Oxoferryl-Porphyrin Radical Catalytic Intermediate in Cytochrome bd Oxidases Protects Cells from Formation of Reactive Oxygen Species* 3

Angela Paulus, Sebastiaan Gijsbertus Hendrik Rossius, Madelon Dijk, and Simon de Vries

From the Department of Biotechnology, Section Enzymology, Delft University of Technology, Julianalaan 67, 2628BC, Delft, The Netherlands

Received for publication, December 14, 2011, and in revised form, January 23, 2012. Published, JBC Papers in Press, January 28, 2012, DOI 10.1074/jbc.M111.333542

Background: Cytochrome bd oxidases are proposed to reduce O2 to H2O via a peroxide intermediate.

Results: Kinetic studies detected, however, an oxoferryl-porphyrin radical intermediate and established insignificant production of reactive oxygen species.

Conclusion: Cytochrome bd oxidases, like heme-copper oxidases, reduce O2 in a single four-electron transfer reaction.

Significance: Both classes of terminal oxidases converged independently to minimize the production of reactive oxygen species.

The quinol-linked cytochrome bd oxidases are terminal oxidases in respiration. These oxidases harbor a low spin heme b558 that donates electrons to a binuclear heme b595/heme d center. The reaction with O2 and subsequent catalytic steps of the Escherichia coli cytochrome bd-1 oxidase were investigated by means of ultra-fast freeze-quench trapping followed by EPR and UV-visible spectroscopy. After the initial binding of O2, the O–O bond is heterolytically cleaved to yield a kinetically competent heme d oxoferryl porphyrin π-cation radical intermediate (compound I) magnetically interacting with heme b595. Compound I accumulates to 0.75–0.85 per enzyme in agreement with its much higher rate of formation (~20,000 s⁻¹) compared with its rate of decay (~1,900 s⁻¹). Compound I is next converted to a short lived heme d oxoferryl intermediate (compound II) in a phase kinetically matched to the oxidation of heme b558 before completion of the reaction. The results indicate that cytochrome bd oxidases like the heme-copper oxidases break the O–O bond in a single four-electron transfer without a peroxide intermediate. However, in cytochrome bd oxidases, the fourth electron is donated by the porphyrin moiety rather than by a nearby amino acid. The production of reactive oxygen species by the cytochrome bd oxidase was below the detection level of 1 per 1000 turnovers. We propose that the two classes of terminal oxidases conserved independently to minimize the production of reactive oxygen species.

The quinol-linked cytochrome bd oxidases are terminal oxidases in respiration. These oxidases harbor a low spin heme b558 that donates electrons to a binuclear heme b595/heme d center. The reaction with O2 and subsequent catalytic steps of the Escherichia coli cytochrome bd-1 oxidase were investigated by means of ultra-fast freeze-quench trapping followed by EPR and UV-visible spectroscopy. After the initial binding of O2, the O–O bond is heterolytically cleaved to yield a kinetically competent heme d oxoferryl porphyrin π-cation radical intermediate (compound I) magnetically interacting with heme b595. Compound I accumulates to 0.75–0.85 per enzyme in agreement with its much higher rate of formation (~20,000 s⁻¹) compared with its rate of decay (~1,900 s⁻¹). Compound I is next converted to a short lived heme d oxoferryl intermediate (compound II) in a phase kinetically matched to the oxidation of heme b558 before completion of the reaction. The results indicate that cytochrome bd oxidases like the heme-copper oxidases break the O–O bond in a single four-electron transfer without a peroxide intermediate. However, in cytochrome bd oxidases, the fourth electron is donated by the porphyrin moiety rather than by a nearby amino acid. The production of reactive oxygen species by the cytochrome bd oxidase was below the detection level of 1 per 1000 turnovers. We propose that the two classes of terminal oxidases converged independently to minimize the production of reactive oxygen species.

Cytochrome bd oxidases are membrane-bound heterodimeric terminal oxidases consisting of CydA (57 kDa) and CydB (43 kDa) (1). These oxidases occur in bacteria and archaea and catalyze the oxidation of ubiquinol or menaquinol (2). This reaction is coupled to the generation of a protonotive force because the four chemical protons consumed per O2 are taken from the cytoplasmic side of the membrane, whereas the QH2 substrate protons are ejected into the periplasm (3, 4). Cytochrome bd oxidases bear no sequence homology to heme-copper oxidases (1) and, because they do not pump protons, have a lower bioenergetic efficiency than heme-copper oxidases (3, 4). Cytochrome bd oxidases generally have a high affinity for oxygen and are suggested to act further as oxygen scavengers and as a protection against H2O2 and NO stress (5–7).

Although three-dimensional structures are lacking for cytochrome bd oxidases, studies suggest that its three heme groups are all located in CydA. The low spin heme b558 is coordinated by His186–Met393, the high spin heme b595 by His19, and the chlorin heme d to Glu99 depending on the redox state (1, 8–15). The heme normals of b558 and b595 are parallel to the plane of the membrane (16), whereas the heme normal of heme d makes an angle of ~55° with those of the two other hemes (16, 17). A quinone-binding domain has also been identified (18, 19) that stabilizes a semiquinone (20). Spectroscopic studies suggest that hemes d and b595 are within 10 Å (21) and form a functional binuclear active site that receives electrons from heme b558, proposed as the direct electron acceptor of QH2 (13, 17, 22–25). Raman spectroscopy has identified heme d2−/O−2 (Oxy3)3 and heme d4+ = O (F, oxoferryl or compound II) intermediates indicating that heme d is the site for binding and conversion of O2 (26–28).

The current catalytic mechanism, which has been proposed on the basis of flow-flash and stopped-flow kinetic experiments of the reaction between fully reduced enzyme and oxygen, suggests an initial binding of O2 to heme d to form the Oxy3 or A
Compound I in Cytochrome bd Oxidase Prevents ROS Formation

state (29–31). Oxy$^3$ is subsequently converted to a peroxy intermediate, $P$, with heme $b_{595}$ and heme $d$ oxidized to their ferric states, while heme $b_{558}$ remains reduced. In the next step ($P \to F$) electron transfer from heme $b_{558}$ and heme $d$ leads to scission of the O–O bond followed by H$_2$O release (29, 30). The further donation of one electron and a proton to the active site would restore the enzyme to its fully oxidized form O$^0$, with a hydroxo-bound heme $d$ iron. However, this form of the enzyme is probably not part of the normal catalytic cycle (31). Instead, and under physiological conditions, it is more likely that the two-electron donor QH$_2$ reduces $F$ to Oxy$^1$ (heme $d^{2+}$–O$_2$) followed by reduction by a second QH$_2$ (yielding Oxy$^3$) to provide the necessary electrons to enter the next catalytic cycle. The as isolated or resting enzyme is usually a mixture of $F$ and heme $d^{2+}$–O$_2$ (26–28).

According to the mechanism described above, the O–O bond is broken in two sequential two-electron transfer steps via a peroxy intermediate. This mechanism differs fundamentally from that of the functionally equivalent heme-copper oxidases, which catalyze a single four-electron O–O bond splitting without a peroxy intermediate (32). The physiological advantage of the latter mechanism is the possible prevention of ROS. Indeed, the production of ROS by the heme-copper oxidases has been found to be undetectably low (33, 34); in fact (all mitochondrial) ROS production is due to side reactions with O$_2$ of other respiratory enzymes in their reduced state, notably complex I and complex III (33–37). Whether cytochrome bd oxidases produce ROS is not known. If they do so, how much ROS is produced and would this be due in consequence to the formation of a peroxy intermediate?

The assignment of a catalytic peroxy intermediate was based solely on the UV-visible spectrum (29) and lacks a solid biophysical underpinning further preventing conclusions about its possible structure as a side-on, end-on, or heme-bridged peroxy species. To characterize the structure of $P$, the catalytic mechanism of the cytochrome bd-I oxidase from *Escherichia coli* was investigated using an ultrafast mixing and freeze-quenching technique (MHQ) that in addition to UV-visible enables EPR spectroscopic analyses (38, 39).

Our results indicate that cytochrome bd oxidases split the O–O bond like the heme-copper oxidases in a single four-electron transfer reaction. However, in cytochrome bd oxidases a compound I intermediate is formed, unlike the heme-copper oxidases. The amount of ROS produced by cytochrome bd oxidase was below the detection level of 1 per 1000 turnovers. We propose that both classes of terminal oxidases have converged evolution to enzymes in which the O–O bond is broken in a single four-electron transfer reaction to minimize the cellular production of ROS.

**EXPERIMENTAL PROCEDURES**

**Overexpression of Cytochrome bd-I Oxidase from E. coli—**

The cytochrome bo and bd-II knock-out strain MB30 was a donation by M. Bekker (40). MB30 was transformed with plasmid pACYC177 containing the *E. coli* CydAB operon overproducing cytochrome bd-I. Precultures were grown aerobically in LB medium with ampicillin (50 µg/ml) in a shaking incubator at 37 °C (~175 rpm). Liter flasks of basal glycerol/fumarate minimal medium (41) containing ampicillin (50 µg/ml) were inoculated with 5% of the LB culture, filled to the rim, and closed, creating semi-anaerobic conditions. Cells were allowed to grow at 37 °C in a shaking incubator for ~20 h. These starter cultures were used to inoculate (4%) four 25-liter glass vessels with basal glycerol/fumarate medium and ampicillin (5 µg/ml). Cells were grown under hypo-aerobic conditions after nitrogen flushing, while stirring at 30 °C for ~65 h.

**Purification of Cytochrome bd-I Oxidase—**

After a 25-fold concentration of the cells cultures in a cross-flow filtration system, the cells were harvested by centrifugation (4 °C, 10 min, 9000 × g) and washed once with 50 mM Tris-HCl buffer, pH 8. The washed cell pellets were resuspended in the same buffer prior to cell disruption at 1.8 kbar. The resulting suspension was centrifuged (4 °C, 10 min, 3000 × g) to remove cell debris. The supernatant was then centrifuged in a Beckman ultracentrifuge (4 °C, 1 h, 100,000 × g) to spin down the cell membranes containing the bd-I oxidase. Membranes were resuspended in 25 mM MOPS, pH 6.8, 1 mM EDTA and washed once or twice. The enzyme was extracted from the *E. coli* membranes by addition of 1% lauryl maltoside to the solution and incubating while stirring on ice for 15 min. Purification of the membrane-extracted enzyme consisted of a single column chromatography step (Q-Sepharose FastFlow) with 25 mM MOPS buffer, pH 6.8, as the running buffer. Diluted fractions were pooled by activity, concentrated, and stored at ~80 °C.

**Freeze-quench Experiments—**

MHQ, EPR, UV-visible experiments, and kinetic simulations were performed as described previously (38, 39) using the IGOR Pro software from Wavemetrics, Inc. The MHQ setup was modified just before the mixer entry with a stainless steel tubing extension immersed in ethylene glycol at −5 °C to bring the reaction temperature to 1 ± 1 °C. For kinetic experiments, purified enzyme (150 or 300 µM) in 50 mM sodium phosphate buffer, pH 7.8, 5 mM EDTA, 0.05% lauryl maltoside was made anaerobic, reduced with 2 mM sodium dithionite, and subsequently mixed with the same buffer saturated with O$_2$. EPR spectra were normalized at the intensity of heme $b_{595}$ and in separate experiments using an internal CuClO$_4$ (0.1 mM) standard in the oxygenated buffer before mixing. Data in Fig. 5 represent the average of four independent experiments. UV-visible averaged spectra were corrected for scatter and base line as described previously (38, 39) and normalized as follows. Normalization is necessary because for the UV-visible experiments, the amounts of cold freeze-quenched powder in the low temperature cuvette is variable. Low temperature reference spectra (not prepared by MHQ) of fully reduced and “as isolated” enzyme (10 µM) were recorded in buffer, and the Soret band maxima relative to 490 nm were determined (1.0 and 0.46, respectively). The major difference in the measured maximal amplitude of the Soret band absorbance is due to the relatively sharp peak of reduced heme $b_{558}$ in particular for samples after 100 µs. The relatively broad Soret peaks of the oxidized hemes contribute mainly to the difference 450–490 nm, in particular for samples after 100 µs. With these two parameters, the relative intensities of the spectra shown in Fig. 4 were calculated. From these, the fractional amount of reduced heme $b_{558}$ was calculated from the spectra in the α-band region. The error in this calculation amounts to ~0.1.
Compound I in Cytochrome bd Oxidase Prevents ROS Formation

heme $b_{558}$ per enzyme. For the absorbance at 680 nm, the error was 0.35 per enzyme. The maximal absorbance at 680 nm was taken the same as that of oxidized heme $d$ (cf. Ref. 29).

Determination of ROS—Spin trapping assays were performed with 25 mM DEPMPO (42) in the same buffer as above. The reaction was started by addition of 0.1 $\mu$M bd-I oxidase or 200 $\mu$M dQH$_2$, both, or both in the presence of either catalase (1 unit) or superoxide dismutase (1 unit). The 200 $\mu$M dQH$_2$ is fully oxidized in 20 s. Superoxide was prepared from solid KO$_2$ in 1 m NaOH. The DEPMPO superoxide adduct has a half-life time of 17 min (42). Room temperature EPR spectra were recorded in a 100-$\mu$L aqueous sample cell 120 s after addition of the reagents and subsequently after 240 and 360 s. The spectra in Fig. 7 are the average of these three spectra. At longer reaction times, a background DEPMPO radical developed in the cavity, and the sharp signal at $g \approx 2.00$ remained. The (partial) upper spectrum shows the $g$ resonance of the low spin heme $b_{558}$ at $g \approx 3.58$ from a 25-fold concentrated as isolated enzyme solution; the peak at $g \approx 3.58$ is too weak to be detected in freeze-quench-oxidized samples. The peak at $g = 4.3$ is from adventitious iron, the broad peak around 3200 G from adventitious Cu$^{2+}$ in the cavity, and the sharp signal at $g \approx 3.380$ G is due to the freeze-quench procedure. EPR conditions are as follows: Microwave frequency, 9.45 GHz; modulation amplitude, 0.5 millitesla; microwave power, 20 microwatts; temperature, 4.2 K. Full traces are displayed at the same gain.

RESULTS

Heme $d$ Oxoferryl Porphyrin $\pi$-Cation Radical Intermediate (Compound I) Detected by EPR Spectroscopy—To study the mechanism of O–O bond splitting by the cytochrome bd oxidase, single turnover experiments were performed at 1 °C to slow down the reaction. After the reaction between reduced enzyme and O$_2$, intermediates were trapped by means of freeze-quenching at times $\geq 100$ $\mu$s and analyzed by EPR and low temperature UV-visible spectroscopy.

The EPR spectrum of as-isolated cytochrome bd oxidase displays resonances from two high spin heme species, the axial heme $d$ ($g \approx 6$) and the rhombic heme $b_{595}$ ($g_\text{ax} \approx 6.2$, and $g_\text{av} \approx 5.7$) and a third signal from the low spin heme $b_{558}$ ($g_\text{av} = 3.58$) (cf. Fig. 1) (24, 43). The reduced enzyme is EPR-silent. After reacting for 100 $\mu$s, heme $b_{595}$ became fully oxidized (Fig. 1, middle trace), whereas only $0.1$–$0.2$ heme $d$ had converted to the ferric state. The middle trace in Fig. 1 further shows a previously undetected intermediate at 3100–3500 G, which is argued below to be a compound I derivative of heme $d$. The new EPR signal (Fig. 2 and supplemental Fig. S1) consists of three overlapping signals arising from three rhombic $S = \frac{1}{2}$ spin systems when recorded at 4.2 K. At higher temperatures the line shapes of the three EPR signals change and coalesce at 77 K into a single rhombic signal with $g$ values that are the average of the...
individual signals (Table 1 and supplemental Fig. S1). At 4.2 K the integrated intensity of the three signals together accounts for 0.75–0.85 spins per enzyme. The unusual temperature dependence of the EPR signals is due to a magnetic dipolar interaction between oxidized heme $b_{595}$ and the compound I, a conclusion that will be explained below.

The magnetic properties of compound I are well understood (supplemental material) (44, 45). Briefly, compound I comprises an $S = 1$ heme oxoferryl center ($\text{Fe}^{4+} = \text{O}$) that is magnetically coupled to a $S = \frac{1}{2}$ porphyrin $\pi$-cation radical. The coupling of the two spins yields three Kramer’s doublets (cf. Fig. 3B) yielding either an $S = \frac{1}{2}$ or $S = 3/2$ ground state, which depends on the relative magnitudes and signs of the Heisenberg exchange interaction ($J$) between the $\text{Fe}^{4+} = \text{O}$ and the porphyrin radical and further on the zero-field splitting ($D$) of the $S = 1$ species. The finding here that the three $g$ values are close to $g = 2$ indicates a total spin of $S = \frac{1}{2}$ for the ground state of the compound I. The Kramer doublets are separated in energy by an amount $\Delta$ ($-D$) and $J$. Because $\Delta$ is usually quite small, 20–40 cm$^{-1}$, compound I species follow a two-phonon Orbach relaxation mechanism. The presence of a low-lying first excited state will also result in significant loss of spin intensity at temperatures greater than $\Delta$ (i.e. above $\sim 30$ K). Hence, to validate the assignment as compound I, both the relaxation behavior and the ground state population were determined (Fig. 3). Both these experiments should yield a similar value for $\Delta$ (46).

The increase of the relaxation rate upon increasing the temperature follows an Orbach relaxation mechanism at $T > 4.2$ K (Fig. 3A) for a first excited state at $\Delta = 36.8 \pm 4.8$ cm$^{-1}$. The decrease of spin intensity corresponds to the presence of excited states that are $32.2 \pm 10.4$ cm$^{-1}$ ($\Delta$) and $33.9 \pm 11.7$ cm$^{-1}$ ($\Delta + J$), respectively, above the ground state (Fig. 3B). The latter two values indicate a small value for $J$ of $\sim 2$ cm$^{-1}$ or $\Delta < 0.1 J$. Such a small value for $J$ (either negative or positive) relative to $\Delta$ (or $D$) is consistent with $g$ values close to $g = 2$ (Table 1), and in fact is quite similar to those calculated for the isolated $S = 1$ $\text{Fe}(\text{IV})$ system for which $J = 0$ (47). The value $g_z = 1.973$ determined here is consistent with a calculated value for $D$ (or $\Delta$) of $\sim 30$ cm$^{-1}$ (47) and close to that determined here. The decrease of the ground state spin population rules out that the EPR signal is derived from a ferric heme peroxo center for which the first excited state lies at $>700$ cm$^{-1}$, determined by the strength of the crystal field (supplemental material) (48). In addition, the $g$ values would be very unusual for low spin heme centers.

The observation that the compound I EPR signal is split into three signals (Figs. 1 and 2 and supplemental Fig. S1) with similar intensities suggests that compound I is coupled to a nearby anisotropic magnet for which at 4.2 K the relaxation is much slower than that of compound I, whereas at higher temperature the reverse holds. At $T > 60$ K, the relaxation of this magnet is so fast that the splitting averages out resulting at 77 K in a compound I signal with $g$ values that are the average of those at 4.2 K (Table 1). Previous studies have provided evidence for magnetic interactions between ferric heme $d$ and heme $b_{595}$ (9, 24, 43). We therefore propose an anisotropic magnetic dipolar interaction between the heme $d$-derived compound I and heme $b_{595}$. At high temperatures, the relaxation of the ferric heme $b_{595}$ is much faster than that of compound I, consistent with the detection of an EPR signal of the latter at 77 K but not of heme $b_{595}$ (see supplemental material). Interestingly, the splitting is much more pronounced in the $g_x,y$ resonances than in the $g_z$ peak. The $g_z$ is directed along the $\text{Fe}^{4+} = \text{O}$ bond, perpendicular to the plane of heme $d$. Because the angle between the heme $d$ and heme $b_{595}$ normals is $\sim 55^\circ$ (16, 17).

### Table 1

<table>
<thead>
<tr>
<th>Compound I EPR parameters</th>
<th>$g_x$</th>
<th>$g_y$</th>
<th>$g_z$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signal at 77 K</td>
<td>2.157</td>
<td>2.112</td>
<td>1.973</td>
</tr>
<tr>
<td>Signal 1 at 4.2 K</td>
<td>2.173</td>
<td>2.092</td>
<td>1.973</td>
</tr>
<tr>
<td>Signal 2 at 4.2 K</td>
<td>2.158</td>
<td>2.128</td>
<td>1.973</td>
</tr>
<tr>
<td>Signal 3 at 4.2 K</td>
<td>2.146</td>
<td>2.131</td>
<td>1.973</td>
</tr>
</tbody>
</table>

FIGURE 3. Saturation behavior (A) and ground state population (B) of compound I as a function of temperature. Data in A were fitted to $\ln(P_{1/2}) = \ln(\Delta T + B(e^{\Delta T} - 1)^{-1})$, where $\Delta T$ represents the direct process ($A = 22$ microwatts/K) and the second term the Orbach relaxation ($B = 4.2$ megawatts). Ground state population in B was calculated with the equation: $(1 + e^{-\Delta T/B} + e^{-(\Delta + J)/B})^{-1}$. Values of $\Delta$ and ($\Delta + J$) are given in the text. The three Kramer’s doublets and their energy separations are shown in the lower inset in B.
Compound I in Cytochrome bd Oxidase Prevents ROS Formation

which is close to the magic angle of 54.7° at which the magnetic dipolar coupling is zero, the small splitting in the g₁ resonance is consistent with this angle determined by other methods. We conclude that the new EPR signal is from a heme d oxoferryl porphyrin π-cation radical in dipolar magnetic interaction with heme b₅₉₅³⁺.

Compound I Species Detected by UV-visible Spectroscopy—Fig. 4 shows low temperature UV-visible spectra of the reaction between cytochrome bd-I oxidase and O₂. After 100 μs, the peak of heme d at 624 nm has shifted to 641 nm and broadened considerably, whereas heme b₅₉₅ has remained largely reduced (75–85%). In agreement with the EPR spectra (Fig. 1), heme b₅₉₅ is completely oxidized after 100 μs indicated by the disappearance of the broad absorbance around 595 nm and the appearance of a negative peak in the Soret region (439 nm) in the "0–100-μs" difference spectrum (49). This difference spectrum further indicates the appearance of a broad absorbance at 404 nm. The peak at 404 nm (and that at 641 nm) is ascribed to that of the heme d Compound I intermediate. In agreement with this are the blue shifts from ~430 nm for the ferrous state to 404 nm and the low extinction, ~25–30% of the intensity of the Soret band of heme b₅₉₅, two features also observed for compound I from horseradish peroxidase (50). The compound I absorbance is also directly visible in the absolute spectra of Fig. 4 as a shoulder at 404 nm on the Soret peaks of hemes b₅₉₅ and b₅₅₈. This shoulder disappears as the reaction proceeds. Difference spectra calculated for times >100 μs did not resolve the 404-nm band as well as after 100 μs because of spectral interference from hemes b₅₅₈ and d, the latter changing to the ferric state at longer reaction times. In contrast, the intermediate state obtained after 100 μs is quite pure, i.e. full oxidation of heme b₅₅₈ after 2 ms are explained by the slight excess of reduc-tant present (sodium dithionite), which renders the oxidation kinetics not pure single turnover; instead, the enzyme reaches a quasi steady state. Here, heme d is ~80% oxidized with the remainder present as F and a small amount of heme d²⁺. O₂ represented by the absorbance at 646 nm (cf. 26–28). This electronic distribution is in agreement with experiments that show that F and Oxy¹ are dominant steady-state species (51). Note that the 680 nm band is also present in the enzyme as isolated
Compound I in Cytochrome bd Oxidase Prevents ROS Formation

TABLE 2

Rate constants at 1 °C for various intermediate steps of the cytochrome bd oxidase catalytic cycle

<table>
<thead>
<tr>
<th>Species</th>
<th>k</th>
</tr>
</thead>
<tbody>
<tr>
<td>( b_{558}^{3+} ) oxidation ( ^{a} )</td>
<td>20,000 ± 2000</td>
</tr>
<tr>
<td>Compound I decay</td>
<td>1950 ± 200</td>
</tr>
<tr>
<td>( b_{553}^{2+} ) oxidation</td>
<td>1850 ± 400</td>
</tr>
<tr>
<td>Compound II decay</td>
<td>7300 ± 2500</td>
</tr>
<tr>
<td>( d^{2-} ) formation</td>
<td>1250 ± 100</td>
</tr>
</tbody>
</table>

\( ^{a} \) Data include the rate of \( O_{2} \) binding. Estimated from Ref. 29 and this work.

(Fig. 4) (26–28) but that heme \( d^{2-} \)-\( O_{2} \) is absent in our preparation.

EPR spectroscopy (supplemental Fig. S2) shows that as compound I disappeared and heme \( d^{3+} \) is formed, the line shape of heme \( b_{553} \) in particular the g ~ 5.7 derivative-like resonance, shifts by ~ 10 G. The small shift is interpreted as a change in magnetic interaction between heme \( b_{553} \) and heme d, as the latter changes from the compound I state to the ferric state. After 2 ms, the EPR spectrum of the high spin heme centers is similar to that of the “as-isolated enzyme.”

The kinetic profiles of the various intermediates determined from the UV-visible and EPR spectra are shown in Fig. 5, and the calculated rate constants are listed in Table 2. The rates of oxygen binding, heme \( b_{553} \) oxidation, and compound I formation were too fast to be determined directly in this study, even at the reaction temperature of 1 °C, where these reactions appear completed within 100 \( \mu \)s. Flow-flash experiments (29) indicate a 10-fold lower rate of oxidation of heme \( b_{558} \) than the preceding reactions suggesting a (combined) rate of ~20,000 s \(^{-1} \) for oxygen binding, oxidation of heme \( b_{553} \) and compound I formation (cf. Table 2). The accumulation of compound I to 0.75–0.85 per enzyme is consistent with the ~10-fold higher rate of its formation than its rate of decay (Table 2).

Significantly, the rates of compound I decay and oxidation of heme \( b_{558} \) are the same, but the formation of heme \( d^{3+} \) is slower (Table 2). The similar rates of compound I decay and heme \( b_{558} \) oxidation are consistent with the view that electron transfer from heme \( b_{558} \) leads to direct reduction of the porphyrin \( \pi \)-cation radical, thus producing the ferryl form of heme \( d \) or \( F \). In the subsequent reaction, \( F \) which barely accumulates, is rapidly reduced further, by excess reductant, yielding heme \( d^{3+} \). The rate of compound II reduction is calculated at ~7300 s \(^{-1} \) based on the experimental time delay between compound I decay and heme \( d^{3+} \) formation and the accumulation to 0.1 per enzyme estimated from the 680 nm absorbance.

DISCUSSION

Catalytic Mechanism—The reduction of \( O_{2} \) by reduced cytochrome \( bd \) oxidase will in general not yield clean (pseudo-) first-order traces because the complete reaction needs four electrons, and the enzyme can store only three. In our experiments, the small excess reductant leads to rapid net 4–5 electron transfer leaving some reduced enzyme after 2 ms that is slowly oxidized in a quasi-steady state in which excess reductant and remaining oxygen are exhausted. In the flow-flash experiments, \( F \) was formed almost stoichiometrically in 47 \( \mu \)s, apparently corresponding to a net three-electron reaction (29). In the next step (~1.1 ms), \( F \) was converted to a mixture of oxidized and oxygenated enzyme as observed here. The transient kinetics of \( F \) in the flow-flash experiments show that it is a true intermediate. The nontransient kinetics of \( F \) in our experiments might suggest that it is not part of the main catalytic pathway but, for example, in rapid equilibrium with another/unknown intermediate. However, because the reaction proceeds to a quasi-steady state, also a true intermediate may show nontransient kinetics.

The optical and kinetic properties of the intermediate formed after 100 \( \mu \)s with peaks at 404 and 641 nm are the same as those observed for the peroxo intermediate \( P \) formed after 4.5 \( \mu \)s (peak at 635 nm at 20 °C (29)). The assignment as a peroxo intermediate by the authors was based solely on UV-visible spectroscopy and on the notion that formation of compound I is not very likely, because it would require oxidation of a nearby amino acid residue or the porphyrin ring that is energetically unfavorable in the presence of the reduced heme \( b_{558} \) in the proximity of the catalytic center (29). Here, we provide both EPR and UV-visible spectroscopic evidence that the intermediate labeled \( P \) in Ref. 29 is in fact compound I. We therefore propose a new reaction mechanism for the cytochrome \( bd \) oxidase (Fig. 6). Accordingly, after binding of \( O_{2} \) to the fully reduced enzyme (Red\( ^{3} \)) in which Oxy\( ^{3} \) is formed, the \( O–O \) bond is split in a single four-electron reaction producing within 100 \( \mu \)s the compound I intermediate (CpdI or F\( ^{3+} \)) without formation of a peroxo state. Oxygen bond breaking is accomplished in an apparently concerted electron transfer reaction from heme \( b_{553} \), heme \( d \) (two electrons), and the heme d porphyrin moiety. The obligatory proton donor needed for \( O–O \) bond splitting remains unknown. The subsequent internal electron transfer from heme \( b_{558} \) (525 \( \mu \)s) converts CpdI into CpdII (or \( F \)). Rapid electron transfer from dithionite or from endogenous QH\( _{2} \) (1–2 electrons per enzyme) produces a largely oxidized enzyme after 2 ms and a mixture of some heme \( b_{558}^{2+} \), Oxy\( ^{3} \), and CpdII. The formation of kinetically competent compound I and II intermediates by the cytochrome \( bd \) oxidases resembles the mechanism of plant peroxidases and eukaryotic catalases. The catalytic mechanism of cytochrome \( bd \) oxidases is similar to that of heme-copper oxidases, which also break the \( O–O \) bond in a single four-electron transfer. However, in the heme-copper oxidases, \( P_{\Delta \text{q}} \) rather than CpdI is formed initially, i.e. a heme a\( _{3} \) oxoferryl intermediate plus an amino acid radical, most likely a tyrosine radical (52).

ROS Production—Breaking the \( O–O \) bond in a single four-electron transfer represents a mechanism to protect the cell from ROS production during aerobic growth. Possible production of ROS was determined by the EPR spin trapping assay with DEPMPO (42) and the UV-visible based Amplex Red reagent assay. The sensitivity of the Amplex Red assay is limited by a slow background reaction and the spin trapping method by the DEPMPO-derivative stability, its EPR detection limit, and the \( \Delta \text{H}_{2} \text{O} \) solubility of 200 \( \mu \)M limiting the number of turnovers. Both methods indicated that the production of ROS by cytochrome \( bd \) oxidase was below the detection limit of < 1 per 1000 turnovers, i.e. less than 0.1 \( \mu \)M \( O_{2} \) or \( H_{2}O_{2} \) produced per 100 \( \mu \)M \( O_{2} \) consumed (cf. Fig. 7). The production of ROS by purified \( P. \text{denitrificans} \) cytochrome aad\( _{3} \) oxidase was determined at < 1 per 250 turnovers, a relatively high value because
of the presence of a background signal. However, the production of ROS by the similar mitochondrial cytochrome *aa₃* oxidase has previously been estimated to be much lower than 1/250 turnovers (33–35, 53). In fact ROS production is too low to detect mainly due to the contribution by other respiratory enzymes. *In vivo* ROS production by mitochondria is estimated at 1/100 per 1000 turnovers of the respiratory chain (53) and possibly similar in bacteria (54). If terminal oxidases indeed produce negligible amounts of ROS, as is generally believed, their contribution should be below 1/10,000 turnovers, a value too low to measure directly with current techniques. Because both classes of terminal oxidases break the O–O bond in a single four-electron transfer, significant production of H₂O₂ and OH⁻ is considered unlikely, but production of O₂⁻ cannot be completely prevented because of the Fe²⁺-O₂ ↔ Fe³⁺-O₂ equilibrium in the initial oxy-derivative. Resonance Raman spectroscopy on the two classes of terminal oxidases and oxymyoglobin indeed shows considerable superoxide character for the oxy-complexes (27, 55, 56). The release of O₂⁻ by oxymyoglobin is, however, very slow (t½ ~ 10 h (57)) among others because of its high midpoint potential (~0.1 V) (58). In the terminal oxidases the midpoint potentials of the hemes are even higher (~0.3 V) (43, 52), which would most likely result in even slower release of O₂⁻. In addition, the oxy-complexes of the terminal oxidases are rapidly (~<10 µs) converted to Cpd1 or to P₅₇ yielding a calculated O₂⁻ production of <1 per 10⁹ turnovers, far below current detection levels and far below that of other respiratory complexes.

It appears that the cytochrome *bd* oxidases and heme-copper oxidases have evolved independently to minimize if not prevent production of ROS by very rapidly breaking the O–O bond in an apparently concerted single four-electron transfer and protonation reaction. To do so, both classes of terminal oxidases harbor a compact bi-metallic center integrated with a nearby proton donor. This bi-metallic center contains one metal ion able to attain the Fe⁴⁺ state and an additional redox center, either the porphyrin ring itself, or a nearby amino acid as donor of the fourth electron. In the single heme-containing peroxi-

![Diagram of the catalytic cycle of cytochrome *bd* oxidase](http://example.com/diagram)

**FIGURE 6. Proposal for the catalytic cycle of cytochrome *bd* oxidase.** Half-lives (in microseconds) of the various steps are indicated. Protons needed in some of the reactions are omitted from the figure. The heme normals of heme *d* and heme *b* make an angle of ~55°, suggested here by the nonparallel porphyrin plane of heme *d*, although not drawn at 55°. See text for further explanation of the catalytic mechanism. The superscripts in O¹, Oxy¹, Oxy³, and Red³ refer to the total number of electrons in the heme centers of the enzyme.

![Room temperature EPR spectra](http://example.com/spectra)

**FIGURE 7. Room temperature EPR spectra of DEPMPO-O₂⁻ adduct (upper three spectra) and of cytochrome *bd* oxidase reacted in various ways to detect possible formation of the DEPMPO-O₂⁻ or other (e.g. OOH⁻) adducts during the reaction.** None are seen in the complete reaction (*bd/dQH₂*), or in the controls with superoxide dismutase (*bd/dQH₂/SOD*), or catalase (*bd/dQH₂/Cat*), or in the presence of only enzyme (*bd*) or substrate (*dQH₂*). The DEPMPO-O₂⁻ adduct at 1.5 µM was prepared as described under “Experimental Procedures.” The traces of 0.4 and 0.1 µM were calculated from the 1.5 µM spectrum by multiplication of 0.27 and 0.067, respectively, and then adding a random noise function with the same noise as the experimental spectrum. The detection DEPMPO-O₂⁻ limit is below ~0.1 µM, because in this (calculated) spectrum the S/N ratio is ~<1. Microwave frequency, 9.79 GHz, modulation amplitude, 0.1 millitesla, microwave power, 20 milliwatt.
Compounds in Cytochrome bd Oxidase Prevents ROS Formation

14. Tsubaki, M., Uno, T., Hori, H., Mogi, T., Nishimura, Y., and Anraku, Y.

Acknowledgments—We thank Prof. Dr. W. R. Hagen (Delft University of Technology) for discussions on EPR, Prof. Dr. I. Schröder (UCLA) for suggestions regarding the overexpression of the cytochrome bd oxidase, and Dr. H. R. C. Dietrich, Ing. M. J. F. Strampraad, and Ing. E. Yıldırım for various contributions to this research.

REFERENCES

Compound I in Cytochrome bd Oxidase Prevents ROS Formation


