Discovery of the True Peroxy Intermediate in the Catalytic Cycle of Terminal Oxidases by Real-time Measurement*

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The sequence of the catalytic intermediates in the reaction of cytochrome bd terminal oxidases from *Escherichia coli* and *Azotobacter vinelandii* with oxygen was monitored in real time by absorption spectrosopy and electrometry. The initial binding of O2 to the fully reduced enzyme is followed by the fast (5 μs) conversion of the oxy complex to a novel, previously unresolved intermediate. In this transition, low spin heme \( b_{558} \) remains reduced while high spin heme \( b_{595} \) is oxidized with formation of a new heme d-oxygen species with an absorption maximum at 635 nm. Reduction of O2 by two electrons is sufficient to produce (hydro)peroxide bound to ferric heme \( d \). In this case, the O-O bond is left intact and the newly detected intermediate must be a peroxo complex of heme \( d \) (Fe\( _{\text{d}^{2+}}-\text{O-O}-\text{H} \)) corresponding to compound 0 in peroxidases. The alternative scenario where the O-O bond is broken as in the \( P_{\text{M}} \) intermediate of heme-copper oxidases and compound I of peroxidases 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. The formation of the peroxy intermediate is not coupled to membrane potential generation, indicating that hemes \( d \) and \( b_{595} \) are located at the same depth of the membrane dielectric. The lifetime of the new intermediate is 47 μs; it decays into oxoferryl species due to oxidation of low spin heme \( b_{558} \) that is linked to significant charge translocation across the membrane.

Heme enzymes such as peroxidases, catalases, cytochromes P450, and terminal oxidases are suggested to share the key catalytic intermediates, namely peroxy and oxoferryl. Reaction of heme enzymes with \( \text{H}_2\text{O}_2 \) suggests sequential formation of a peroxo complex (compound 0, Fe\( ^{3+} \)-OOH), and the two oxoferryl species, compound I (Fe\( ^{4+} = \text{O} \ R^+ \), where \( R^+ \) is a porphyrin or amino acid radical) and compound II (Fe\( ^{4+} = \text{O} \)) (Ref. 1 and references therein). Compound 0 could be detected at low temperatures (2) but not at room temperature (3). Studies on heme-copper terminal oxidases reported the intermediates analogous to compound I (\( P_{\text{M}} \)) and compound II (\( P_{\text{R}} \) and F) (4–23). A transient formation of an intermediate equivalent to compound 0 has been proposed but never documented in heme-copper oxidases.

Cytochromes bd comprise a peculiar class of terminal oxidases performing a number of vitally important functions in bacteria (Ref. 24 and references therein). Like heme-copper oxidases, cytochrome bd conserves energy in the form of electrochemical proton gradient (\( \Delta \mu_{\text{H}^+} \)) across the membrane accumulated in the reaction of the reduction of molecular oxygen to water (25–29). Cytochrome bd, however, is not a proton pump (30), and the formation of \( \Delta \mu_{\text{H}^+} \) occurs only due to the vectorial chemistry where the protons from quinol oxidation are released into the positive (periplasmic) side of the membrane whereas protons required for water formation are taken up from the negative (cytoplasmic) side. Cytochrome bd uses ubiquinol or menaquinol, but never cytochrome c, as a natural respiratory substrate. Cytochrome bd contains three hemes \( (b_{558}, b_{595}, \text{and} \ d) \) but no copper (27). The low spin heme \( b_{558} \) seems to be directly involved in quinol oxidation, whereas the high spin hemes \( b_{595} \) and \( d \) likely form a di-heme catalytic site for binding and activation of \( \text{O}_2 \), reducing it further to \( \text{H}_2\text{O} \) (31–37).

The reaction of the fully reduced (\( R \)) cytochrome bd with oxygen showed the sequential formation of the 650- and 680-nm absorbing species (38, 39). The 680-nm species can also be generated by the addition of excess \( \text{H}_2\text{O}_2 \) to the “as-isolated” or fully oxidized (O) form of the enzyme (39–43). The 650- and 680-nm species were identified by resonance Raman spectroscopy as the ferrous oxy (A) and oxoferryl (F) intermediates, respectively. It was reported that during the reaction of the R enzyme with \( \text{O}_2 \), A decays directly into F without any in-between intermediate (38, 39).

In this work, we studied the reaction of the R cytochromes bd from *Escherichia coli* and *Azotobacter vinelandii* with oxygen, using the flow-flash method by means of spectroscopic and electrometric techniques that allow the recording of absorption spectra and membrane potential development with 1-μs time resolution. We were able to detect a transient formation of a spectrally discernible intermediate between A and F.

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* This work was supported by grants from Biocentrum Helsinki, the Sigrid Juselius Foundation, the Academy of Finland, the Russian Foundation for Basic Research (Project 05-04-48096), Howard Hughes Medical Institute (Project 55005615), and the Civilian Fund for Research and Development (Project RUB-1-2836-MO-06). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: O, fully ferric species; A, ferrous oxy species; P, peroxy species; F, oxoferryl species; R, fully ferrous species; \( \tau \), time constant, reciprocal of rate constant; \( \text{CcO} \), cytochrome c oxidase; MOPS, 4-morpholinepropanesulfonic acid.
Peroxy Intermediate of Cytochrome bd Oxidase

Four Sequential Steps of the Catalytic Cycle—The flow-flash method is a powerful tool for the study of terminal oxidases (51). We used this approach to examine the reaction of cytochrome bd oxidases from *E. coli* and *A. vinelandii* in the R state with oxygen on the microsecond time scale at +21 °C. To resolve the transient formation and decay of the reaction intermediates, we recorded the optical changes with a time resolution of 1 spectrum/μs. The spectra on Fig. 1A show the development of the optical changes during the first 100 μs of the reaction with the *E. coli* enzyme. The changes at 560, 595, and in the range of 630–680 nm indicate that all of the three hemes, b$_{558}$, b$_{595}$, and d, are involved in this reaction. Fig. 1B shows a time course of the absorbance changes at 654–635 nm. The kinetic behavior at the selected pair of wavelengths clearly shows all sequential phases, because each next phase has an opposite direction of the signal development. The initial unresolved decrease of absorbance reflects the photolysis of CO from the reduced enzyme (R-CO → R transition) and is followed by the three more transitions that can be well resolved. Global analysis of the surface of the spectra reveals that these transitions can be fitted as three sequential steps with time constants of 1.4, 4.5, and 47 μs (Fig. 1B, main panel) and the corresponding kinetic difference spectra can be obtained (Fig. 1C). The spectrum of the 1.4-μs phase has a trough at 630 nm and a peak at 654 nm (Fig. 1C, dotted line), which is typical of O$_2$ binding to ferrous heme d (52). Thus, the 1.4-μs phase can be clearly assigned to the formation of the A intermediate (R → A transition). It has to be noted that the rate of the A formation (*τ* = 1.4 μs at [O$_2$] = 365 μM) linearly depends on the concentration of O$_2$ (data not shown; see also Refs. 38, 49).

The spectrum of the 4.5-μs phase shown in Fig. 1C (solid line) has a minimum at 654 nm (decay of the A intermediate) and a maximum at 635 nm that reflects the formation of an intermediate that has not been resolved in earlier studies (38, 39). We denote this new reaction intermediate as P and the 4.5-μs phase as the A → P transition. The rate of the P formation does not depend on the concentration of O$_2$. The spectrum of the 47-μs phase (Fig. 1C, dashed line) has troughs at 561 nm (due to the oxidation of heme b$_{558}$) and at 640 nm (decay of the P intermediate) and a peak at 680 nm. The 680-nm peak is diagnostic of the heme d oxoferryl species (F) (45); thus, the 47-μs phase corresponds to the P → F transition.

As shown in the inset of Fig. 1B, the oxygen-induced absorption changes of the *A. vinelandii* cytochrome bd are very similar to those of the *E. coli* enzyme. With the *A. vinelandii* enzyme,

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**EXPERIMENTAL PROCEDURES**

Carbon monoxide, oxygen, nitrogen, and argon gases were from AGA; plant l-lecithin was from Avanti Polar Lipids (Alabaster, AL). Other basic chemicals and biochemicals were from Sigma-Aldrich, Merck, Anatarce, Fluka, and Serva. *E. coli* cells were obtained from GO105/pTK1 strain according to Ref. 46, and cytochrome bd oxidase was isolated from cell membranes as described (37, 47). Cytochrome bd from *A. vinelandii* strain MK8 was isolated as reported in Ref. 48. Concentration of the enzymes from *E. coli* and *A. vinelandii* was determined from the dithionite-reduced minus as isolated difference absorption spectra using $\Delta\varepsilon_{628-607}$ of 10.8 mm$^{-1}$ cm$^{-1}$ (33) and $\Delta\varepsilon_{628-605}$ of 9.5 mm$^{-1}$ cm$^{-1}$ (49), respectively.

*Time-resolved Spectrophotometric Measurements—* Time-resolved spectrophotometric measurements were performed using a home-built CCD-based instrument. The setup allows acquiring absorption changes surfaces with a time resolution of 1 μs between the spectra. Details of the methodology can be found in Ref. 50. To obtain the CO complex of the R enzyme, the as-isolated enzyme was (i) deoxygenated by argon equilibration, (ii) reduced under anaerobic conditions with 2.5 mm sodium ascorbate and 5 μM N,N,N',N'-tetramethyl-1,4-phenylenediamine, and (iii) equilibrated with 1% CO. The R-CO enzyme was transferred to the stopped flow module in a gastight Hamilton syringe preflushed with argon and then mixed with oxygen. CO photolysis was initiated by a laser flash (Brilliant B; Quantel, Les Ulis, France; frequency-doubled YAG, 532 nm; pulse energy, 120 mJ).

*Time-resolved Measurement of Electric Potential Generation—* Reconstitution of cytochrome bd into liposomes, the anaerobic sample preparation, and time-resolved electrometric measurements were made as reported (Refs. 37, 39 and references therein).

Software for Experiments and Data Analysis—Instrumental software for experimental setups was written by Dr. N. Belevich (Helsinki, Finland). MATLAB (The Mathworks, South Natick, MA) was used for data analysis and presentation.
The Role of the Heme Groups in the Formation of the Catalytic Cycle Intermediates—When the complex of the ferrous heme d with oxygen forms (R → A transition), the b-type hemes remain reduced, as evidenced by the lack of the distinct troughs at 560 and 595 nm in the spectrum of the 1.4-µs phase (Fig. 1C). In contrast, the spectrum of the 4.5-µs phase corresponding to the A → P transition has minima at 560 and 595 nm, which are typical features of the oxidized-minus-reduced spectrum of heme b₅₉₅ (37, 53, 54). Further support for this conclusion that heme b₅₉₅ gets oxidized during the A → P transition comes from the fact that the difference spectrum of the 4.5-µs phase in the Soret is dominated by the bleaching at 440 nm (Fig. 2, solid line). The 440-nm band was identified as the Soret band of ferrous heme b₅₉₅ (34, 55). Although the heme d absorption change also contributes to the Soret difference spectrum, its contribution to the Soret is usually minor compared with a b-type heme (56). It is clear that the P formation is accompanied by the oxidation of heme b₅₉₅. At the same time, there is no evidence for the oxidation of heme b₅₅₈ at this stage.

The oxidation of heme b₅₅₈ occurs during the next transition, the P → F, transition as evidenced by a prominent trough at 561 nm in the spectrum of the 47-µs phase (Fig. 1C, dashed line).

Furthermore, the P → F transition is accompanied by a loss of absorbance at 430 nm and its increase at 414 nm (Fig. 2, dashed line). The peaks at 430 and 560 nm were reported to be the Soret and α bands of ferrous heme b₅₉₅, respectively (29). There is no indication that the oxidation of heme b₅₉₅, observed during the P formation, contributes also to the F formation. A trough at 589 nm (Fig. 1C, dashed line) is most probably a specific spectral feature of the compound F. A similar feature was observed in the F-minus-O difference absorption spectrum where F was generated by the addition of excess hydrogen peroxide to cytochrome bd in the O state (41).

The Distribution of the Charge Translocation Events along the Catalytic Cycle—An electrometric recording of the reaction of the fully reduced enzyme from E. coli with oxygen is shown in Fig. 3, dashed trace. It consists of an initial non-electrogenic phase (the lag) followed by the electrogenic phase, in agreement with previous reports (37, 39). The fit of the electrometric trace with the rate constants obtained in spectrophotometric measurements revealed that the first two processes, which were assigned to the formation of compound A followed by the transition from A to P, are electrically silent. Thus, both R → A and A → P transitions are not coupled to any charge translocation across the membrane plane.

In the three electron-reduced cytochrome bd (no bound quinol), the electrically silent lag is followed by an electrogenic (Fig. 3, dashed phase) that develops concurrently with the 47-µs phase observed in the spectrophotometric experiment (Fig. 3, solid trace). Thus, it corresponds to the transition of the P intermediate to the F state (P → F transition). When the enzyme contains bound quinol, an additional electrogenic phase with τ of 0.6–1.1 ms is observed. This slow phase matches the corresponding spectral phase reflecting the F → A transition (37).
**Peroxy Intermediate of Cytochrome bd Oxidase**

When such a correction with the O₂ on-rates is introduced into analysis, the lag in cytochrome bd also must be fitted as two sequential non-electrogenic phases (Fig. 3).

**Identity of P**—In the course of the reaction of the reduced cytochrome bd with O₂, compound A decays to an intermediate with a maximum at 635 nm (denoted P) with τ = 4.5 µs (Fig. 1). The A → P transition coincides with the oxidation of heme b₉₉₅ (Figs. 1C and 2). Reduction of O₂ by two electrons is sufficient to generate a two-electron-reduced form of O₂ at the active site, i.e. bound (hydro)peroxide. It is not clear, however, whether the O-O bond is broken at this stage of the reaction. If the O-O bond is still intact, P corresponds to compound 0 in peroxidases, i.e. a true peroxy complex (Fe₃⁺-O-O-·H). Compound 0 was monitored in horseradish peroxidase at subzero temperatures (2). Although an intermediate analogous to compound 0 was postulated for CcO, it has never been trapped.

If the O-O bond is already broken, P is an oxoferryl species. Formation of the oxoferryl species requires four electrons to break the O-O bond. In the P state, two electrons may be taken from heme d and one from heme b₉₉₅. However, the forth electron cannot be taken from heme b₅₅₈ because no oxidation of heme b₅₅₈ is observed at this stage (Figs. 1C and 2). Hence, if P is oxoferryl, the fourth electron should come from a nearby group, most likely amino acid residue (Fe₃⁺ = O₆⁺·R'). In the latter case, P would be analogous to compound I of cytochrome c peroxidase or Pₘ species of CcO. However, withdrawal of an electron from an amino acid residue requires rather high redox potential, typically higher than +0.8 V (58), and the oxidation of heme b₅₅₈ (Eₘ₉ ≈ +0.15 V) (27) would be much more favorable (−0.65 V potential difference, or 10 orders difference in the equilibrium constant). At the same time, in our experiments the formation of P is not coupled to the oxidation of the low spin heme, in contrast to that detected during the formation of the Rₘ oxoferryl species in heme-copper oxidases. The oxidation of the low spin heme b₅₅₈ is observed only during decay of P, i.e. in the next transition. This is the reason why we suggest that in the case of cytochrome bd P is a true peroxy intermediate. Nevertheless, some further studies are required to establish its precise chemical structure.

**Reaction Scheme**—The scheme describing the reaction reported in this work is shown in Fig. 4. The initial complex of the R cytochrome bd with CO (R-CO) is photolyzed in the presence of oxygen. The unliganded R enzyme generated by the photolysis binds O₂ very rapidly, forming the ferrous heme d oxo species (A). The R → A transition is not electrogenic, and its rate is proportional to [O₂] (kₘ₉ = 1.9 × 10⁹ M⁻¹ s⁻¹) (38, 49). The A formation is followed by electron transfer from heme b₉₉₅ to form P. The A → P transition occurs with k = 2.2 × 10⁷ s⁻¹ and is also non-electrogenic. The finding that electron transfer from heme b₉₉₅ to heme d is not coupled with

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**DISCUSSION**

*Why P Has Not Been Detected Previously*—In this work, we examined the reaction of cytochromes bd from *E. coli* and *A. vinelandii* in the R state with oxygen. Earlier studies of this reaction reported that the initially formed intermediate A decays directly to F without any transient intermediate between A and F (38, 39). This apparently contradicts the identical reaction catalyzed by cytochrome c oxidase (CcO), where under the same conditions a transient formation of P intermediate between A and F was documented (7). It was concluded that in the reaction of cytochrome bd with O₂ there is either no intermediate between A and F or this intermediate is too short-lived to be detected (39). In this study, we were able to clearly resolve the transient formation of a spectrally discernible intermediate between A and F with a time constant of 4.5 µs at +21 °C (Fig. 1), which was denoted P. We suggest that P was not detected in earlier studies (38, 39) due to some limitations of the methodologies used. In the work of Hill *et al.* (38), the measurements were performed on the appropriate time scale of a few hundred microseconds; however, the measurements were done at selected wavelengths (Fig. 3 in Ref. 38) where the contribution of P was small and therefore could not have been trapped. Jasaitis *et al.* (39) tried to catch P both in the reaction of the R enzyme with O₂ at −20 °C and in the reaction of the O enzyme with excess H₂O₂ at room temperature. Despite recording the entire spectra, the time resolution (1 ms) appeared to be not sufficient to detect P in that study, even at low temperature (39). A question may arise why the P formation was not found in the electrometric measurement (39). The present work shows that the initial electrically silent lag phase is actually a sum of the R → A and A → P transitions (Fig. 3). This lag phase was also observed in Ref. 39 and compared with the similar lag phase in CcO. It was established that in CcO the lag can be reasonably modeled as two sequential steps corresponding to the R → A and A → Pₕₐₗ transitions (15). Based on the fact that the lag in cytochrome bd is markedly shorter than that in CcO, the former was fitted as a single step (39). However, it was not taken into consideration that binding of O₂ to cytochrome bd (R → A transition) is ~10 times faster compared with CcO (38, 57). Much faster R → A transition in the bd oxidase obviously reduces the entire non-electrogenic lag in cytochrome bd.
membrane potential generation is fully consistent with a recent study (37). The chemical structure of P needs to be established. As depicted in the scheme, it is likely that P is a peroxy complex of ferric heme d. If this is the case, the bound peroxide is likely not in the anionic form but at least singly protonated. The proton may come from one of the two protonatable groups linked to the b_{595}/d binuclear site (37) upon its oxidation. We cannot exclude, however, that at the P state the O-O bond is already broken and P could be similar to P_M in CcO. The latter would be apparently inconsistent with the same reaction catalyzed by the R CcO where no P_M-like intermediate preceding the formation of P_R has been observed (7, 16, 17). The P intermediate is further converted into F with k = 2.1 × 10^4 s^-1. This is accompanied by the oxidation of heme b_{558}. In agreement with previous studies (37, 39), formation of F is coupled to generation of a membrane potential. There is no doubt that at the F state the b_6-type hemes are in the ferric state and heme d is in the oxoferryl state. When cytochrome bd contains bound quinol, the reaction proceeds further to form the oxidized enzyme (O). The F → A transition occurs with k = 0.9 × 10^3 s^-1 (Fig. 1C, gray solid line) and is electrogenic (37).

The fact that in the course of the reaction the oxidation of heme b_{595} precedes that of heme b_{558} may indicate that heme b_{595} is located closer to heme d than heme b_{558}. This is in line with the proposal of physical proximity of hemes d and b_{595} and their functional cooperation in the O_2-reducing site (31–37).

Because an intermediate spectrally and kinetically very similar to P of the E. coli cytochrome bd is also observed with the bd enzyme from A. vinelandii, P is most likely a catalytic intermediate inherent in all members of the bd-family of terminal oxidases.

Acknowledgments—We thank Dr. R. B. Gennis and Dr. R. K. Poole for their generous gift of the bacterial strains E. coli GO105/pTK1 and A. vinelandii MK8, respectively. V. B. B. thanks Dr. A. A. Konstantinov for invaluable help, support, and many stimulating discussions.

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