance changes at 445 nm after photodissociation of CO (at time = 0) from the fully reduced ML(II-263) mutant enzyme in H_2O and D_2O, respectively. The traces are scaled to 1 μM reacting enzyme as determined from the CO-dissociation absorbance change, using an absorption coefficient of 67 mM^{-1} cm^{-1}. (B) The same reaction monitored at 590 nm with the *R. sphaeroides* wild-type enzyme. The initial increase with a time constant of ~8 μs is due to oxygen binding (R → A). The following decrease in absorbance is due to formation of P_r. The slow decrease in absorbance after P_r formation ($\tau \cong$ 1 ms) is due to spectral changes mainly associated with the F → O transition. Experimental conditions: 0.1 M Hepes, pH_{obs} 7.5, 0.05% dodecyl-β-D-maltoside, (A) ~2 μM and (B) ~1 μM reacting enzyme, 22 °C, 1 mM O_2.

In D_2O, the P_r formation kinetics was slowed to ~130 μs, i.e., the deuterium isotope effect was 1.8 (± 0.2), which is about the same as that found previously with the bovine enzyme [~1.4 (*39*, *41*)].

At 445 nm, the P → F transition ($\tau \cong$ 140 μs) contributes slightly to the absorbance change, but with less than 10% of that associated with oxidation of the hemes (*42*, *43*).

The temperature dependence of P_r formation was also investigated (Figure 2). The activation energies, E_a, in H_2O and D_2O were 20 ± 3 and 25 ± 1 kJ/mol, respectively. As seen in Table 1, the activation entropies ($\Delta S^{\#}$) in H_2O and D_2O were about the same.

DISCUSSION

Before discussing the mechanisms of P_m/P_r formation in detail, we briefly consider the O_2-binding kinetics to the mixed-valence and fully reduced enzymes. The slower binding of O_2 to heme a_3 in the mixed-valence than in the fully reduced enzyme can be explained in terms of the "backflow" of electrons from heme a_3 to heme *a* after dissociation of CO (*44*, *45*). With CO bound to the binuclear center the reduced form of heme a_3 and Cu_B is stabilized and therefore in essentially 100% of the enzyme population heme a_3 and Cu_B are reduced while Cu_A and heme *a* are oxidized. However, after flash-induced dissociation of CO from heme a_3, the apparent redox potential of heme a_3 decreases, which results in a rapid ($\tau \cong$ 3 μs) equilibration of electrons at the binuclear center with heme *a*. Consequently, in the mixed-valence enzyme, the A intermediate is not formed with the fully reduced binuclear center because in a fraction of the enzyme population, before formation of the intermediate there is a partial oxidation of heme a_3. With the *R. sphaeroides* enzyme the fraction of heme a_3 oxidation on the microsecond time scale is ~40% (*45*). Since the electron equilibration between the hemes is faster than O_2 binding to heme a_3 (measured with the fully reduced enzyme), formation of the A intermediate is slowed by a factor of ~0.6 due to the fractional oxidation of heme a_3.

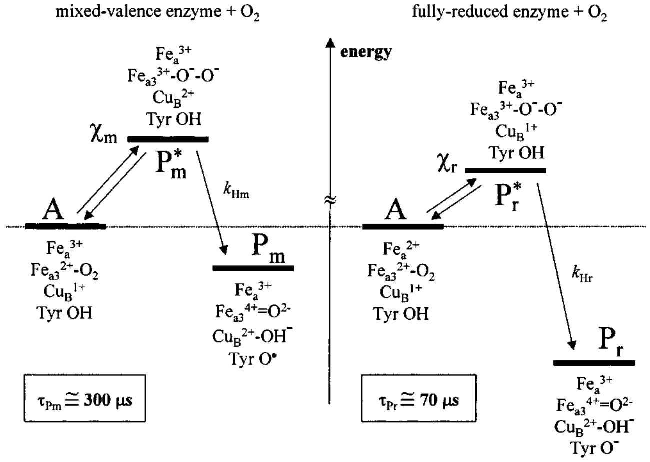

FIGURE 4: Schematic diagram showing the reaction mechanisms of formation of the P_m and P_r intermediates during reaction of the mixed-valence and fully reduced enzymes, respectively, with molecular oxygen. The A state is formed upon O_2 binding to the reduced binuclear center (R, not shown). The P_m*/P_r* states are transient high-energy states. The equilibrium between A and P_m*/P_r* is rapid (much faster than formation of P_m/P_r), and the states P_m*/P_r* are formed in fractions χ_m/χ_r of the enzyme populations. The apparent formation rates of the P_m and P_r intermediates (time constants τ_{Pm} and τ_{Pr}) are described by eq 2. It is assumed that the proton needed to form P_m*/P_r* is taken from the tyrosine [Y(I-288)], but the general model and conclusions are independent of the identity of the proton donor. A H_2O molecule may donate a proton transiently to states P_m*/P_r* (*25*). The energy scale is arbitrary.

As discussed in the introduction section, the O−O bond is most likely cleaved in the P intermediate, a scenario that requires four electrons and at least one proton to be transferred to dioxygen (see eq 1 and Figure 4). In the mixed-valence enzyme (P_m) the fourth electron is presumably transferred from Y(I-288) (see ref *13*, *23*−*25*), covalently linked to H(I-284) (*28*, *29*), one of the ligands of Cu_B. The tyrosine is situated in close vicinity of heme a_3, and it may participate in activation of the heme a_3-bound dioxygen by donating both the electron and the proton (hydrogen atom) (*13*, *21*, *23*, *25*). The final product would thus be $Fe_{a3}^{4+}{=}O_2^{2-}$, $Cu_B^{2+}{-}OH^-$, and a tyrosyl radical (see Figure 4). In the fully reduced enzyme, formation of the P_r intermediate is associated with electron transfer from heme *a* to the binuclear center, and the end product (P_r) most likely does not have the tyrosyl radical.

The kinetic phases associated with formation of both the P_m and P_r intermediates display the same absorbance-difference spectra (*10*), which indicates that their basic structures are the same. Since the formation rates of both P_m and P_r are independent of pH and display about the same deuterium isotope effects [cf. also results with the bovine enzyme (*39*)], the formation of both intermediates is presumably rate-limited by an internal proton transfer (note that there is no proton uptake from the bulk solution upon formation of P_m/P_r). In Table 1 are summarized the characteristics of the formation of the "peroxy" intermediates with the mixed-valence (P_m) and fully reduced enzymes (P_r), respectively.

As found previously with the bovine enzyme, formation of the P intermediate was about a factor of ∼5 faster with the fully reduced than with the mixed-valence enzyme. Assuming that the same proton/hydrogen atom transfer is rate-limiting for formation of both P_m and P_r, the question that needs to be addressed is why formation of P_r is faster than that of P_m and what is the origin of the deuterium isotope effect.

One possible scenario that explains the difference in the rates as well as the observed kinetic deuterium isotope effect is illustrated in Figure 4. Upon binding of O_2 to the reduced binuclear center (intermediate A), transiently a true peroxy intermediate (P*, $Fe_{a3}^{3+}{-}O^{-}{-}O^{-}$) is formed. Formation of this intermediate is highly endergonic, and it is not populated during reaction of O_2 with the fully reduced enzyme. However, formation of P* is followed by an exergonic reaction in which the O−O bond is cleaved (see Figure 4), which drives formation of P_m/P_r. One of the electrons in P* is donated to O_2 by heme a_3, while the second electron is donated either by heme *a* (P_r*, in the fully reduced enzyme) or by Cu_B (P_m*, in the mixed-valence enzyme). In both cases, the electron transfer is much faster than the following proton/hydrogen transfer from Y(I-288) during O−O bond cleavage [e.g., the transfer rate from heme *a* to heme a_3 displays a rate of $\sim 10^5\ s^{-1}$ (*45*)]. Thus the formation rate, k_P ($= \tau_P^{-1}$, see Figure 4), is

$$k_P = \chi k_H \tag{2}$$

where χ is the enzyme fraction in which the transient true peroxy intermediate P* is formed, and k_H is the internal proton/hydrogen-transfer rate.

According to the model in Figure 4, different P formation rates with the mixed valence and fully reduced enzymes, respectively, is a consequence of a difference in the fractions (χ_m and χ_r, see Figure 4) or a difference in the proton-transfer rates (k_{Hm} and k_{Hr}, see Figure 4), where the difference in the fractions χ_m and χ_r is determined by the difference in the redox potentials of the electron donors Cu_B and heme *a*, respectively.

According to the model in Figure 4, the measured activation energy of P_m/P_r formation is an apparent activation energy, and it has contributions both from the temperature dependence of the equilibrium constant for electron transfer to P_m^*/P_r^* (i.e., the temperature dependence of χ) and from the temperature dependence of the proton/hydrogen-transfer rate k_H (cf. eq 2).

Recently, on the basis of theoretical calculations, Blomberg et al. (*25*) presented a quantitative model describing the formation mechanism of the P_m intermediate. According to this model, P* is a transition state of the reaction with an activation energy of ∼38 kJ/mol, i.e., in a reasonable agreement with the experimental value found in this work.

The relatively small kinetic deuterium isotope effect of <2 for formation of both the P_m and P_r intermediates is characteristic of a proton-transfer reaction that is rate-limited by hydrogen bond breaking and/or reorientations of hydrogen bonds and does not involve proton "hopping" between two heavy sites (see e.g., ref *46*). As discussed above, this internal proton may be donated by Y(I-288). It should also be noted that the electron-transfer rate and equilibrium between hemes *a* and a_3 as well as CO recombination do not display any deuterium isotope effects (*39*), which indicates that the protein environment of the redox centers does not undergo any structural changes upon incubation in D_2O.

The assignment of Y(I-288) as an electron/proton donor is consistent with results from studies of mutants of the *R. sphaeroides* enzyme in which the protonatable amino acid residues K(I-362) and T(I-359) in the same pathway as Y(I-288) were modified. In these mutant enzymes, formation of the P_r intermediate was slowed by at least a factor of 2 (*47*).

As seen in Figure 4, in the P_m but not in the P_r intermediate, the tyrosyl radical is built up to significant concentrations, and the free energy "stored" in the radical can be used for proton pumping. It has been debated in the literature whether both intermediates P_r and P_m are relevant for the catalytic turnover of cytochrome *c* oxidase. It was pointed out that the fully reduced enzyme is not likely to be formed during turnover, and therefore the P_r intermediate may not be a "natural" state, relevant for the catalytic mechanism of the enzyme (see e.g., refs *12*, *48*, *49*). Investigation of proton release on the outside of cytochrome *c* oxidase proteoliposomes during reaction of the fully reduced enzyme with oxygen indeed showed that at most two (not four) protons are released to the outside, which takes place only during the F → O transition (*50*). Recent data indicate that during reaction of the fully reduced enzyme with oxygen the free energy used for proton pumping is stored in the enzyme structure; only two charges are pumped during the oxidative part of the cycle and the additional two charges are pumped only after rapid rereduction of recently oxidized enzyme (*49*). Thus, the loss of free energy during P_r formation discussed above may be apparent as the energy may instead be stored by means of protein structural changes.

Previous studies have shown that the K pathway [including amino acid residues Y(I-288), T(I-359), and K(I-362)] is used for proton transfer during reduction (formation of state R) of the binuclear center, while after initiation of the reaction of the reduced enzyme with O_2 (steps P → F → O) exclusively the D pathway [including amino acid residues D(I-132) and E(I-286)] is used (reviewed in ref *51*). Thus, the reaction investigated in the present study involves a change in the proton accessibility through the two proton-transfer pathways, which is most likely one of the key steps in the proton-pumping mechanism of cytochrome *c* oxidase.

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