Electron Transfer Processes in Subunit I Mutants of Cytochrome bo Quinol Oxidase in Escherichia coli

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Received February 9, 2009; Accepted March 12, 2009; Online Publication, July 7, 2009
[doi:10.1271/bbb.90105]

Cytosochrome bo is a terminal quinol oxidase in the aerobic respiratory chain of Escherichia coli. Subunit I binds all four redox centers, and electrons are transferred from quinols to high-spin heme o and CuB through a bound ubiquinone-8 and low-spin heme b. To explore the role of conserved charged amino acid residues, we examined the one-electron transfer processes in subunit I mutants. We found that all the mutants examined increased the electron transfer rate from the bound quinone to heme b more than 40-fold. Tyr288 and Lys362 are key residues in the K-channel for charge compensation of the heme o-CuB binuclear center with protons. The Tyr288Phe and Lys362Gln mutants showed 100-fold decreases in heme b-to-heme o electron transfer, accompanied by large increases in the redox potential of heme o. Our results indicate that electro-magnetic coupling of hemes is important for facilitated heme-heme electron transfer in cytochrome bo.

Key words: cytochrome bo; electron transfer; pulse radiolysis; quinol oxidase; site-directed mutagenesis

Cytochrome bo (CyoABCD) is one of terminal ubiquinol oxidases in the aerobic respiratory chain of Escherichia coli, and is predominantly expressed under highly aerated growth conditions.1,2 Subunit I (CyoB) binds all four redox centers, the high-affinity ubiquinone-binding site (QH1), low-spin heme b, high-spin heme o, and CuB.2,3 Cytochrome bo shows absorption peaks at 428, 532, and 563 nm and at 409 nm at the fully reduced state and the oxidized state respectively. Hemes b and o contribute equally to the Soret (γ) peak, but α peak absorption has been attributed mostly to heme b. Quinols are oxidized at the low-affinity quinol-oxidation site (QL) in subunit II,2,3 and electrons are sequentially transferred through QH1 and heme b to the heme o-CuB binuclear center4–7 where dioxygen reduction takes place. The release of four protons to the periplasm by the two-electron oxidation of two molecules of ubiquinol-8 is coupled to the uptake of four protons by the four-electron reduction of dioxygen to water. Accordingly, four chemical protons are apparently translocated from the cytoplasm to the periplasm, generating an electrochemical proton gradient across the membrane. In addition, by a pump mechanism, cytochrome bo can vectorially translocate four protons across the cytoplasmic membrane per dioxygen reduction. Site-directed mutagenesis1,2,5–11 and X-ray crystallographic3,12–14 studies of heme-copper terminal oxidases suggest that D- and K-channels in subunit I are operative during redox-coupled proton pumping. D-channel contains Asp135 (E. coli cytochrome bo numbering) at the proton entry site and Glu286 near heme b and the binuclear center (Fig. 1). K-channel is characterized by Lys362 at the entry site, and ends at Tyr288 below the binuclear center. Iwata et al.12 proposed that D-channel participates in the translocation of four pumped protons and K-channel in the delivery of four chemical protons to the binuclear center from the cytoplasm. It is now assumed that K-channel delivers only one chemical proton to the binuclear center at the initial reductive phase of dioxygen reduction, and that D-channel translocates all other chemical and pumped protons.15,16 Uptake and release of protons and the intramolecular proton translocation within the oxidase appear to be coupled to the intramolecular electron transfer processes.17–20

In E. coli cytochrome bo, the C1=O of the bound ubiquinone-8 at the QH1 site has been suggested to hydrogen bond to both His98 and Gln101 of transmembrane helix II, where His106 ligates low-spin heme b along with His421 in transmembrane helix X.3,5 Hemes b and o are connected through the heme axial ligands, His421 and His419 respectively, and peptide bonds in helix X.1–3 Accordingly, electron transfer from the QH1 site to heme o through heme b can be facilitated by a covalent bond system20 consisting of the ligands for the redox centers and connecting peptide bonds in helices II and X. The reduction of metal centers within enzymes is assumed to couple with the uptake of protons to compensate for an increased negative charge within the protein.17 Conserved charged amino acid residues in the heme-copper terminal oxidases participate in proton uptake and/or proton translocation.

Pulse radiolysis is a powerful tool for investigating the electron transfer process within proteins, often allowing one electron to be introduced rapidly and selectively into one redox center of the enzymes.7,19,20

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Abbreviations: NMA, N-methyl nicotinamide; QH1, the high-affinity quinone binding site
We applied pulse radiolysis to E. coli cytochrome bo, where all four redox centers are in the oxidized state, and demonstrated direct electron transfer from the Q$_{1}$ site to the hemes.$^{19}$ Further, we found that Cu$_{b}$ plays a crucial role in heme b-to-heme o intramolecular electron transfer.$^{20}$ We have shown that substitution of Asp135 and Glu286 in D-channel, Tyr288 and Lys362 in K-channel, Arg257 and Asp407 on the periplasmic surface, and Glu540 on the cytoplasmic surface of subunit I eliminated the catalytic activity$^{11}$ (Fig. 1). To explore the role of conserved charged amino acid residues in the subunit I mutants, we examined the intramolecular one-electron transfer processes in mutant enzymes by pulse radiolysis. We found that all the mutations accelerated electron transfer from the Q$_{1}$ site to heme b, and that the K-channel mutations (Tyr288Phe and Lys362Gln) reduced the heme b-to-heme o electron transfer two orders of the magnitude due to a large increase in the $E_m$ value of the high-spin heme. Our data suggest that electromagnetic coupling of the two hemes is important for facilitated heme-heme electron transfer in cytochrome bo, and that it was lost in the Tyr288Phe and Lys362Gln mutants.

Materials and Methods

Enzyme preparation. Purification of the wild-type cytochrome bo from E. coli GO103/pHN3795-1 (cyo$^{-}$, ΔcydA::Km$^r$/cyo$^{+}$ Amp$^{r}$) was carried out as described previously.$^{11}$ Subunit I mutations on the single copy expression vector$^{11}$ were subcloned into pHN3795-1, a pBR322 derivative, and mutant enzymes were expressed in cytochrome bo-deficient E. coli strain ST4785 (Δcyo$^{-}$/cyb$^{+}$ cyd$^{-}$), a derivative of GO103. Mutant enzymes were prepared as for the wild-type enzyme.

Pulse radiolysis. Pulse radiolysis experiments were performed under anaerobic conditions with a linear accelerator at the Institute of Scientific and Industrial Research of Osaka University.$^{2,9,20}$ The pulse width and energy were 8 ns and 27 MeV. The sample was placed in a quartz cell with an optical path length of 0.2 or 1 cm. The temperature of the sample was maintained at 25 °C. Mutant enzymes were diluted with 10 mM potassium phosphate (pH 7.4) containing 0.1% sucrose monolaurate (SML; Mitsubishi-Kagaku Foods, Tokyo) and 2 mM N-methylisocitramide (NMA) as electron mediator. The concentration of NMA radicals generated by pulse radiolysis was adjusted by varying the dose of the electron beam. The spectral data after the first or the second pulse were collected, since many pulses inflict damage on proteins.

Potentiometric titrations. Spectroscopic titrations were performed essentially as described by Dutton,$^{22}$ using a U-3000 UV/vis spectrophotometer (Hitachi, Tokyo) and a custom-made anaerobic cuvette. The oxidized enzyme in 20 mM potassium phosphate buffer (pH 7.4) containing 1.0% n-octyl-β-D-glucoside (Dojindo, Kumamoto, Japan) was mixed with redox mediators; potassium ferricyanide ($E_m = +430$ mV), quinhydrone ($E_m = +280$ mV), 1,2-naphthoquinone ($E_m = +143$ mV), phenazine methosulfate ($E_m = +80$ mV), duroquinone ($E_m = +10$ mV), 2-hydroxy-1,4-naphthoquinone ($E_m = -145$ mV), and riboflavin ($E_m = -390$ mV). Reductive titrations were performed anaerobically at 20 °C by the addition of small aliquots of 5 mM sodium hydrosulfite; for subsequent oxidative titrations, 5 mM potassium ferricyanide was used as titrant. Changes in the absorbance at 563 nm (o peak) were monitored during the titration and were corrected for the dilution effect.

Results and Discussion

Electron transfer process in proton channel mutants

Pulse radiolysis experiments involve the almost instantaneous generation of NMA radicals, which in turn reduce redox centers within a protein.$^{23,24}$ The NMA radical gave a rapid and specific reduction of the bound ubiquinone-8 at the Q$_{1}$ site (mid-point redox potential ($E_m$), about 0 mV) in the wild-type cytochrome bo$^{23,24}$ and the subunit I mutants (data not shown), and produced the ubisemiquinone anion radical at the Q$_{1}$ site. Reduction of the bound ubiquinone was too fast to be time-resolved with our instrument, but the rate of quinone reduction ($K_Q$) was presumably $>10^4$ s$^{-1}$ (Table 1). Electron transfer from the ubisemiquinone radical to hemes can be monitored by the absorbance changes at 440 (ubisemiquinone) and 560 nm (ferrous hemes b and o). In the wild-type enzyme (Fig. 2), events following the initial rapid reduction of the bound ubiquinone-8 at the Q$_{1}$ site was a simultaneous reduction of hemes b and o with a rate constant of $1.5 \times 10^3$ s$^{-1}$, which was coupled to a monophasic decay of the ubisemiquinone radical.$^{25}$ Because of a kinetic effect, we were unable to estimate the rate of heme b-to-heme o electron transfer, which may have

Fig. 1. Locations of Mutations Introduced in Subunit I (taken from Kawasaki et al.$^{11}$).
corresponded to 5.1 \times 10^4 \text{ s}^{-1}, as estimated by flow-flash experiments.\(^{26}\)

Among the subunit I mutants examined in this study, only Glu286Asp retained partial activity (31% of the wild-type enzyme).\(^{27}\) Upon pulse radiolysis, we found that a reduction of hemes occurred in two phases in all the mutants (Fig. 2, Table 1). The rate constants of the faster and slower phases (\(k_1\) and \(k_2\) respectively) were independent of the enzyme concentration (data not shown). The faster phase was associated with decay of ubisemiquinone radical, and was attributable to the reduction of heme \(b\). We found that not only mutations in proton channels but also mutations at the enzyme surface resulted in 26-to-250-fold acceleration of electron transfer. In the K-channel mutants (Tyr288Phe and Lys362Gln), such an effect appears to dominate over the defect in the delivery of a chemical proton to the heme \(o\)-Cu\(_B\) binuclear center from the cytoplasm, for compensation for an increased negative charge within the protein.\(^{17}\)

**Potentiometric analysis of hemes in proton channel mutants**

Due to the heme \(b\)-to-heme \(o\) and heme \(o\)-to-Cu\(_B\) interactions in cytochrome \(bo\),\(^{21}\) redox titration curves were composite and could be fitted by a two-component model. On the basis of the effect of the bound quinone at the Q\(_{I}\) site on redox potentials,\(^{25}\) \(E_{m1}\) (+67 mV) and \(E_{m2}\) (+123 mV) were assigned to redox potentials of hemes \(b\) and \(o\) respectively in the wild-type enzyme (Fig. 4, Table 1). In His333Ala,\(^{20}\) Tyr288Phe, and Lys362Gln (Table 1), where we found large decreases in the heme-\(b\)-to-heme \(o\) electron transfer rate (\(k_2\)), the \(E_{m2}\) value increased by 110 to 140 mV. The electron transfer pathway and X-ray structure of cytochrome \(bo\)\(^{3}\) suggest that these mutations resulted in larger increases in the \(E_{m}\) value of the high-spin heme. Electromagnetic interactions between heme \(b\) and heme \(o\) and/or between heme \(o\) and Cu\(_B\) should lower the \(E_{m}\) value for the high-spin heme. We found a linear correlation between \(k_2\) and the redox potential difference between the two hemes (Fig. 5). Electromagnetic interactions may be essential to facilitate the electron transfer from heme \(b\) to heme \(o\) by decreasing the energy gap between the two hemes. It should be noted that in bovine heart
cytochrome c oxidase, the heme-heme interaction can lower the redox potential of the heme by about 135 mV. 

**pH-dependence of intramolecular electron transfer processes in the K-channel mutants**

To test the coupling of the intramolecular electron transfer to proton uptake, we examined the pH-dependence of heme o reduction in the K-channel mutants (Fig. 6). In the wild type, heme o reduction was much faster than heme b reduction in the pH range examined, and thus the pH-dependence of the k2 value could not be estimated. The k2 values of Asp135Asn and Asp407Asn were relatively constant between pH 5.6 and 7.0 (data not shown). In contrast, the k2 values of Tyr288Phe and Lys362Gln showed pH-dependence (Fig. 6), and approached $2 \times 10^4$ s$^{-1}$ of Asp135Asn and Asp407Asn at pH 5.6. This indicates that both Tyr288 and Lys362 are involved in proton uptake via K-channel. These observations indicate that the reduction in the high-spin heme is coupled to proton uptake from the cytoplasm and the delivery of the first chemical proton to the heme o-CuB binuclear center through K-channel, as reported for cytochrome c oxidase.3,16) Similar pH-dependence in the light-driven proton pump activity of bacteriorhodopsin has been reported for the proton uptake channel mutant.33–35) Facilitation of proton translocation via charged amino acid residues in proton uptake channels appears to be a feature shared by bacteriorhodopsin,33–35) heme-copper terminal oxidases (viz., cytochrome c oxidase and cytochrome bo quinol oxidase),3,36,37) and cytochrome bd quinol oxidase.38–40)

**Conclusion**

Pulse radiolysis studies of the subunit I mutants of cytochrome bo$^{11)}$ revealed that defects in Tyr288Phe and Lys362Gln were associated with slower heme b-to-heme o electron transfer and an increased $E_m$ value of the high-spin heme. Both suppressed dioxygen reduction at the heme o-CuB binuclear center. This study also indicates that the substitutions of the conserved charged amino acid residues in proton channels and on the protein surface affected reduction of the bound ubiquinone ($K_Q$ in Table 1) and the QH-to-heme b electron transfer. Possible cause of such long-range interactions could be examined by future X-ray crystallographic studies of mutant enzymes or by monitoring changes in the protonation state of their side chains during catalytic turnover by Fourier-transform infrared spectroscopy.

**Acknowledgments**

We thank Eri Mizuochi-Asai and Sachiko Endou (ERATO, JST) for technical assistance, and the members of the Radiation Laboratory of the Institute of Scientific and Industrial Research (Osaka University) for assistance in operating the linear accelerator. This work was supported in part by Grants-in-Aid for Scientific Research (B) (14380318) and (C) (20570124) and Creative Scientific Research (18GS0314) from the Japan Society for the Promotion of Science.
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