Electron Transfer Process in Cytochrome *bd*-Type Ubiquinol Oxidase from *Escherichia coli* Revealed by Pulse Radiolysis[†]

Kazuo Kobayashi,[‡] Seiichi Tagawa,[‡] and Tatsushi Mogi*[,§]

Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047, and Department of Biological Sciences, Graduate School of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

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ABSTRACT: Cytochrome *bd* is a two-subunit ubiquinol oxidase in the aerobic respiratory chain of *Escherichia coli* and binds hemes b_{558}, b_{595}, and *d* as the redox metal centers. Taking advantage of spectroscopic properties of three hemes which exhibit distinct absorption peaks, we investigated electron transfer within the enzyme by the technique of pulse radiolysis. Reduction of the hemes in the air-oxidized, resting-state enzyme, where heme *d* exists in mainly an oxygenated form and partially an oxoferryl and a ferric low-spin forms, occurred in two phases. In the faster phase, radiolytically generated *N*-methylnicotinamide radicals simultaneously reduced the ferric hemes b_{558} and b_{595} with a second-order rate constant of 3×10^8 M^{-1} s^{-1}, suggesting that a rapid equilibrium occurs for electron transfer between two *b*-type hemes long before 10 μs. In the slower phase, an intramolecular electron transfer from heme *b* to the oxoferryl and the ferric heme *d* occurred with the first-order rate constant of $4.2-5.6 \times 10^2$ s^{-1}. In contrast, the oxygenated heme *d* did not exhibit significant spectral change. Reactions with the fully oxidized and hydrogen peroxide-treated forms demonstrated that the oxidation and/or ligation states of heme *d* do not affect the heme *b* reduction. The following intramolecular electron transfer transformed the ferric and oxoferryl forms of heme *d* to the ferrous and ferric forms, respectively, with the first-order rate constants of 3.4×10^3 and 5.9×10^2 s^{-1}, respectively.

Cytochrome *bd* is a two-subunit ubiquinol oxidase in the aerobic respiratory chain of *Escherichia coli* and catalyzes the two-electron oxidation of ubiquinol-8 at the periplasmic side and the four-electron reduction of dioxygen at the cytoplasmic side, thus establishing an electrochemical proton gradient across the membrane via scalar reactions (see refs *1*, *2* for recent reviews). On the basis of spectroscopic and ligand binding properties, three distinct redox metal centers have been identified as hemes b_{558}, b_{595}, and *d* (*1*−*3*). Heme b_{558} is a *hexa*-coordinated low-spin heme which exhibits absorption peaks at 562, 532, and 428 nm in the reduced state (*3*−*5*), and is a primary electron input site from quinols. Heme b_{595} is a *penta*-coordinated high-spin heme which shows peaks at 595, 562, and 442 nm in the reduced state (*3*−*6*) and serves as a low-affinity ligand binding site (*7*, *8*). Heme *d* is a primary ligand binding site and has an absorption maximum at 628 nm in the fully reduced state and at 647 nm in the one-electron-reduced, oxygenated state (Fe(II)-O_2) (*3*−*5*, *9*), whereas the ferric heme *d* does not show significant absorbance at around 650 nm (*10*). In the air-oxidized, resting-state enzyme, heme *d* exists in mainly an oxygenated form (*11*−*13*) due to an extremely high affinity for dioxygen (K_d, 20 nM) (*14*), and partially an oxoferryl (10−25%; ref *15*) and a cyanide-sensitive ferric low-spin (10%; ref *16*) forms. The oxoferryl heme *d* (Fe(IV)═O) can be prepared as a stable product in the reaction with hydrogen peroxide and shows a peak at 680 nm (*10*, *15*, *17*). Heme *d* and heme b_{595} appear to form a heme−heme binuclear center (*8*, *18*, *19*).

In the reaction of the fully reduced enzyme with dioxygen at low temperature, the first intermediate is the oxygenated species (*20*, *21*). The decay of the oxygenated form was associated with oxidation of heme b_{595} followed by oxidation of heme b_{558}, suggesting that heme b_{595} is a direct electron donor to heme *d* (*22*). Hill et al. (*14*) studied the reaction of the reduced enzyme with dioxygen by the flow-flash technique at room temperature and found that the ferrous heme *d* yields the oxygenated form with the second-order rate constant of 2×10^9 M^{-1} s^{-1}. The oxygenated form then decays to the oxoferryl intermediate concomitant with oxidation of heme b_{558} (*14*). These observations are consistent with electron flow from heme b_{558} to heme *d* through heme b_{595}.

A powerful approach for investigating electron transfer within proteins is that of pulse radiolysis through which one electron can be introduced rapidly and selectively into one redox center of enzymes (*23*−*28*). Here we report the application of this method to the *E. coli* cytochrome *bd*. In contrast to the heme-copper terminal oxidases, the molecular structure of the redox metal centers and intramolecular electron-transfer processes in cytochrome *bd* are poorly understood. Taking advantage of spectroscopic properties of three hemes which exhibit distinct absorption peaks, we

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* To whom correspondence should be addressed. Fax: 81-3-5841-4464. E-mail: mogi@biol.s.u-tokyo.ac.jp.

[‡] Osaka University.

[§] University of Tokyo.

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carried out pulse radiolysis studies on the air-oxidized, resting-state enzyme using transiently generated *N*-methylnicotinamide (NMA[1]) radicals as a reductant. We found that the ferric heme *b* is reduced by the NMA radical with the second-order rate constant of 3×10^8 M^{-1} s^{-1}. Electrons are rapidly equilibrated between hemes b_{558} and b_{595} with a rate constant of $>10^4$ s^{-1}, and subsequently the ferrous *b*-hemes reduce heme *d* with a rate constant of $4.2-5.6 \times 10^2$ s^{-1}. In addition, effects of the oxidation and ligation states of the hemes on intramolecular electron transfer were examined.

MATERIALS AND METHODS

Purification of Cytochrome bd. The enzyme was isolated from the cytochrome *bd*-overproducing strain GR84N/pNG2 (*29*), a generous gift from R. B. Gennis, as described previously (*30*), and was stored at −80 °C in 50 mM sodium phosphate (pH 7.4) containing 0.1% sucrose monolaurate (Mitsubishi-Kagaku Foods Co., Tokyo). The concentration of the enzyme was calculated from the heme B content determined by pyridine ferrohemochromogen method using an extinction coefficient of 20 700 (31), assuming that the enzyme contains two heme B molecules (*19*, *30*).

Preparation of Various Redox and Ligation Forms. A fully oxidized form was obtained by addition of dithionite and ferricyanide sequentially to the air-oxidized, resting-state form (*24*). The sample thus obtained was passed through an Ampure SA column (Amersham) equilibrated with 10 mM potassium phosphate (pH 7.4) containing 0.1% sucrose monolaurate to remove excess reagents. An oxoferryl form was obtained by the addition of an equimolar amount of hydrogen peroxide to the air-oxidized form.

Pulse Radiolysis. Pulse radiolysis experiments were performed under anaerobic conditions with an linear accelerator at the Institute of Scientific and Industrial Research, Osaka University (*23*−*27*). The pulse width and energy were 8 ns and 27 MeV, respectively. The source for monitoring light was a 150 W halogen lamp or a 1 kW xenon lamp. After passing through an optical path, the transmitted light intensity was analyzed and monitored by a fast spectrophotometric system composed of a Nikon monochromator, an R-928 photomultiplier, and a Unisoku data analyzing system. The concentration of the NMA radicals generated by pulse radiolysis was determined by absorbance change at 420 nm using an extinction coefficient of 3200 M^{-1} cm^{-1} (*32*), and was adjusted by varying the dose of the electron beam.

Samples for pulse radiolysis were prepared as follows. The enzyme solutions, which contain 10 mM potassium phosphate (pH 7.4), 0.1% sucrose monolaurate, and 2 mM NMA, were subjected to repeated deaeration followed by flushing with Ar gas. Sucrose monolaurate acts as a scavenger for OH radicals; therefore, a radical scavenger such as *tert*-butyl alcohol was not added to the solution. After these treatments, the sample showed the α peak of heme *d* at 647 nm with a shoulder at 680 nm, indicating that it still remains as the air-oxidized, resting-state form (*13*). The quartz cells had a light path of 0.3 or 1 cm. For each pulse, a fresh sample was used, even though pulse radiolysis did not give any damage to the sample as judged by its visible absorption spectrum. Static absorption spectra were recorded with a Hitachi U-3000 spectrophotometer.

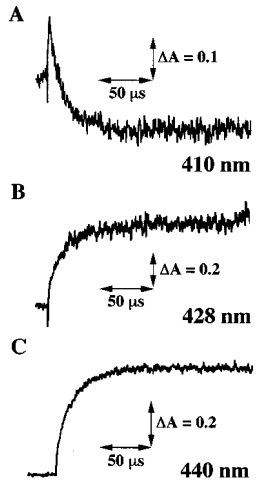


FIGURE 1: Absorption changes after pulse radiolysis of the air-oxidized, resting-state enzyme monitored at 410 (A), 428 (B), and 440 nm (C). Samples contained 181 μM enzyme, 2 mM NMA, 0.1% sucrose monolaurate, and 10 mM potassium phosphate (pH 7.4). The light path was 3 mm.

RESULTS

Reaction with Air-oxidized, Resting-State Form. Since hydrated electrons (e_{aq}^-), generated by pulse radiolysis were unable to directly reduce the hemes in the air-oxidized, resting-state cytochrome *bd* (data not shown), NMA was used as an electron mediator. All of the e_{aq}^- stoichiometrically reacted with NMA to form NMA radicals at a maximum concentration of 20−30 μM. The NMA radical reacted very rapidly with cytochrome *bd*, resulting in the reduction of *b*-type heme. A simultaneous increase in absorbance at 428 and 440 nm and a decrease at 410 nm reflected the increase of the ferrous heme b_{558} and b_{595}, respectively, and the decrease of the air-oxidized form (Figure 1). The kinetic difference spectrum in the Soret region at 0.1 ms after pulse radiolysis showed broad absorption around 435 nm and is nearly identical to the dithionite-reduced *minus* air-oxidized redox difference spectrum (Figure 2). Since the contribution of heme *d* to the Soret absorbance change is relatively small, it can be concluded that both hemes b_{558} and b_{595} were reduced but not kinetically resolved.

Reduction of heme *b* in the air-oxidized form and also in the fully oxidized form followed pseudo-first-order kinetics, and the rate constants linearly increased with the concentration of the enzyme up to 180−200 μM where the concentration of the NMA radical was fixed at about 3 μM (data not shown). Accordingly, reduction of heme *b* is a consequence of a bimolecular reaction of the NMA radical with the enzyme. The second-order rate constant was calculated to be 3×10^8 M^{-1} s^{-1}.

Subsequently, the initial changes in the Soret absorption reversed, as shown in Figure 3, indicating the reoxidation of hemes b_{558} and b_{595}. Concomitantly, the absorption at 680 and 630 nm, associated with heme *d*, decreased and increased, respectively. Figure 4 shows the kinetic difference spectra in the visible region at 0.1 and 5 ms after the pulse. The kinetic difference spectrum at 0.1 ms, which has an

[1] Abbreviations: e_{aq}^-, hydrated electron; NMA, *N*-methylnicotinamide; NIR, nitrite reductase.

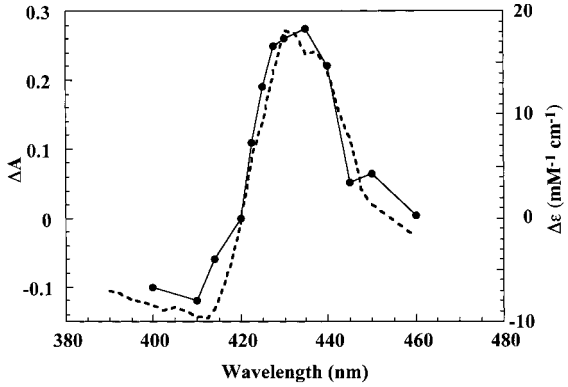


FIGURE 2: Comparison of the kinetic difference spectrum at 0.1 ms after pulse radiolysis of the air-oxidized, resting-state enzyme with the fully reduced *minus* air-oxidized difference spectrum. Note that the left-hand ordinate is in units of Δ*A* (absorbance) corresponding to the kinetic difference spectrum, while the right-hand ordinate is in units of extinction coefficients corresponding to the static redox difference spectrum (---). Experiments were carried out as described in the legend to Figure 1.

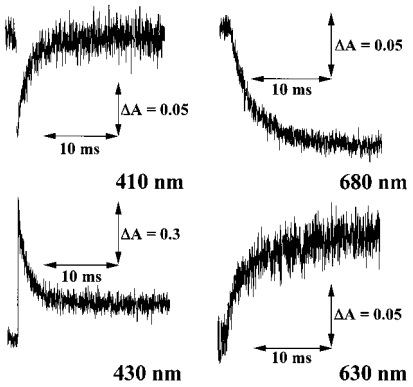


FIGURE 3: Slower absorbance changes after pulse radiolysis of the air-oxidized, resting-state enzyme monitored at 410, 430, 680, and 630 nm. Pulse radiolysis was carried out as described in the legend to Figure 1 except that the enzyme concentration was changed to 90 μM.

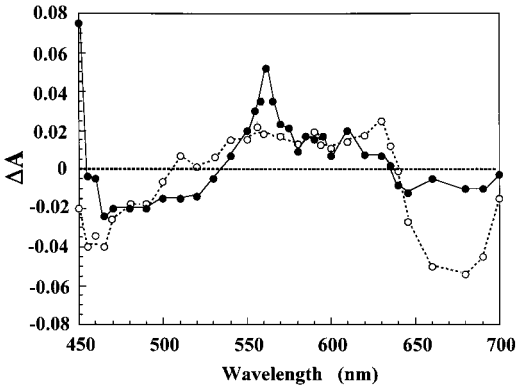


FIGURE 4: Kinetic difference spectra at 0.1 (●) and 5 ms (○) after pulse radiolysis of the air-oxidized, resting-state enzyme. Pulse radiolysis was carried out as described in the legend to Figure 3.

absorption maximum at 560 nm, corresponds to the reduction of heme b_{558}. The kinetic difference spectrum at 5 ms has an absorption maximum at 630 nm and a minimum at 680 nm; these are characteristic for the ferrous heme *d* and the oxoferryl heme *d*, respectively. Thus both hemes b_{558} and b_{595} were reduced at the faster phase, and subsequently, reoxidation of heme *b* occurred with concomitant reduction of heme *d* at the slower phase.

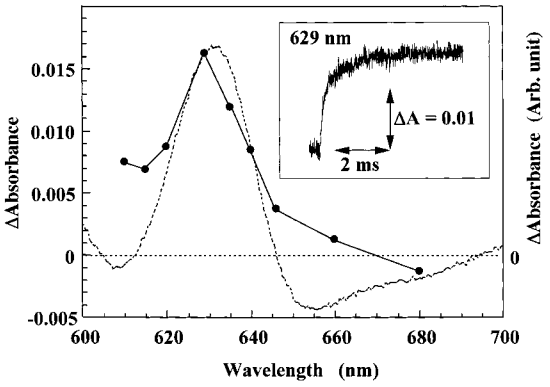


FIGURE 5: Slower absorbance change after pulse radiolysis of the fully oxidized enzyme monitored at 629 nm (A). Pulse radiolysis was carried out as described in the legend to Figure 1 except that the enzyme concentration was changed to 67 μM. Comparison of the kinetic difference spectrum at 5 ms after pulse radiolysis of the oxidized enzyme (●) with the fully reduced *minus* fully oxidized difference spectrum (—) (B). Note that the left-hand ordinate is in units of Δ*A* (absorbance) corresponding to the kinetic difference spectrum, while the right-hand ordinate is in units of absorbance corresponding to the static redox difference spectrum.

The absorption changes at 428, 460, 620, and 680 nm followed first-order kinetics, and these rate constants were independent, within experimental error, of the enzyme concentration, indicating that these processes are attributable to intramolecular electron transfer (data not shown). Rate constants for the reoxidation of heme b_{558} and b_{595} were estimated to be 5.6×10^2 s^{-1} at 428 nm and 4.2×10^2 s^{-1} at 460 nm, respectively, and a rate for reduction of heme *d* was estimated to be 4.2×10^2 s^{-1} at 620 and 680 nm, respectively. Accordingly, electron transfer from heme *b* to heme *d* occurs with a rate constant of $4.2-5.6 \times 10^2$ s^{-1}.

Reactions with Fully Oxidized and Hydrogen Peroxide-Treated Forms. Upon pulse radiolysis of the fully oxidized form under anaerobic conditions, where the ferric heme *d* does not have appreciable absorbance for the α peak (*33*), the NMA radical rapidly reduced hemes b_{558} and b_{595} with the second-order rate constant as the same as that of the air-oxidized form (data not shown). In the slower phase, only an increase in the absorption at 629 nm, characteristic for the ferrous heme *d*, was observed (inset to Figure 5). The kinetic difference spectrum at 5 ms after the pulse, which has a peak at 630 nm, is similar to the dithionite-reduced *minus* fully oxidized redox difference spectrum (Figure 5). The increase in absorbance at 630 nm (i.e., ferrous heme *d*) and the decrease in absorbance at 440 nm (ferrous heme b_{595}) followed first-order kinetics, and a rate constant of 3.4×10^3 s^{-1} was independent of the concentration of the enzyme (data not shown). Thus, the slower process is attributable to intramolecular electron transfer from the ferrous heme b_{595} to the ferric heme *d* within the enzyme.

Reaction of the air-oxidized enzyme with a stoichiometric amount of hydrogen peroxide resulted in the conversion to an oxoferryl form (*11*). Upon pulse radiolysis of the oxoferryl form obtained as above, the absorption at 620 and 680 nm was decreased as shown in Figure 6A. The kinetic difference spectrum at 50 ms after the pulse, which exhibits a trough at 670 nm but no obvious peak, is almost identical to the hydrogen peroxide-treated *minus* air-oxidized difference spectrum (Figure 6B). This indicates that the one-electron reduction by pulse radiolysis changed the oxoferryl heme *d*

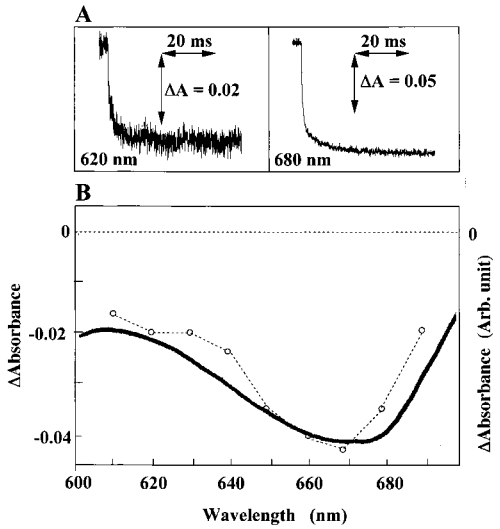
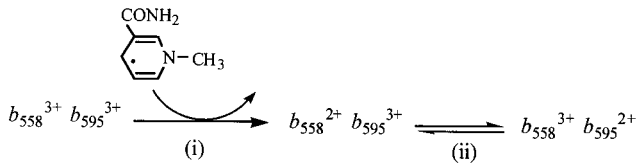


FIGURE 6: Slower absorbance change after pulse radiolysis of the hydrogen peroxide-treated enzyme monitored at 620 and 680 nm (A). Pulse radiolysis was carried out as described in the legend to Figure 1 except that the enzyme concentration was changed to 73 μM. Comparison of the kinetic difference spectrum at 50 ms after pulse radiolysis of the hydrogen peroxide-treated enzyme with the air-oxidized *minus* hydrogen peroxide-treated difference spectrum (—) (B). Note that the left-hand ordinate is in units of ΔA (absorbance) corresponding to the kinetic difference spectrum, while the right-hand ordinate is in units of absorbance corresponding to the static difference spectrum.

Scheme 1



to the ferric heme *d*, as expected (*34*). The rate for intramolecular electron transfer from the ferrous heme b_{595} to the oxoferryl heme *d* was estimated from the absorption changes at 680 and 440 nm, which followed the first-order kinetics, and was 5.9×10^2 s^{-1} at both wavelengths.

DISCUSSION

Early Electron-Transfer Processes. Present experiments showed that hemes b_{558} and b_{595} were reduced by the NMA radical simultaneously even under the condition of [enzyme] $\gg$ [NMA radical]. However, the reaction scheme after the reduction of cytochrome *bd* by the NMA radical can be interpreted by the following sequential events, as Scheme 1. An initial step is a bimolecular reaction of the NMA radical with the primary redox site in step (i). Subsequently an electron is transferred to the electron-accepting site by intramolecular migration until equilibrium is reached in step (ii). In this scheme, we cannot discriminate which heme is the primary accepting site for the NMA radical, but it is very likely that heme b_{558} at the periplasmic surface of subunit I (*9*) can be directly reduced by the NMA radical. The reaction of the NMA radical is assigned mainly to a direct reaction proceeding via the site accessible to solvent. Previous reports showed that NMA radical can selectively reduce Cu_A of bovine cytochrome *c* oxidase (*23*), the type-I Cu of copper-containing nitrite reductase (NIR) (*26*), and heme *c* of cytochrome cd_1 NIR (*25*), respectively. Their electron entry points are located near the surface of protein molecules, according to their X-ray structures (*35*−*37*). The second-order rate constant for the reaction of the NMA radical with heme b_{558} (3×10^8 M^{-1} s^{-1}) is comparable to the reduction with the NMA radical of an Fe−S center of milk xanthine oxidase (6.5×10^7 M^{-1} s^{-1}) (*24*) and the type-I Cu of copper-containing NIR (3.4×10^8 M^{-1} s^{-1}) (*26*).

If the rate-determining step in Scheme 1 is the reduction by the NMA radical ($k_{ii} > k_i$ [enzyme]), step (ii) cannot be observed and two *b*-type hemes are apparently reduced simultaneously. In the present experiments we could not discriminate the kinetic difference spectra even at a high concentration of the enzyme (>200 μM). Similarly, electron transfer from heme b_{558} to heme b_{595} was also unable to be time-resolved in the reaction of the fully reduced cytochrome *bd* with dioxygen (*14*). These results indicate that electron transfer between two *b*-type hemes occurs long before 10 μs, which is the time scale range available to observe. The rate constant of k_{ii} would be greater than 10^4 s^{-1} and comparable to electron transfer from Cu_A to heme *a* in cytochrome *c* oxidase in pulse radiolysis studies (1.8×10^4 s^{-1}) (*23*) and electron transfer between hemes *b* and *o* in cytochrome *bo* in the flow-flash and reverse electron-transfer experiments ($(1-5) \times 10^4$ s^{-1}) (*38*, *39*). In the present experiments, the transient spectrum obtained in the faster phase is almost identical to the fully reduced *minus* air-oxidized redox difference spectrum. This is consistent with the previous observation that there is no difference between the redox potentials of heme b_{558} (+140 mV) (*12*) and b_{595} (+136−154 mV) (*6*, *40*).

Late Electron-Transfer Process. The one-electron reduction of the fully oxidized and oxoferryl forms by pulse radiolysis resulted in the increase of the absorbance at 630 nm and the decrease in the absorbance at 680 nm, respectively, in the slower phase. These changes were accompanied by concomitant decrease in the absorbance at 440 nm with the same rate constant. Since the ferric (high-spin) heme *d* does not have significant absorbance at around 650 nm (*33*), the final products are the ferrous heme *d* and the ferric heme *d*, respectively. Rates for electron transfer from the ferrous heme b_{595} to the ferric heme *d* and to the oxoferryl heme *d* were 3.4×10^3 and 5.9×10^2 s^{-1}, respectively.

In contrast, reduction of the air-oxidized, resting-state enzyme by the ferrous *b*-hemes showed the complexity in absorbance changes for the slower phase (Figures 3 and 4). A larger decrease in the absorbance at 680 nm is attributable to the reduction of the oxoferryl population in the air-oxidized enzyme (*24*) to the ferric high-spin heme *d*, and a smaller increase in the absorbance at 630 nm to that of the ferric low-spin species (*25*). The rates for the one-electron reduction of the oxoferryl and the ferric low-spin heme *d* in the air-oxidized enzyme ($4.2-5.6 \times 10^2$ s^{-1}) and for the oxoferryl heme *d* in the hydrogen peroxide-treated enzyme (5.9×10^2 s^{-1}) and the ferric high-spin heme *d* in the fully oxidized enzyme (3.4×10^3 s^{-1}) were much slower than the rate for the reduction of the oxygenated heme *d* in the flow-flash studies with the fully reduced enzymes (1.0×10^4 s^{-1}) (*14*). The absorbance change at 647 nm does not support the reduction of the oxygenated form of the air-oxidized, resting-state cytochrome *bd*. However, the spectrum of the air-oxidized enzyme 10 min after pulse radiolysis clearly showed that the oxoferryl species was formed as a

final product of the reaction of the oxygenated heme *d* (data not shown). This indicates that electron transfer from the ferrous *b*-hemes to the one-electron-reduced oxygenated heme *d* could occur with a much slower rate due to the requirement of two electron transfers to yield the ferryl state.

Conclusion. Pulse radiolysis studies showed that heme b_{558} is reduced by the NMA radical with a second-order rate constant of 3×10^8 M^{-1} s^{-1}. Following rapid electron redistribution between hemes b_{558} and b_{595} with a rate constant of $>10^4$ s^{-1}, the ferric low-spin heme *d* and the oxoferryl species in the air-oxidized, resting-state enzyme were reduced by the ferrous heme b_{595} with a rate constant of $4.2-5.6 \times 10^2$ s^{-1}. These data are consistent with the electron-transfer pathway starting from heme b_{558} to heme *d* via heme b_{595} (*22*).

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