Heme-copper oxidases catalyze the four-electron reduction of O$_2$ to H$_2$O at a catalytic site that is composed of a heme group, a copper ion (Cu$_b$), and a tyrosine residue. Results from earlier experimental studies have shown that the O–O bond is cleaved simultaneously with electron transfer from a low-spin heme (heme a/b), forming a ferryl state ($P_b$ Fe$^{IV}=$O$^2^-$, Cu$_b$ $^{2+}$$-$OH$^-$). We show that with the *Thermus thermophilus* ba$_3$ oxidase, at low temperature (10°C, pH 7), electron transfer from the low-spin heme b to the catalytic site is faster by a factor of $\approx$10 ($\tau$$\approx$11 µs) than the formation of the $P_b$ ferryl ($\tau$$\approx$110 µs), which indicates that O$_2$ is reduced before the splitting of the O–O bond.

Application of density functional theory indicates that the electron acceptor at the catalytic site is a high-energy peroxy state [Fe$^{IV}$$-$O$$^2^-$–O$^-$–O($H^+$)], which is formed before the $P_b$ ferryl. The rates of heme b oxidation and $P_b$ ferryl formation were more similar at pH 10, indicating that the formation of the high-energy peroxy state involves proton transfer within the catalytic site, consistent with theory. The combined experimental and theoretical data suggest a general mechanism for O$_2$ reduction by heme-copper oxidases.

**INTRODUCTION**

Cytochrome c oxidase (CytcO) catalyzes the reduction of O$_2$ to H$_2$O in the respiratory chain of aerobic organisms using reduced cytochrome (cyt.) c as an electron donor. The enzyme is a transmembrane protein that is composed of two or more subunits, which harbor four redox-active metal sites. During turnover, electrons from cyt. c are transferred first to the primary electron acceptor Cu$_{a_1}$, then to the low-spin heme intermediate electron acceptor, and finally to the catalytic site, which consists of a heme group and a copper ion, Cu$_b$, in close proximity, as well as a redox-active Tyr residue (Fig. 1) for a review of the structure and function of oxidases, see related studies (1–14). The heme-copper oxidases typically pump, on average, 0.5 to 1 proton across the membrane per electron transferred from cyt. c to the catalytic site.

The oxidases are classified based on their structural details, particularly the architecture of the proton pathways, as members of one of three classes denoted by letters A, B, and C (15–17). The type of heme groups varies in different oxidases, and it is not strictly related to the class of the CytcO. In the B-type CytcO from *Thermus thermophilus*, the low-spin intermediate electron acceptor is heme b, whereas heme a$_3$ resides in the catalytic site; that is, the CytcO is a cyt. ba$_3$. The well-studied A-type CytcOs from *Rhodobacter sphaeroides*, *Paracoccus denitrificans*, and mitochondria are all aa$_3$ CytcOs.

The A-family bacterial oxidases use two proton pathways starting at the surface on the negative (n) side of the membrane, namely, the K and D pathways. The ba$_3$ CytcO from *T. thermophilus* harbors only one functional proton pathway (18), which approximately overlaps in space with the K pathway in the A-type oxidases, and it is therefore referred to as the K pathway analog. Structures of the ba$_3$ CytcO have been determined at atomic resolution using x-ray crystallography (19–21).

Detailed kinetic and mechanistic information for the *T. thermophilus* ba$_3$ CytcO has been obtained, for example, from studies of the reaction of the reduced CytcO with O$_2$ and the comparison of this reaction sequence to that in the well-studied A-type oxidases. With the A-type oxidases, dioxygen binds first to heme a$_3$ at the catalytic site, forming the oxo-ferrous intermediate called A, first described by Chance et al. (22). It was assumed originally that after O$_2$ binding to CytcO, a peroxy intermediate (Eq. 1) is formed at heme a$_3$ [see related studies (23–26)]

$$[\text{Fe}^{IV}_{a_3}$$–O$_2$]+Cu$_b^+$$+$e$^-$+H$^+$ $\rightarrow$[Fe$^{IV}_{a_3}$–O$^-$–O($H^+$)]+Cu$_b^+$ (1)

Hence, this state was referred to as P. However, on the basis of an analysis of the optical absorption spectra of the P state, Weng and Baker (27) suggested that the O–O bond is broken in the P state, indicating that a ferryl state (Fe$^{IV}_{a_3}$=O$^2^-$) is formed (Eq. 2). Further support for this suggestion was obtained from resonance Raman experiments (28–31). In 1999, Fabian et al. (32) demonstrated that one of the oxygen atoms of the O$_2$ molecule bound to the two-electron reduced CytcO was released to solvent, which was interpreted to show that the O–O bond is broken upon formation of P

$$[\text{Fe}^{IV}_{a_3}$$–O$_2$]+Cu$_b^+$$+$e$^-$+H$^+$ $\rightarrow$[Fe$^{IV}_{a_3}$=O$^2^-$]$+$[Cu$_b^{2+}$–OH$^-$] (2)

The source of the electron and proton at the left-hand side of the reaction varies depending on the initial state of the CytcO. If only the catalytic site is reduced, then the electron and proton are transferred from a Tyr residue (Tyr$^{388}$ or Tyr$^{337}$ in *R. sphaeroides* aa$_3$ CytcO or *T. thermophilus* ba$_3$ CytcO, respectively) (26, 29, 33–35), which forms a tyrosyl radical, TyrO$^*$. The state that is formed is denoted P$_M$ (Eq. 3)

$$[\text{Fe}^{IV}_{a_3}$$–O$_2$]+Cu$_b^+$$+$TyrOH $\rightarrow$[Fe$^{IV}_{a_3}$=O$^2^-$]$+$[Cu$_b^{2+}$–OH$^-$]$+$TyrO$^*$ (3)

The time constant of this reaction is 200 to 300 µs (36, 37), and the P$_M$ state is stable over a time scale of minutes (38).
When the \( P \) state is formed in the fully reduced CytcO, the electron is transferred from the low-spin heme \( \alpha_3 \), and the Tyr residue donates only a proton. The state is referred to as \( P_R \) (Eq. 4).

\[
\begin{align*}
[F_{\alpha_3}^{\text{Fe}^3+} - O_2] + Cu_b^+ + TyrOH + Fe_{\alpha}^{\text{Fe}^3+} & \rightarrow [F_{\alpha_3}^{\text{Fe}^4+} = O^{2-}] \\
+ [Cu_b^{\text{Fe}^2+} - OH^-] + TyrO^- + Fe_{\alpha}^{\text{Fe}^3+} & \\
\end{align*}
\]

The \( P_R \) formation time constants were found in the range 30 to 70 \( \mu \)s (37, 39–44), depending on the species from which the CytcO is isolated. The summary above shows that the \( P_M \) and \( P_R \) states have the same structure; the only difference is the donor of the "fourth" electron, which is Tyr \( \text{Fe}^3 \) or the low-spin heme, respectively [reviewed by Kaila et al. (9)]. Note that the formation of \( P_M/P_R \) is not associated with any proton uptake from the solution to the catalytic site (45–47); the Tyr residue is the hydrogen or proton donor. Although a peroxy state \( [Fe^{\text{Fe}^3 - O^\cdot - O^\cdot (H^+)]} \) has not been previously observed upon reaction of the reduced CytcO with \( O_2 \), on the basis of theoretical studies, it has been predicted that this state (called \( I_p \) is formed transiently on the reaction path leading from state \( A \) to state \( P_M \) (48–50). \( I_p \) has not been observed because it is higher in energy than state \( A \). Here, we investigated the formation of the \( P_R \) state in the \( T. \) thermophilus \( \alpha_3 \) CytcO as a function of temperature at neutral and high pH (10). The data indicate that at neutral pH and low temperature (\( 10^\circ \)C), electron transfer from heme \( \alpha_3 \) to the catalytic site is about 10 times faster than the formation of the \( P_R \) state, which suggests that a true peroxy state is formed transiently before the O–O bond is broken in \( P_R \). This difference in rate constants was not observed at higher temperatures (\( \sim 45^\circ \)C), where the electron transfer and \( P_R \) formation displayed the same rate constants, just as in the A-type CytcOs at room temperature. Furthermore, at high pH (10), the difference in rate constants (at low temperature) was significantly smaller than at neutral pH, which suggests that formation of a transient peroxy state \( [\text{Fe}^{\text{Fe}^{\text{Fe}^3 - O^\cdot - O^\cdot (H^+)]}] \) involves internal proton transfer within the catalytic site.

**RESULTS**

**Experimental results**

The reduced cyt. \( \alpha_3 \) CytcO, with CO bound to heme \( \alpha_3 \), was mixed with an \( O_2 \)-saturated solution after which the CO ligand was removed by means of a laser flash to allow \( O_2 \) to bind to heme \( \alpha_3 \). Absorbance changes were monitored at 560 and 610 nm. At 560 nm, there is a maximum in the contribution of the redox changes of heme \( \alpha_3 \), whereas at 610 nm, the binding of \( O_2 \) and the formation of the "peroxy" (P) intermediate are observed [a broad trough near 615 nm and a peak at 610 to 612 nm (51, 52)]. Figure 2 shows absorbance changes as a function of time after CO dissociation at \( \tau = 0 \). The starting level of the traces just after the flash (adjusted to zero) reflects the absorbance of the reduced CytcO relative to that of the CytcO-CO complex.

We first discuss data at \( 10^\circ \)C and pH 7. At 610 nm (Fig. 2A), the first decrease in absorbance is associated with the binding of \( O_2 \) to heme \( \alpha_3 \) with a rate constant of \( 2.8 \times 10^5 \pm 0.8 \times 10^5 \text{ s}^{-1} \) (\( \tau \approx 3.6 \mu \text{s} \)). The increase in absorbance is associated with the formation of the \( P_R \) state with a rate constant of \( 9 \times 10^5 \pm 1 \times 10^5 \text{ s}^{-1} \) (\( \tau \approx 110 \mu \text{s} \)), whereas the slowest decrease in absorbance is associated with oxidation of the CytcO with a rate constant of \( 400 \pm 60 \text{ s}^{-1} \) (\( \tau \approx 2.5 \mu \text{s} \)). At 560 nm (Fig. 2A), the initial decrease in absorbance is associated with oxidation of heme \( \alpha_3 \) with a rate constant of \( 9 \times 10^4 \pm 4 \times 10^4 \text{ s}^{-1} \) (\( \tau \approx 11 \mu \text{s} \)), which is followed in time by re-reduction of heme \( \alpha_3 \) (\( \tau = 180 \pm 30 \mu \text{s} \)) and oxidation (\( \tau = 2.6 \pm 0.2 \mu \text{s} \)). The data indicate that at \( 10^\circ \)C and pH 7, formation of heme \( \alpha_3 \) (decrease in absorbance at 560 nm, \( \tau = 11 \mu \text{s} \)) was ~10 times faster than the formation of state \( P_R \) (increase in absorbance at 610 nm, \( \tau = 110 \mu \text{s} \)).

At high temperature (\( 45^\circ \)C) and pH 7 (Fig. 2B), the decrease in absorbance at 560 nm (oxidation of heme \( \alpha_3 \)) and the increase in absorbance at 610 nm (formation of \( P_R \)) displayed rate constants of \( 1.9 \times 10^5 \pm 0.2 \times 10^5 \text{ s}^{-1} \) (\( \tau = 5.3 \mu \text{s} \)) and \( 1.5 \times 10^5 \pm 0.5 \times 10^5 \text{ s}^{-1} \) (\( \tau = 6.7 \mu \text{s} \)), respectively, that is, the same within the error. Figure 2 (C and D) shows absorbance changes at pH 10 and at \( 10^\circ \)C and \( 45^\circ \)C, respectively. At higher \( pH \), the ratios of the rate constants of heme \( \alpha_3 \) oxidation and \( P_R \) formation were ~3 and ~1.3 at \( 10^\circ \)C and \( 45^\circ \)C (the rate constant values are given in Table 1), respectively; that is, the difference in rates at a low temperature was smaller than at neutral pH.

Figure 3A shows the temperature dependence of the rate constant for the absorbance change attributed to \( P_R \) formation at 610 nm (black trace) and those attributed to heme \( \alpha_3 \) oxidation at 560 nm (red trace) at pH 7. As seen in the figure, the difference between these rate constants was significantly larger at low than at high temperature. Figure 3 also shows the temperature dependence of the kinetic component associated with the binding of \( O_2 \) (blue trace, \( \tau = 2 \to 4 \mu \text{s} \)). This time constant was essentially temperature-independent, presumably because the slower \( O_2 \) binding at lower temperatures is compensated for by the increased solubility of \( O_2 \).

Figure 4 shows absorbance changes at 610 nm associated with the reaction of the mixed-valence \( \alpha_3 \) CytcO (that is, reduced heme \( \alpha_3 \) and...
Fig. 2. Absorbance changes during reaction of the fully reduced ba3 CytcO with O2. (A and B) pH 7 at 10° and 45°C, respectively. (C and D) pH 10 at 10° and 45°C, respectively. At 610 and 560 nm, the absorbance changes are mainly attributed to redox changes at heme a3 and heme b, respectively. The black lines are fits of the data with a model that is described by a sum of three exponential functions. The rate constants obtained from the fit are given in the text and in Table 1. The difference between the fit and the data (that is, the residuals) is shown below each panel. In addition, in (A), we show the residuals of a fit with a single rate constant (5 × 10^4 s\(^{-1}\)) for electron transfer from heme b to the catalytic site and PR formation (gray lines). Experimental conditions after mixing: 0.6 to 0.8 mM CytcO (scaled to 1 mM), 0.05% DDM, 90 mM Hepes (pH 7) or 90 mM CAPS (pH 10), and ~1 mM O2. The cuvette path length was 1.00 cm. The 560-nm traces are shifted up by 1.7 × 10^-3 units for clarity.

Table 1. Rate constants for the early steps of O2 reduction at low and high temperature for pH 7 and 10, respectively. Errors are the SDs for \( n = 3 \) (pH 10 data) or \( n = 4 \) (pH 7 data) measurements.

| T (°C) | pH 7 | pH 10 |
|-------|------|-------|
| **O2 binding (610-nm decay)** | | |
| 10 | \((2.8 \pm 0.8) \times 10^5\) (3.6) | \((2.9 \pm 0.1) \times 10^5\) (3.4) |
| 45 | \((2.7 \pm 0.8) \times 10^5\) (3.7) | \((4 \pm 1) \times 10^5\) (2.5) |
| **Heme b oxidation (k_b) (560-nm decay)** | | |
| 10 | \((9 \pm 4) \times 10^4\) (11) | \((2.6 \pm 0.6) \times 10^4\) (38) |
| 45 | \((1.9 \pm 0.2) \times 10^2\) (5.3) | \((1.9 \pm 0.2) \times 10^2\) (5.3) |
| **PR formation (k_P) (610-nm increase)** | | |
| 10 | \((9 \pm 1) \times 10^3\) (110) | \((8.7 \pm 0.3) \times 10^3\) (120) |
| 45 | \((1.8 \pm 0.5) \times 10^3\) (5.5) | \((1.5 \pm 0.5) \times 10^3\) (6.7) |
| **Ratio (k_b/k_P)** | | |
| 10 | \(10\) | \(3\) |
| 45 | \(1.1\) | \(1.3\) |
of this reaction is pH-independent in the pH range 6.1 to 9.2 (Fig. 5). Previous computational studies (see Introduction), have suggested a two-step mechanism for the O

\[ \text{O}_2 \rightarrow \text{Cytc}_0 + 2e^- + \text{H}^+ \rightarrow \text{CytcO} + \text{O}_2 \]

formation are shown as indicated in the graphs for (A) pH 7 and (B) pH 10. Conditions were the same as in Fig. 2.

**Fig. 3. Temperature dependence of the rate constants.** The rates of O\(_2\) binding (Arrhenius plots), heme b oxidation, and P\(_f\) formation are shown as indicated in the graphs for (A) pH 7 and (B) pH 10. Conditions were the same as in Fig. 2.

Hence, it is reasonable to assume that the electron transfer step to the catalytic site? The present calculations show that the electron affinity of intermediate \( A \) is significantly lower than that of the electron donor heme b [also compare the study of Blomberg (54)], which is expected because there is no strong electron acceptor at the catalytic site. On the other hand, in the peroxide intermediate \( I_p \), an electron acceptor, either Cu\(^{2+}\) or the tyrosyl radical, is created, resulting in an increase of the calculated electron affinity by about ~50 kJ/mol (not shown in Fig. 5). This result indicates that the product of the electron transfer is a reduced peroxide intermediate, \( I_p^- \) (Fig. 5), that relaxes to P\(_R\) upon cleaving the O–O bond.

The transition state for O–O bond cleavage (TS\(_{O-O}\)) was determined for both \( I_p \) and \( I_p^- \) intermediates. The calculated barrier heights are quite similar, 38.5 and 36.8 kJ/mol, respectively (from \( I_p \) and \( I_p^- \), respectively, to TS\(_{O-O}\) in Fig. 5). The rate constants for P\(_R\) and P\(_M\) formation are determined by the energy difference between intermediate \( A \) and the highest point on the energy profiles. A transition state was determined for the initial proton transfer step (TS\(_{H_2} \)), but because the calculated barrier height depends on the number of water molecules included in the model, a definite value could not be determined. However, it is estimated that TS\(_{H_2} \) is similar to or lower than TS\(_{O-O} \), and therefore, the position of TS\(_{O-O} \) (that is, the highest point) determines the formation rate constant of both P\(_R\) and P\(_M\). The height of this rate-limiting barrier is the sum of the endergonicity for the formation of the peroxide intermediate (\( I_p \) or \( I_p^- \)) and the O–O bond cleavage barrier relative to the peroxide.

Using the present model without considering any changes in the K pathway, we found the peroxide intermediate \( I_p \) to be 30.5 kJ/mol above intermediate \( A \) (this level is not shown in Fig. 5), which would lead to a total barrier for P\(_M\) formation of almost 70 kJ/mol. This is much higher than the activation free energy of about 50 kJ/mol, corresponding to the experimentally observed rate constant. Therefore, in previous computational studies, we investigated factors that could influence the barrier height. In particular, it was shown that a positive charge, for example, at Lys\(^{362}\) of the R. sphaeroides CytcO (55), in the vicinity of the negatively charged tyrosinate, significantly stabilizes the peroxide intermediate \( I_p \) relative to \( A \) (48, 50). Therefore, it can be suggested that the positive charge would yield an estimated difference between \( I_p \) and \( A \), as shown in Fig. 5, yielding an overall barrier of about 50 kJ/mol for P\(_M\) formation (highest barrier from \( A \) to P\(_M\); see Fig. 5). Note as well that with the smaller energy difference between \( A \) and \( I_p \), the latter would remain unobservable (populated to ~5%), in agreement with the experiments. Furthermore, the calculated electron affinity of intermediate \( I_p \) is similar to the calculated electron affinities of other intermediates of the catalytic cycle, which precede electron transfer to the catalytic site (54). Hence, it is reasonable to assume that the electron transfer step to form state \( I_p^- \) is somewhat exergonic, putting this state close enough in energy to intermediate \( A \) to become observable, in accordance with the present experiments (see Fig. 5). Because the O–O bond cleavage barrier relative to the preceding peroxide state is rather similar for \( I_p \) and \( I_p^- \) (see above), the difference in rate constants for P\(_M\) and P\(_R\) formation is mainly the result of the exergonicity of the electron transfer from heme b to \( I_p \), forming \( I_p^- \). In summary, the qualitative picture of the O–O bond cleavage step obtained from the quantum chemical calculations is used to construct the energy profiles presented in Fig. 5, wherein the detailed relative energies are adapted to fit the present experimental data at low temperatures and neutral pH.

**DISCUSSION**

As described in detail in the Introduction, early models assumed that immediately after \( \text{O}_2 \) binding to heme a\(_3\), a peroxo state is formed,
In earlier studies, the value for another transition (previously discussed in depth at that time. Furthermore, on the basis of results and Poiana et al. (57), we did not observe this interference from CO dissociation. As discussed previously (56), we did not observe this interference from CO dissociation.

The Arrhenius activation energy for $P_R$ formation in the *R. sphaeroides* CytC_O is ~20 kJ/mol (37), that is, lower than that for $P_R$ formation with the *ba*$_3$ CytC_O but about the same as that for heme b oxidation (see Table 2). In earlier studies, the value for another transition (F→O) was determined [~42 kJ/mol (58)]. This reaction involves both electron transfer from heme b to the catalytic site and proton transfer from the solution. Previous experience has shown that the type of computational studies used in this study to interpret the data yields better agreement with the experiment for free energies than for the partitioning into enthalpy and entropy contributions, which may be related to the fact that the source for this partitioning is not always located in the active site (49, 59). Therefore, we present the parameters in Table 2 without further discussion.

When heme b becomes oxidized before state $P_R$ is formed, a question regarding the identity of the electron acceptor arises. Upon binding of $O_2$, that is, forming of state $A$, in the fully reduced CytC_O, none of the redox sites can accept any additional electrons. State $P_R$ is formed over a time scale of 110 μs at 10°C (Fig. 2A and Table 1). Consequently, if $P_R$ was the first intermediate to be formed at the catalytic site after binding of $O_2$ (intermediate $A$), there would be no available electron acceptor over the time scale wherein we observed oxidation of heme b ($τ$ ≈ 11 μs at 10°C; see Fig. 2A). The $P_M$ state was formed with a time constant of ~140 μs at ~20°C (Fig. 4); that is, this state was not formed before electron transfer from heme b. To explain the data, we must therefore consider mechanisms to create an electron acceptor after binding of $O_2$ but before breaking of the O–O bond. One plausible possibility is to form a transient peroxide intermediate over the same time scale as electron transfer from heme b. This intermediate was suggested by Blomberg et al. (48–50), and a reaction scheme including this state ($I_P$) is presented in Fig. 5. The absorbance change associated with the $A→I_P$ reaction would then correspond to the difference between states heme b$^{2+}$ Fe$^{2+}$–$O_2$, and heme b$^{3+}$ Fe$^{3+}$–$O$–$O^−$ (H$^+$), which would be dominated by the absorbance change associated with oxidation of heme b. This is because the O–O bond length in state Fe$^{2+}$–$O_2$ is in between that of dioxygen and peroxide, similar to a superoxide coordinated to a ferric iron (Fe$^{3+}$–$O_2^−$) (60), and we assume that no major absorbance changes occur at heme a$_3$ during the transition from a superoxide-like state to a peroxide state.

According to the theoretical model (48–50), in state $I_P$, the heme a$_3$ iron is oxidized and an electron is transferred to $O_2$ from either Cu$_b$ or Tyr$^{357}$, wherein the latter in both cases is also the proton donor (Fig. 5). In the absence of an electron at heme b, state $I_P$ would relax to $P_M$. However, if heme b is reduced, as in our experiments, then the formation of $I_P$ would be accompanied by electron transfer from heme b to the catalytic site, forming state $I_P^−$, which is lower in energy than $I_P$. In this model, observation of $I_P^−$ is possible only if the energy level of this state is sufficiently close to that of state $A$. As outlined in Results, the time constant for the overall $A→P_{RM}$ reaction is dependent on the energy difference between state $A$ and the highest transition state $S_{SO}−O$ (Fig. 5). Because $I_P^−$ is lower in energy than $I_P$ and the energy difference between $I_P^−$ and $I_P$ is approximately the same as that between $I_P$ and $S_{SO}−O$, the highest energy barrier along the trajectory from $A$ to $P_R$ is lower than that leading to $P_M$. This difference explains why the formation of $P_R$ occurs over a shorter time scale than the formation of $P_M$. Furthermore, according to the model, the $I_P$ state would not be observed upon the reaction of the mixed-valence CytC_O with $O_2$ because state $I_P$ is higher in energy than $I_P^−$; that is, with the mixed-valence CytC_O, state $I_P$ would not be populated.

Next, we discuss differences in the data obtained at pH 7 and 10, respectively. We note that at high pH, the rate constants of heme b oxidation and $P_R$ formation displayed more similar slopes in the Arrhenius plots than at pH 7 (Fig. 3). The proposed model involves proton transfer from the Tyr residue to stabilize the peroxo state in $I_P$ (Fig. 5) (48), which would also take place at high pH because the Tyr$^{357}$ residue in the *ba* CytC_O is presumably protonated [for example, see the study of Koutsoupakis et al. (52)]. To explain the larger slope in the temperature dependence of heme b oxidation at high pH, we assume that the K pathway “below” Tyr$^{357}$ becomes more negative (or less positive) upon increasing the pH. This change in the charge would stabilize the proton.

Fig. 4. Kinetics of the reaction of mixed-valence CytC_O with $O_2$. The reaction was monitored at 610 nm. Experimental conditions after mixing: 0.6 to 0.8 μM CytC_O (scaled to 1 μM), 0.05% DDM, and 90 mM Hepes (pH 7.4). A laser artifact at $t=0$ has been truncated for clarity.
at Tyr\textsuperscript{237} such that the height of the transition state TSH would increase slightly at high pH. This change in the barrier height would act to slow the initial reaction step A→IP. In the \textit{T. thermophilus} ba\textsubscript{3} CytcO, which lacks a residue equivalent to Lys\textsuperscript{362}, the overall charge may be determined by the protonation state of several groups in the K pathway, including Tyr\textsuperscript{244}, Tyr\textsuperscript{248} (SU I), and Glu\textsuperscript{15} (SU II) (18, 52, 61, 62).

Assuming that the suggested scenario is generally applicable for respiratory oxidases, the data from this study would point to a mechanism for the initial steps of O\textsubscript{2} reduction at the catalytic site: Electron transfer from the low-spin heme to the catalytic site would coincide with formation of a peroxy state, as originally suggested (23, 25), but the peroxy state Fe\textsuperscript{3+}–O\textsuperscript{−}–O\textsuperscript{−}(H\textsuperscript{+}) would only be formed transiently, and it would not always be observed. This is because the intermediate would be observed (that is, populated to a sufficient concentration) only if the energy level of IP− is sufficiently close to that of intermediate A, which may not be the case in all CytcOs.

A question then arises, why is the formation of the “true” peroxy state [Fe\textsuperscript{3+}–O\textsuperscript{−}–O\textsuperscript{−}(H\textsuperscript{+})] observed in the ba\textsubscript{3} CytcO but has not been observed in earlier studies with the A-type CytcOs? One possible explanation is that structural differences in the K pathway, as discussed above, would lead to differences in the transition state TSH (Fig. 5). Another possibility is that the different CytcOs are optimized to operate at different temperatures. With the ba\textsubscript{3} CytcO, electron transfer from heme b to the catalytic site and PR formation are presumably synchronized at temperatures >45°C, but the different slopes in the temperature dependencies of the rate constants lead to a clear separation.

**Table 2. Thermodynamic parameters.** The parameters were determined by fitting the data in Fig. 3 with the expression \( k = A \exp(-E_a/RT) \). The range of A values was 30 to 40% of the values in the table (when taking into account the error bars in Fig. 3).

|                  | pH 7 | pH 10 |
|------------------|------|-------|
| Heme b oxidation (560-nm first decay) | 17 ± 2 | 18 | 40 ± 4 | 27 |
| PR formation (610-nm increase) | 60 ± 5 | 34 | 60 ± 5 | 34 |

**Fig. 5. Mechanism of the initial steps of O\textsubscript{2} reduction catalyzed by the ba\textsubscript{3} CytcO.** Energy profiles for the initial reaction steps after binding of O\textsubscript{2} to heme a\textsubscript{3}, reflecting the approximate rate constants observed at pH 7 and 10°C. The reduced CytcO (state R) binds O\textsubscript{2} to state A. In state IP, a peroxy state is formed upon oxidation of the heme a\textsubscript{3} iron and either Cu\textsubscript{b} or Tyr\textsuperscript{237}. The peroxy state is stabilized by a proton from Tyr\textsuperscript{237}. The reaction sequence below the diagram illustrates the specific electron transfers within the ba\textsubscript{3} CytcO. Two possible configurations that are in resonance are shown for state IP. It is a geometric single minimum with an electronic structure that is a mixture (resonance) of two main electronic configurations.
in the rate constants at 10°C. Other A-type CytcOs are presumably optimized to operate at lower temperatures, and a separation of the two rate constants would be observed only at temperatures significantly lower than 10°C. In other words, we assume the same mechanism but slightly shifted relative energy levels in the diagram in Fig. 5 for the different CytcOs.

**SUMMARY**

Earlier theoretical studies predicted a high-energy peroxy state, $I_p$ \([Fe^{3+}–O−O(H^+)]\), formed along the reaction pathway leading to O–O bond breaking at the catalytic site of the heme-copper oxidases. Here, we showed that at low temperatures, with the $b_{3}$ *T. thermophilus* CytcO, an electron acceptor is formed at the catalytic site after ~11 μs, before the O–O bond breaking ($\tau \approx 110 \mu$s). This electron acceptor is suggested to be the predicted $I_p$ state, which, after electron transfer to the catalytic site (state $I_p$), becomes significantly populated in the *T. thermophilus* CytcO at low temperature.

**MATERIALS AND METHODS**

**Purification of $b_{3}$ CytcO**

*T. thermophilus* HB8 strain YC 1001 (with a deletion of the *cba* gene) was transformed with a plasmid encoding the $b_{3}$ CytcO gene with a 7-His tag at the N terminus of subunit I and cultivated at 60°C with mild shaking (125 rpm), as described by Chen *et al.* (63) (see also the study of Keightley *et al.* (64)). The harvested cells were suspended in 100 mM Hepes (pH 8) at a ratio of about 1:3, together with a small amount of deoxyribonuclease. The cells were broken using a constant flow cell disrupter at 190 MPa (Constant Systems). The protease inhibitor phenylmethylsulfonyl fluoride was added but after breaking the cells to avoid excessive foaming during the breaking procedure. The solution was centrifuged for 1 hour at 205,000g at 16°C. The pellet was homogenized in 50 ml of 50 mM Hepes (pH 8) and 2.5% Triton X-100 for each 10 g of cell membrane, and the sample was incubated overnight at 4°C. The sample was centrifuged for 1 hour at 170,000g, and the obtained supernatant was supplemented with 10 mM imidazole and loaded onto a prepacked 5-mL Ni–nitrilotriacetic acid affinity chromatography column equilibrated with 10 mM Hepes (pH 8), 150 mM NaCl, 10 mM imidazole, and 1% Triton X-100. After binding of the CytcO, the column was washed with 10 mM Hepes (pH 8), 150 mM NaCl, 40 mM imidazole, and 1% Triton X-100, until the solution passing through the column was clear. Elution of the enzyme was obtained with 10 mM Hepes (pH 8), 150 mM NaCl, 250 mM imidazole, and 1% Triton X-100. The brown elution fractions were pooled and dialyzed overnight at 4°C in 1 liter of 5 mM Hepes (pH 8) and 0.05% Triton X-100 first and then in 1 liter of 5 mM Hepes (pH 8) and 0.05% dodecyl-β-d-maltoside (DDM). The enzyme was stored in this last buffer at 4°C until use.

The concentration of heme $a_3$ was determined from the fully reduced spectrum using the extinction coefficient $e(613–658) = 6.3 \text{mM}^{-1} \text{cm}^{-1}$. The concentration of heme b was calculated from the fully reduced spectrum using $e(560–590) = 26 \text{mM}^{-1} \text{cm}^{-1}$.

**Flow-flash measurements**

Purified $b_{3}$ CytcO was diluted to a final concentration of ~10 μM with 5 mM Hepes and 0.05% DDM (pH 7.4) and placed in a Thunberg cuvette. The sample was made anaerobic on a vacuum line, and air was replaced for N$_2$. The CytcO was then reduced by 2 mM sodium ascorbate using 0.5 μM phenazine methosulfate as a mediator. Incubation under 150 kPa N$_2$ for approximately 1 hour led to the formation of the fully reduced state. Last, the atmosphere was exchanged for CO, and complete binding to the catalytic site was observed after ~20 min of incubation under 100 kPa CO.

The flow-flash measurements were performed using a modified stopped-flow setup (Applied Photophysics) (65, 66). Briefly, the $b_{3}$ CytcO was mixed at a ratio of 1:5 with oxygen-saturated buffer [100 mM Hepes and 0.05% DDM (pH 7) or 100 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) and 0.05% DDM (pH 10)]. After ~30 ms, a 10-ns laser flash (200 mJ, 532 nm, Nd-YAG laser Quantel) was used to dissociate the CO ligand, which initiated the catalytic reaction. Absorbance changes were monitored at specific wavelengths (see text and figure legends). The temperature in the optical chamber was varied using a thermostated water bath.

To prepare the mixed-valence CytcO, the oxidized CytcO (~10 μM in 5 mM Hepes (pH 7.4) and 0.05% DDM) was transferred to a Thunberg cuvette, after which air was replaced by N$_2$. Next, the gas was exchanged for CO, after which the sample was incubated for 2 to 3 hours. Formation of the mixed valence state was confirmed from the optical absorption spectrum (oxidized heme b and reduced heme $a_3$).

**Data handling and analysis**

The data were collected using a digital oscilloscope in which 10$^7$ sampling points were averaged to ~10$^5$ points using a pseudo-logarithmic oversampling function available in the LKS software (Applied Photophysics). If necessary, the traces were smoothed by averaging nine points in a moving time window. Data points in the time window 0 to 1 μs were removed to facilitate the analysis by eliminating a laser artifact (because of incomplete shielding of the detector from the laser flash). The signals were fitted to a model describing irreversible, sequential reactions using KinTek software (KinTek Corp.).

**Computational details**

Density functional theory was used to study the O–O bond cleavage in CytcO. The same model and methodology were used as those described by Blomberg and Siegbahn (67). The model of the catalytic site was based on the crystal structure of CytcO from *R. sphaeroides* (I). It contained heme $a_3$ and the CuB complex, including the cross-linked tyrosine. This part of the catalytic site is very similar to that of the $b_{3}$ CytcOs, which means that the same model could be used to describe the details of the O–O bond cleavage step in both systems. The hybrid density functional B3LYP-D3 [with 15% exact exchange and dispersion correction (68–70)] was used together with a polarized double zeta basis set (lacvp*) for the geometry optimizations and together with the large cc-pvtz (−f) basis plus laccv!* for the metals for the energy calculations (71). Polarizing effects from the surrounding protein were included using the self-consistent reaction field approach, and zero-point corrections to the energies were obtained from Hessian calculations. Entropy effects on the relative energies were found to be negligible for the investigated part of the reaction. For details on the model and the methods, see the study of Blomberg and Siegbahn (67). As previously noted (54), the computational results yield a qualitative picture of the reaction mechanism. In particular, spin states and redox properties of heme groups may not be correctly reproduced by density functional theory. Another difficulty concerns the description of water molecules in the catalytic site. The number and positions of water molecules seen in the crystal structures of the catalytic
site vary for the different CytoCs. Because this situation is difficult to handle computationally, the most accurate relative energies are obtained when no water molecules are included in the model. An obvious exception is the investigation of proton transfer reactions, where water molecules need to be included in the model. To obtain a more quantitative picture of the energetics of a reaction mechanism, a careful combination of computational and experimental results has to be used (54, 72).

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