Role of a bound ubiquinone on reactions of the *Escherichia coli* cytochrome *bo* with ubiquinol and dioxygen[1]

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Abstract To probe the functional role of a bound ubiquinone-8 in cytochrome *bo*-type ubiquinol oxidase from *Escherichia coli*, we examined reactions with ubiquinol-1 and dioxygen. Stopped-flow studies showed that anaerobic reduction of the wild-type and the bound ubiquinone-free (ΔUbiA) enzymes with ubiquinol-1 immediately takes place with four kinetic phases. Replacement of the bound ubiquinone with 2,6-dibromo-4-cyanophenol (PC32) suppressed the anaerobic reduction of the hemes with ubiquinol-1 by eliminating the fast phase. Flow-flash studies in the reaction of the fully reduced enzyme with dioxygen showed that the heme *b*-to-heme *o* electron transfer occurs with a rate constant of $\sim 1 \times 10^4$ s^{-1} in all three preparations. These results support our previous proposal that the bound ubiquinone is involved in facile oxidation of substrates in subunit II and subsequent intramolecular electron transfer to low-spin heme *b* in subunit I.

Key words: Bound quinone; Cytochrome *bo*; Quinol oxidase; Time-resolved visible spectroscopy; *Escherichia coli*

1. Introduction

Cytochrome *bo* is one of three terminal ubiquinol oxidases in the aerobic respiratory chain of *Escherichia coli* and is expressed predominantly under highly aerated growth conditions [1]. It catalyzes the two-electron oxidation of ubiquinol-8 (Q_8H_2) at the periplasmic side of subunit II and the four-electron reduction of molecular oxygen to water at the heme-copper binuclear center in subunit I [1]. The enzyme belongs to the heme-copper respiratory oxidase superfamily [2], and vectorially translocates protons not only via scalar reactions but also via proton pumping [3].

Subunit I binds all the redox metal centers, low-spin heme *b*, high-spin heme *o* and Cu_B, and serves as a reaction center for proton pumping and dioxygen reduction [1]. Photoaffinity cross-linking studies using an azido-ubiquinone [4,5] and site-directed and random mutagenesis studies [6,7] showed that a low-affinity quinol oxidation site (Q_L) [8] resides in the C-terminal hydrophilic domain of subunit II. Subunits III and IV are required for assembly of the redox metal centers in subunit I but are not involved in catalytic functions [1].

Previously, we demonstrated the presence of a high-affinity quinone binding site (Q_H) in cytochrome *bo*, which is close to both the Q_L site and heme *b* [9]. Potentiometric studies showed that the bound Q_8 at the Q_H site lowers the mid-point potential of heme *b* by 20–25 mV, and can be stabilized as a ubisemiquinone radical during the catalytic cycle [10,11]. The bound ubiquinone undergoes double reduction followed by protonation but does not leave the Q_H site [9]. These properties suggest that a unique mechanism is operative for substrate oxidation by bacterial quinol oxidases, and that the Q_H site mediates electron transfer from the Q_L site to heme *b* not only as a transient electron reservoir but also as an electron gate which connects two-electron, two-proton redox components with one-electron transfer system (i.e. heme iron) [10].

In this study, we prepared a bound ubiquinone-free enzyme (ΔUbiA) and its derivative where 2,6-dibromo-4-cyanophenol (PC32) [12] has been introduced at the Q_H site, and examined the reactions with Q_1H_2 and dioxygen by stopped-flow and flow-flash techniques. These observations support our proposal that the bound quinone at the Q_H site mediates electron transfer from the Q_L site to heme *b* [10].

2. Materials and methods

2.1. Purification of cytochrome bo

The wild-type enzyme with a bound Q_8 was isolated from the cytochrome *bo*-overproducing strain GO103/pHN3795-1 (*cyo*$^+$ Δ*cyd ubiA*$^+$/*cyo*$^+$) [13], and stored in 50 mM Tris-HCl (pH 7.4) containing 0.1% sucrose monolaurate (Mitsubishi-Kagaku Foods Co., Tokyo). The ΔUbiA enzyme, which is free from any bound ubiquinones, was purified from the ubiquinone biosynthesis mutant MU1227/pMFO4 (*cyo*$^+$ *cyd*$^+$ Δ*ubiA*/*cyo*$^+$) [9]. Preparation of the ΔUbiA enzyme whose Q_H site has been reconstituted with PC32 (ΔUbiA/ PC32) was carried out as described in [12].

2.2. Spectroscopic analysis

Anaerobic reduction of the air-oxidized enzyme with Q_1H_2 was studied in 50 mM Tris-HCl (pH 7.4) containing 0.1% sucrose monolaurate at 20°C by the stopped-flow technique [14]. The reaction of the Q_1H_2-reduced enzyme with dioxygen was examined using the flow-flash technique in combination with rapid scanning spectrophotometry [15]. The enzymes were reduced anaerobically with 1 mM Q_1H_2 for 5 min under a CO atmosphere.

3. Results and discussion

3.1. Anaerobic reduction of air-oxidized enzyme with Q_1H_2

Anaerobic reduction of the air-oxidized enzymes with Q_1H_2 was investigated by the stopped-flow technique. Within dead time (< 1 ms) the Soret peak of the air-oxidized ΔUbiA en-

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Abbreviations: ΔUbiA, cytochrome *bo* isolated from a ubiquinone-deficient mutant; ΔUbiA/PC32, ΔUbiA reconstituted with PC32; Q_n, ubiquinone-*n*; Q_nH_2, ubiquinol-*n*; Q_H, the high-affinity quinone binding site; Q_L, the low-affinity quinol oxidation site; PC32, 2,6-dibromo-4-cyanophenol

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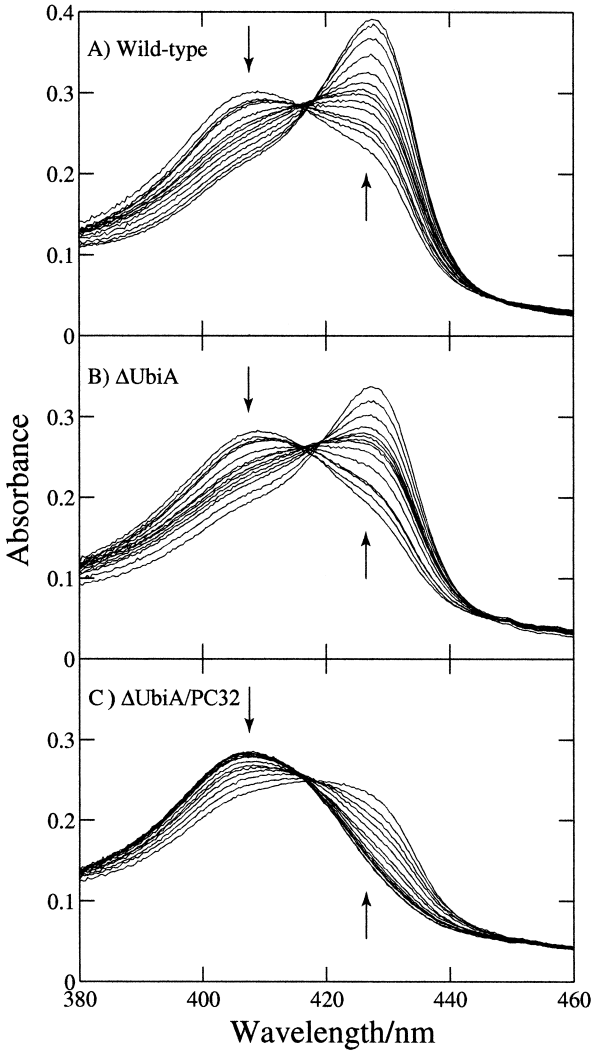

Fig. 1. Effect of the bound ubiquinone on spectral changes at the Soret region after initiation of anaerobic reduction of cytochrome *bo* with Q_1H_2. The wild-type, ΔUbiA and ΔUbiA/PC32 enzymes were diluted to 2.5 μM with 50 mM Tris-HCl (pH 7.4) containing 0.1% sucrose monolaurate, and the reaction was started at room temperature by rapid mixing with Q_1H_2 at a final concentration of 12.5 μM in a stopped-flow apparatus (model RSP-601, Unisoku Instrument). All the solutions were flushed with a stream of nitrogen gas for 20 min prior to the reaction. Spectral changes were recorded at 0.001, 0.006, 0.021, 0.061, 0.176, 0.491, 1.24, 3.00, 7.20, 17.1, 40.7, 96.4, 228, and 541 s after the initiation of the reaction.

zyme shifted from 412 nm to 409 nm of the wild-type enzyme (Fig. 1), indicating the rapid reconstitution of the Q_H site with either Q_1 or Q_1H_2 in the reaction mixture [9,12]. Subsequent spectral changes in the Soret region showed that reduction of hemes *b* and *o* proceeds similarly in the wild-type and ΔUbiA enzymes but is suppressed in the ΔUbiA/PC32 enzyme (Fig. 1).

Absorbance changes at 429 and 405 nm, which are characteristic for reduced and oxidized forms, respectively, in kinetic difference spectra (not shown), were monitored for 10^3 s and were found to be multiphasic for both the wild-type and ΔUbiA enzymes (Fig. 2). The observed rate constants at 429 nm in the reaction of 1.25 μM enzymes with 12.5 μM Q_1H_2 were 50 (1% in relative amplitude), 5 (37%), 0.18 (12%), and 0.004 (50%) s^{-1} for the wild-type enzyme, and 50 (7%), 5 (25%), 0.057 (28%), and 0.008 (40%) s^{-1} for the ΔUbiA enzyme. Since the maximum velocity and the Michaelis constant for Q_1H_2 oxidation under aerobic conditions are about 10^3 e^- s^{-1} and 50 μM [8,12,15], binding and dissociation of substrates at the Q_L site could be complete within 2 ms and an expected overall rate would be below 2×10^2 e^- s^{-1} at 12.5 μM Q_1H_2. Multiphasic kinetics may reflect intramolecular one-electron transfer processes from the Q_L site to the redox centers (Q_H, heme *b*, heme *o*, and Cu_B) and/or direct electron transfer from Q_1H_2 to the heme(s).

In the ΔUbiA/PC32 enzyme, reduction of the hemes was suppressed for about 1 s and the observed rate constants at 429 nm were 0.41 (18%), 0.036 (22%), and 0.001 (60%) s^{-1} (Fig. 2). This is consistent with the marked reduction in the V_{max} value of the ΔUbiA/PC32 enzyme for the Q_1H_2 oxidation under aerobic conditions [12]. A complete lack of the first

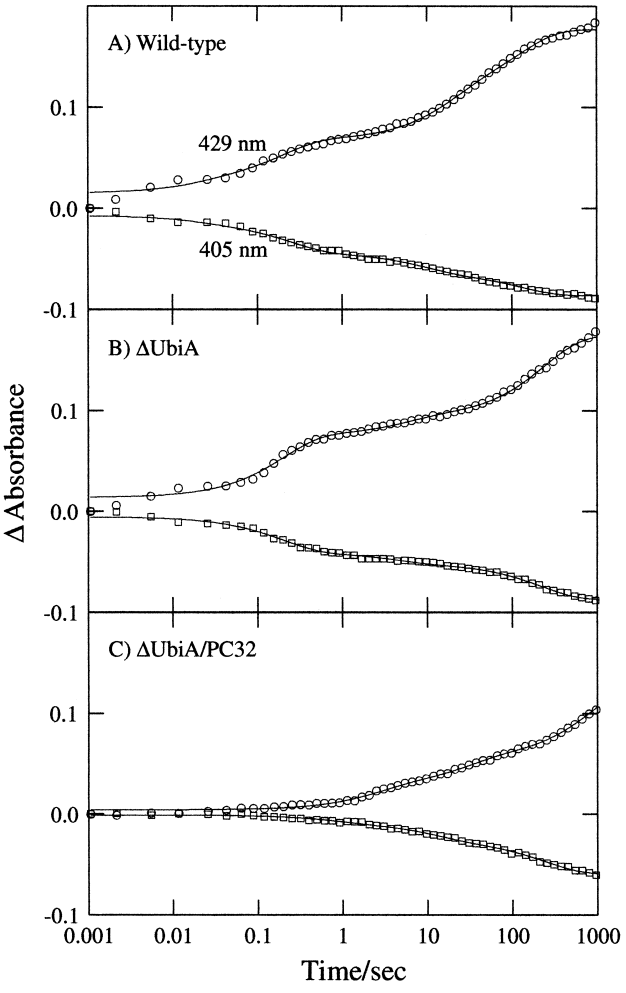

Fig. 2. Time courses for reaction of the air-oxidized wild-type, ΔUbiA and ΔUbiA/PC32 enzymes with Q_1H_2. The enzymes (1.25 μM) were flushed with nitrogen gas for 10 min, and the reaction was started by the stopped-flow method and monitored at 405 (oxidized form) and 429 (reduced form) nm. Concentrations of the enzymes and Q_1H_2 were 1.25 and 12.5 μM, respectively, after mixing and the traces are the averages of four individual transients. Other conditions are as in the legend to Fig. 1. Solid lines indicate best fits, and the observed rate constants at 429 nm were 50, 5, 0.18, and 0.004 s^{-1} for the wild-type enzyme, 50, 5, 0.057, and 0.008 s^{-1} for the ΔUbiA enzyme, and 0.41, 0.036, and 0.001 s^{-1} for the ΔUbiA/PC32 enzyme.

two phases found with the wild-type and ΔUbiA enzymes strongly indicates that the bound ubiquinone is essential for both oxidation of substrates and electron transfer to the hemes. Accordingly, slower phases are attributable to direct reduction of the heme(s) by Q_1H_2 and following intramolecular electron transfer process(es).

3.2. Reaction of reduced enzymes with dioxygen

Fig. 3 illustrates time traces at 429 nm for the reaction of the Q_1H_2-reduced wild-type, ΔUbiA, and ΔUbiA/PC32 enzymes with dioxygen. In the presence of 0.7 mM dioxygen at 20°C, the absorbance changes for the wild-type and ΔUbiA enzymes followed monophasic kinetics with the same apparent rate constant of 1.3×10^4 s^{-1} whereas the ΔUbiA/PC32 enzyme showed biphasic kinetics with apparent rate constants of 1.0×10^4 and 1.6×10^3 s^{-1} (Fig. 3). Since the faster phase in the ΔUbiA/PC32 enzyme can represent a major absorbance change (84%), the presence or absence of the bound ubiquinone (either Q_8 or Q_1) at the Q_H does not affect intramolecular electron transfer from ferrous heme *b* to the binuclear center under flow-flash conditions. In addition, we found biphasic kinetics for the dithionite-reduced enzymes irrespective of the presence (i.e. wild-type) or absence (ΔUbiA and ΔUbiA/PC32) of the bound ubiquinone at the Q_H site (data not shown). Thus the bound ubiquinone did not affect the dioxygen reduction kinetics. Accordingly, electron transfer from the bound ubiquinone in a reduced form to ferric heme *b* does not take place after the heme *b*-to-heme *o* electron transfer (10^4 s^{-1}) in the time range of a few milliseconds, probably due to redox balance between the bound ubiquinone and the redox metal centers.

Svensson and Nilsson [16] studied dioxygen reduction kinetics of the Triton X-100-purified enzyme and found three phases, 4.5×10^4, 5×10^3 and ~1 s^{-1}, after full reduction of the enzyme with ascorbate in the presence of phenazine methosulfate or 2,6-dimethoxy-5-methyl-1,4-benzoquinone. However, the second phase seems attributable to heterogeneity of the enzyme due to the presence of cytochrome oo_3 produced in the over-expressing strain RG145 [17]. Recently, Svensson-Ek and Brzezinski [18] examined dioxygen reduction kinetics of the Q_8-bound, ascorbate/phenazine methosulfate-reduced enzyme, and found three phases, 2.2×10^4, 1.4×10^3 and 2.5×10^2 s^{-1}. The initial phase was attributed to the oxidation of hemes *b* and *o*, and the second and third phases were assumed to be electron transfer from the bound Q_8H_2 to ferric heme *b* and from ferrous heme *b* to the binuclear center, respectively. In contrast, Puustinen et al. [19] reported that removal of the bound Q_8 from the His-tagged version of cytochrome *bo* by Triton X-100 altered kinetics of the dithionite-reduced enzyme from multiphasic to monophasic, and that reconstitution of the Q_H site with Q_8 reversed the kinetics. Solubilization of cytochrome *bo* with Triton X-100 can alter kinetics of the Q_1H_2 oxidation from monophasic to biphasic [12], whereas the ΔUbiA enzyme that is free from any bound ubiquinone was never exposed to any stronger detergents. Discrepancy in dioxygen reduction kinetics may be due to the difference in purification procedures or the gene-engineered modification of subunit II where the Q_L site is located [4–7].

Time-resolved resonance Raman studies showed the formation of the oxoferryl intermediate with a rate constant of about 2×10^4 s^{-1} [20,21] which is comparable to about 5×10^4 s^{-1} for the fast phase observed by visible spectroscopy [13,16,19,22]. The final product of the fast reaction with peaks at 557 and about 420 nm could be the oxoferryl intermediate [19] which then decays to the oxidized state within 1 s [13,16,21,22]. The chemical identity of the reaction intermediates and the redox states of all the redox centers including the bound Q_8 at the Q_H site must be determined for further understanding of the unique molecular mechanism of substrate oxidation by bacterial quinol oxidases.

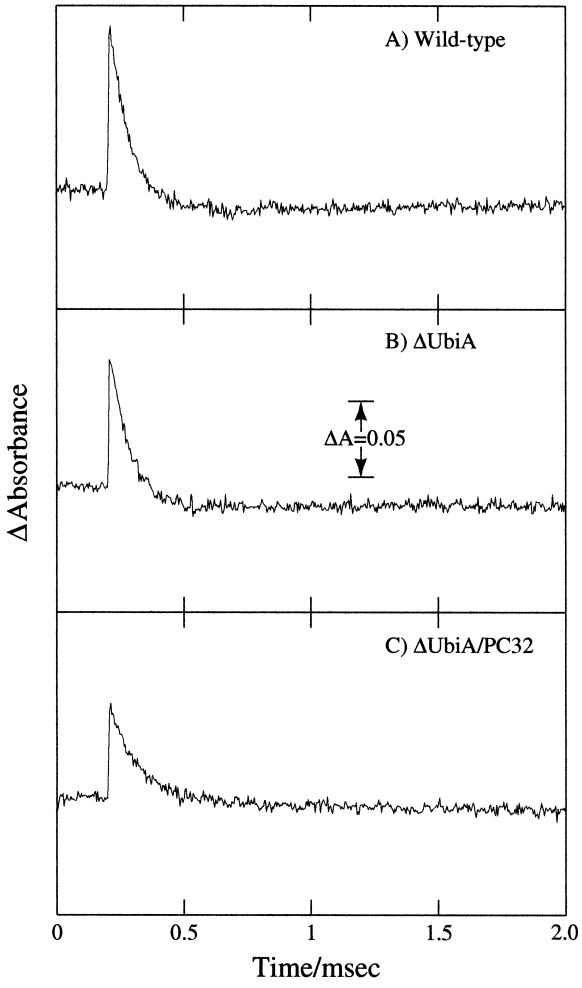

Fig. 3. Time courses for reaction of the Q_1H_2-reduced wild-type, ΔUbiA and ΔUbiA/PC32 enzymes with dioxygen followed at 429 nm. The enzymes were flushed with nitrogen gas for 10 min, and subsequently reduced with 1 mM Q_1H_2 for 5 min under a CO atmosphere. Concentrations of the enzymes, dioxygen and CO were 1.25 μM, 0.7 mM and 50 μM, respectively, after mixing and the traces are the averages of four individual transients. The reaction was initiated by the flow-flash method, and abrupt absorption increase represents a release of the reduced enzyme upon CO photolysis. Other conditions are as in the legend to Fig. 1.

In conclusion, we demonstrated that the bound ubiquinone at the Q_H site of cytochrome *bo* is essential for the catalytic turnover of the oxidase reactions, but it is not necessary for re-reduction of ferric heme *b* after the heme *b*-to-heme *o* electron transfer under flow-flash conditions. These and previous observations [9,10,12] support our proposal for the functional role of the Q_H site as a transient electron reservoir for facile two-electron oxidation of substrates at the Q_L site in subunit II and an electron gate which ensures sequential one-electron

transfer from substrates to low-spin heme *b* in subunit I. Such a mechanism is unique in bacterial quinol oxidases and could facilitate dynamic equilibrium with the membrane quinol pool and prevent abortive catalytic cycle which may release ubisemiquinone radical at the Q_L site.

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