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Effects of Replacement of Low-Spin Haem *b* by Haem O on *Escherichia coli* Cytochromes *bo* and *bd* Quinol Oxidases

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Cytochromes *bo* and *bd* are terminal ubiquinol oxidases in the aerobic respiratory chain of *Escherichia coli* and generate proton motive force across the membrane. To probe roles of haem species in the oxidation of quinols, intramolecular electron transfer and the dioxygen reduction, we replaced *b*-haems with haem O by using the haem O synthase-overproducing system, which can accumulate haem O in cytoplasmic membranes. Characterizations of spectroscopic properties of cytochromes *bo* and *bd* isolated from BL21 (DE3)/pLysS and BL21 (DE3)/pLysS/pTTQ18-cyoE after 4 h of the aerobic induction of haem O synthase (CyoE) showed the specific incorporation of haem O into the low-spin haem-binding site in both oxidases. We found that the resultant haem *oo*- and *obd*-type oxidase severely reduced the ubiquinol-1 oxidase activity due to the perturbations of the quinol oxidation site. Our observations suggest that haem B is required at the low-spin haem site for the oxidation of quinols by cytochromes *bo* and *bd*.

Key words: bacterial terminal oxidase, CyoE, haem O synthase, low-spin haem *b*, quinol oxidation site.

Abbreviations: HPLC, high-performance liquid chromatography; SML, sucrose monolaurate; TMPD, *N,N.N′,N′*-tetramethyl-*p*-phenylenediamine; Q_H, the high-affinity quinine-binding site.

Unlike mitochondria and other aerobic bacteria, the *Escherichia coli* respiratory chain lacks cytochrome bc_1 (Complex III), cytochrome *c*, and cytochrome *c* oxidase; thus quinols reduced by membrane-bound dehydrogenases are directly reoxidized by terminal quinol oxidases for the generation of proton motive force. Cytochrome *bd* is a heterodimeric terminal oxidase (CydAB) and predominantly expressed under microaerophilic growth conditions [see refs (*1*, *2*) for review]. It catalyses dioxygen reduction with two molecules of ubiquinol-8, leading to the release of four protons from quinols to the periplasm. Through a putative proton channel, four protons used for dioxygen reduction are taken up from the cytoplasm and delivered to the dioxygen reduction site at the periplasmic side of the cytoplasmic membrane. During dioxygen reduction, cytochrome *bd* generates an electrochemical proton gradient across the membrane through apparent vectorial translocation of four chemical protons (*3–5*).

Biochemical and mutagenesis studies on the *E. coli* cytochrome *bd* suggest the presence of the quinol oxidation site in loop VI/VII (Q-loop) of subunit I. Binding of monoclonal antibodies to ^{252}KLAAIEAEWET^{262} (*6*) and proteolytic cleavage at Tyr290 or Arg298 (*7*, *8*) suppressed ubiquinol oxidase activity. Photoaffinity labelling studies with azidoquinols identified that Glu280 is a part of the binding site for 2- and 3-methoxy groups on the ubiquinone ring (*9*). Site-directed mutagenesis studies indicated that Lys252 and Glu257, in the *N*-terminal region of Q-loop, are involved in the quinol oxidation by cytochrome *bd* (*10*). On the basis of spectroscopic and ligand-binding studies, three distinct redox metal centres have been identified as haem b_{558}, haem b_{595} and haem *d* [see ref. (*11*) for review]. Haem b_{558} is a low-spin protohaem IX and is ligated by His186 and Met393 of subunit I (*12*, *13*). Reduced haem b_{558} has absorption peaks at 428, 531 and 561 nm at room temperature. Inhibitor-binding studies indicate the close proximity of haem b_{558} to the quinol oxidation site (*10*, *14*). Haem b_{595} is a high-spin protohaem IX bound to His19 of subunit I (*12*) and mediates electron transfer from haem b_{558} to haem *d* (*15–17*), where dioxygen is reduced to water. Ferrous haem b_{595} shows absorption peaks at 440, 560 and 596 nm. Haem *d* is a high-spin chlorin bound to an unidentified nitrogenous ligand (*18–21*) and shows the α peak at 630 nm in the fully reduced form and at 646 nm in the air-oxidized, oxygenated form. Haem *d* forms a dihaem binuclear centre with haem b_{595} (*22*, *23*), and Glu99 and Glu107 in helix III of subunit I are required for the dioxygen reduction at the haem b_{595}-*d* binuclear centre (*24*).

Cytochrome *bo*, an alternative quinol oxidase under highly aerated growth conditions, is a member of the haem-copper terminal oxidase superfamily and generates proton motive force via not only scalar reactions like cytochrome *bd* but also proton pumping [see refs (*2*, *25*) for review]. The *cyoABCDE* operon encodes four subunits of cytochrome *bo* and haem O synthase (CyoE), which

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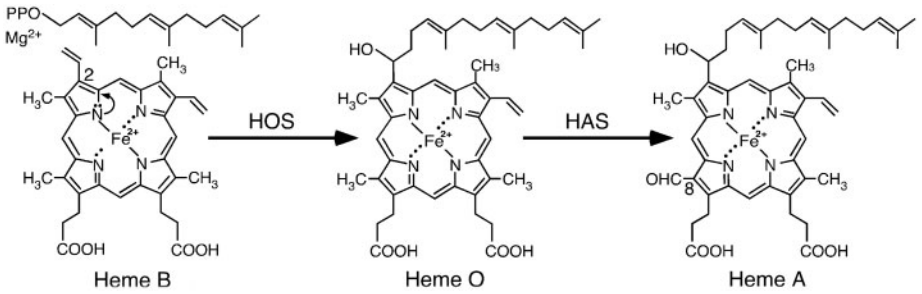

Fig. 1. **Biosynthesis of Haem O and Haem A.** Conversion of haem B to haem O is catalysed by HOS (haem O synthase), which is encoded by the *E. coli cyoE*, *Bacillus subtilis ctaB* and mitochondrial *COX10* genes. HAS (haem A synthase), which is encoded by the *B. subtilis ctaA* and mitochondrial *COX15* genes, oxidizes haem O to haem A. PPO indicates a diphosphoryl group.

supplies haem O (Fig. 1) exclusively to the dioxygen reduction site of cytochrome *bo* (*26–29*). Subunit I binds all four redox centres, the high-affinity ubiquinone binding site (Q_H), low-spin haem *b*, high-spin haem *o* and Cu_B (*2*, *30*). Quinols are oxidized at the low-affinity quinol oxidation site (Q_L) in subunit II (*31–33*), and electrons are sequentially transferred through Q_H and haem *b* to the haem *o*-Cu_B binuclear centre (*34–37*), where dioxygen reduction takes place. Cytochrome *bo* shows the Soret peak at 427 nm and a broad α peak at 561 nm in the fully reduced state, and the Soret peak at 407–412 nm in the air-oxidized state (*38–40*). Both haem *b* and *o* contribute equally to the Soret absorption while haem *b* contributes mostly to the reduced α peak (*41*), which splits into the 559 and 565 nm peaks in the second-order finite difference spectrum at room temperature. Puustinen *et al*. (*42*) proposed that low-spin haem *b* has a relatively sharp α peak at 560 nm while high-spin haem *o* has a very broad α peak centered at 560 nm with the three-fourth of the molar absorptivity. In haem O synthase mutants, high-spin haem *o* was replaced with haem B, and the resultant haem *bb*-type enzyme was found to be non-functional (*26*, *27*, *43*).

Haems bound to terminal oxidases play pivotal roles in the oxidation of quinols, intramolecular electron transfer and the dioxygen reduction, but *in vitro* substitutions of haems are practically impossible in intrinsic membrane proteins. Previously, we have demonstrated that upon the overexpression of the *E. coli* haem O synthase with pTTQ18-cyoE haem O can be accumulated in cytoplasmic membranes (*26*, *28*). In this study, we overexpressed haem O synthase in the protease-deficient *E. coli* strain [BL21(DE3)/pLysS] and examined effects of the overproduction of haem O on the spectroscopic and enzymatic properties of cytochromes *bo*- and *bd*-type quinol oxidases. Our results suggest that haem O can be introduced at the low-spin haem *b*-binding site of both cytochromes *bo* and *bd*, but the resultant *oo*- and *obd*-type oxidases severely reduced the oxidase activity due to perturbations of the quinol oxidation site.

EXPERIMENTAL PROCEDURES

Expression of Haem O Synthase—The *E. coli* BL21(DE3)/pLysS harbouring pTTQ18-cyoE (*26*) was grown aerobically at 37°C in IM medium supplemented with 100 μg/ml Na–ampicillin and trace metals (*44*). The expression of the *E. coli* haem O synthase was induced for 4 h with 0.5 mM isopropyl-β-D-thiogalactopyranoside at the mid-log phase of the growth (OD_{650} = 0.4).

Isolation of Cytochromes bo and bd—Cells were suspended in 50 mM Tris–HCl (pH 7.4) containing 10 mM Na–EDTA, 1 mM phenylmethanelsulfonyl fluoride (Sigma) and 0.5 mg/ml egg white lysozyme (Sigma) and disrupted by sonication or two passages through French Pressure Cell (*10*). Enzymes were solubilized from cytoplasmic membranes with 2.5% sucrose monolaurate (SML) (Mitsubishi-Kagaku Foods Co., Tokyo, Japan) and isolated by anion-exchange high-performance liquid chromatography (HPLC) on a TSKgel SuperQ-5PW column (21.5 mm i.d. × 15 cm; Tosoh, Japan) in 50 mM Na–phosphate (pH 6.8) containing 0.1% SML (*10*). Peak fractions were subjected to rechromatography. Purified enzymes were stored at −80°C until use.

Determination of Haem and Protein Content—Haem B content was determined by pyridine hemochromogen method, and haem *d* content was estimated from redox difference spectra using an extinction coefficient of $\varepsilon_{628-651}$ = 27,900 (*23*). Protein concentration was determined by BCA method (Pierce).

Analysis of Haem Composition—Haems were extracted from membranes with HCl–acetone and subjected to reverse-phase HPLC on an Ultrashere ODS column (4.6 mm i. d. × 25 cm; Beckmann Coulter) in 95% ethanol/acetic acid/water (70:17:7, vol/vol) at a flow rate of 0.5 ml/min (*26*, *41*).

Absorption Spectroscopy—Absorption spectra of the air-oxidized and Na–hydrosulfite-reduced forms of cytochromes *bo* and *bd* were determined with a V-550 UV–vis spectrophotometer (JASCO, Tokyo, Japan) at a final concentration of 10 μM in 50 mM Na–phosphate (pH 7.4) containing 0.1% SML. To probe structural changes at the quinol oxidation site near low-spin haem b_{558}, absolute spectra of the Na–hydrosulfite-reduced enzymes (2 μM) were measured before and after the 10-min incubation with 10 μM aurachin C-1-10 (*45*) or aurachin D 5-10 (kindly gifted by Dr Hideto Miyoshi, Kyoto University).

Spectroscopic Assay of Oxidase Activity—Quinol oxidase activity was determined at 25°C by monitoring the absorbance change at 278 nm and calculated using an extinction coefficient of 12,300 (*46*). The reaction mixture (1 ml) contained 50 mM Na–phosphate (pH 7.4), 0.1% SML and 10 nM enzymes. The enzyme concentration was

estimated from the haem (B *plus* O) content, by assuming that cytochromes *bo* and *bd* contains one each of haem B and haem O and two *b*-haems, respectively. The reaction was started by the addition of a reduced form of ubiquinone-1, a kind gift from Eisai Co. (Tokyo, Japan), at a final concentration of 200 μM. For kinetic analysis, the concentration of ubiquinol-1 was varied from 10 to 400 μM. K_m and V_{max} values were estimated with Kaleidagraph version 3.5 (Synergy Software) (*47*).

Polarographic Assay of Oxidase Activity—Oxygen consumption was determined with a YSI Model 5300 Biological Oxygen Monitor (YSI Inc., Ohio) in a closed stirred glass vessel (3 ml) at 25°C. Reaction was started by the addition of the enzymes (a final concentration of 0.5 μM) to 100 mM Na–phosphate (pH 7.4) containing 0.1% SML, 0.2 mM TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; Nacalai Tesque, Kyoto, Japan), and 5 mM Na–ascorbate. The dissolved oxygen concentration at 25°C was assumed to be 237 μM. Reduced TMPD can donate electrons to haems from a site different the quinol oxidation site.

RESULTS

Accumulation of Haem O in Cytoplasmic Membranes—As reported previously by Saiki *et al*. (*26*), upon overexpression of the *E. coli* haem O synthase (CyoE) in BL21(DE3)/pLysS, haem O was accumulated in cytoplasmic membranes. Reverse-phase HPLC analysis of HCl–acetone extractable haems in the membranes showed that upon overexpression of haem O synthase the haem O:haem B ratio was changed to 83:17 from 9:91 in the control strain. Haem B (*plus* O) content was rather decreased to 1.43 from 3.13 nmol/mg membrane protein of the control strain. We found a similar decrease in haem B (*plus* O) content upon the overexpression of the *B. subtilis ctaB* gene in BL21 CodonPlus (DE3)-RIL/pQE2-ctaB.

Cytoplasmic membranes isolated from the control strain [BL21(DE3)/pLysS] harvested at the mid-log phase showed the intense peaks at 411 nm in the air-oxidized (as prepared) form and at 427, 531 and 560 nm in the fully reduced form (Fig. 2A). These features are attributable to cytochrome *bo* (Fig. 2B), which is predominantly expressed under highly aerated growth conditions (*2*, *38*). The weak features at 648 and 628 nm (Fig. 2A) are originated from the air-oxidized and fully reduced forms, respectively, of cytochrome *bd* (Fig. 2C), whose expression is induced under microaerophylic growth conditions (*2*, *3*). As shown in Fig. 3 for the elution profile of membrane-bound cytochromes in the CyoE-overproducing strain, a major cytochrome species under the growth conditions used was cytochrome *bo*.

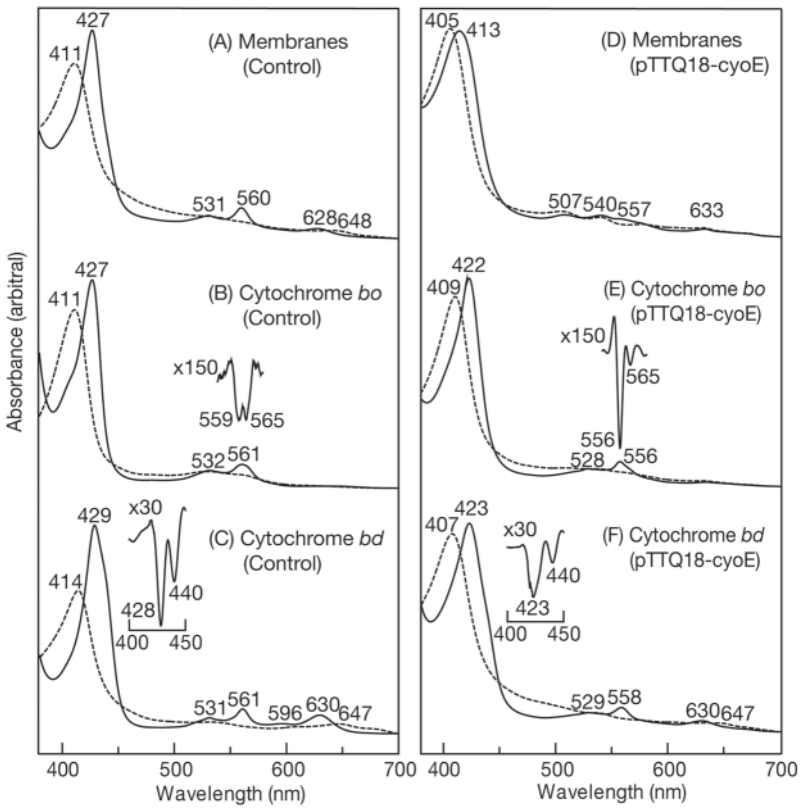

Fig. 2. **Absorption spectra of the air-oxidized (as prepared) (broken line) and fully reduced (solid line) forms of cytoplasmic membranes (A and D) and cytochromes *bo* (B and E) and *bd* (C and F) isolated from the control (*E. coli* BL21/pLysS) and haem O-overproducing strain (*E. coli* BL21/pLysS/pTTQ18-cyoE).** Absolute spectra of the isolated enzymes were recorded in 50 mM Na-phosphate (pH 7.4) containing 0.1% SML before (broken line) and after reduction (solid line) with Na-hydrosulfite. Insets indicate the second-order finite difference spectra of the α peak of cytochrome *bo* and the Soret peak of cytochrome *bd*. The concentration of haem B (*plus* O) was 9.2 (A), 5.0 (B, D and F) and 10 (C, E) μM.

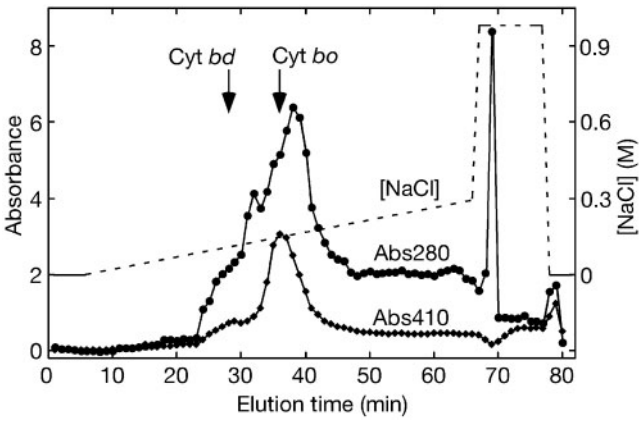

Fig. 3. **Elution profile of membrane proteins from the haem O-overproducing strain on anion-exchange chromatography.** Cytoplasmic membranes isolated from the haem O-overproducing strain were solubilized with 2.5% SML, and the supernatant after ultracentrifugation was applied to a TSKgel SuperQ-5PW column (21.5 mm i.d. × 15 cm). Cytochromes *bo* (Cyt *bo*) and bd (Cyt *bd*) were eluted with 0–0.3 M NaCl gradient for 60 min at the flow rate of 5 ml/min in Na-phosphate (pH 6.8) containing 0.1% SML and 0.1 mM phenylmethanesulfonyl fluoride. Elution profiles of proteins and cytochromes were monitored at 280 and 410 nm, respectively.

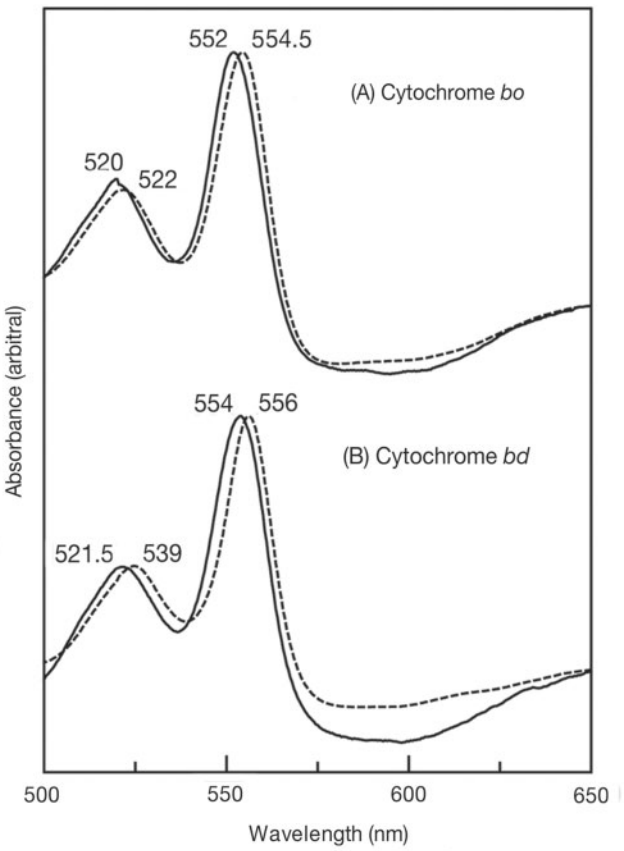

Fig. 4. **Redox difference spectra of pyridine haemchrome of cytochromes *bo* and *bd* isolated from the control (broken line) and haem O-overproducing strain (solid line).**

In contrast, the membranes isolated from the CyoE-overproducing strain [BL21 (DE3)/pLysS/pTTQ18-cyoE] showed the peaks at 405, 504, 539, 577 and 632 nm in the oxidized form and at 413, 507, 540, 557 and 633 nm in the reduced form (Fig. 2D). These spectroscopic features are totally different from those of the membranes from the control strain BL21(DE3)/pLysS (Fig. 2A), and of cytochromes *bo* and *bd* isolated from the control (Fig. 2B, C) and cyoE-overproducing strain (Fig. 2E, F). The periodic peaks in the oxidized form and the new peaks at 504, 507, and 577 nm in the CyoE-overproduced membranes (Fig. 2D) suggest the presence of an unbound population of haem O.

Spectroscopic Properties of Cytochromes bo and bd—To examine the misincorporation of haem O into terminal quinol oxidases of the aerobic respiratory chain, we isolated cytochromes *bo* and *bd* from the haem O synthase-overexpression strain. Cytochromes *bo* and *bd* were eluted at 0.11 and 0.15 M NaCl, respectively, on anion-exchange HPLC (Fig. 3). Since the expression of haem O synthase was induced for 4 h at the mid-log phase of the aerobic growth, the amount of cytochrome *bd* was much less than that of cytochrome *bo*. We also tried the overnight expression to increase the yield of cytochrome *bd*, but we found that such growth conditions were not favourable for the mis-incorporation of haem O into cytochrome *bd*. It should be noted that we were unable to identify a peak corresponding to succinate dehydrogenase, which binds haem b_{556} in the membrane anchor subunits, probably due to the instability of a haem O-bound form during the solubilization of the membranes.

The wild-type cytochrome *bo* binds 1 mol each of haem B and O (*48*). Puustinen *et al*. (*42*) showed that low-spin haem *b* and high-spin haem *o* contribute equally to the Soret peak absorption while the α peak absorption is predominated by low-spin haem *b* and established that the α-peak position was linearly correlated to the haem O to haem B ratio. The reduced *minus* oxidized difference spectrum of the pyridine ferrohemochrome showed the α peak at 554.5 nm in the wild-type cytochrome *bd* (Fig. 4A, broken line), an intermediate value of the α peak for authentic haem B (556 nm) and haem O (552 nm) (*29*, *48*). In cytochrome *bo* isolated from the CyoE-overproduced membranes, the α peak was shifted to 552 nm, indicating that cytochrome *bo* was a haem *oo*-type. The wild-type cytochrome *bd* binds 2 mol of haem B and 1 mol of haem D, and haems extracted from the air-oxidized, oxygenated enzyme showed the α peak of pyridine ferrohemochrome at 556 nm (Fig. 4B), identical to that of haem B. Haems extracted from cytochrome *bd* purified from the CyoE-overproduced membranes showed the α peak at 554 nm, indicating that a half of *b*-haems was substituted by haem O.

Effects of the misincorporation of haem O on spectroscopic properties of quinol oxidases were examined at room temperature. Substitution of low-spin haem *b* by haem O converted cytochrome *bo* to *oo* and resulted in blue shifts of the Soret peak at 411 and 427 nm of the air-oxidized and fully reduced form, respectively, to 409

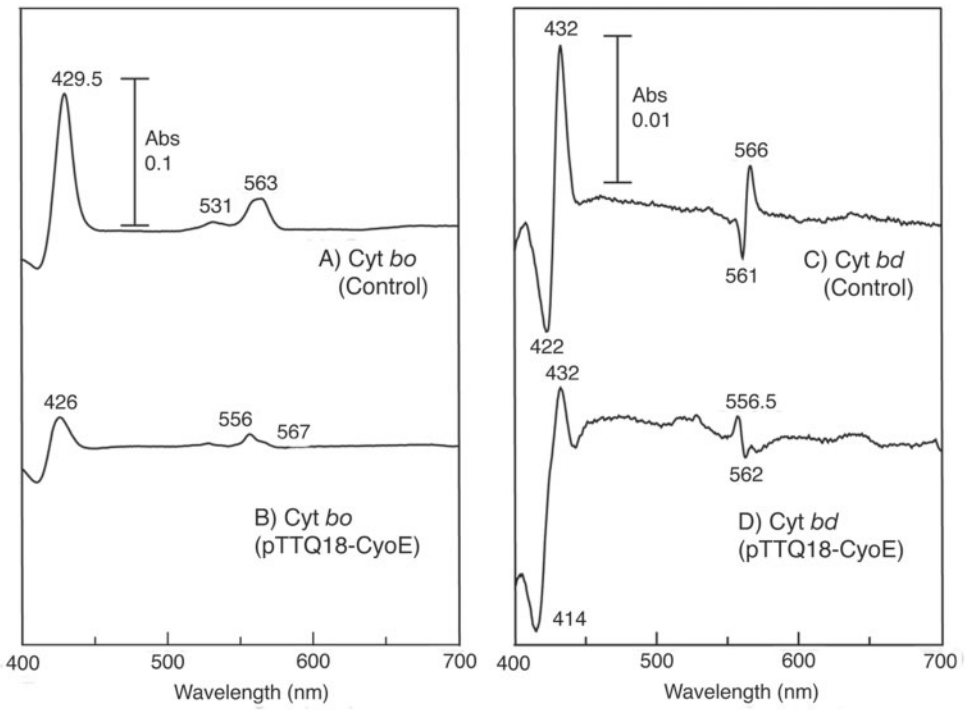

Fig. 5. **Spectral perturbations induced by binding of aurachin C1–10 to cytochrome *bo* (A, B) and aurachin D 5–10 to cytochrome *bd* (C, D) isolated from the control (A, C) and haem O-overproducing strain (B, D).** Before and after the addition of 10 μM aurachin C1–10 or D5–10, absolute spectra of the Na-hydrosulfite reduced enzymes (2 μM) were recorded in 50 mM Na-phosphate (pH 7.4) containing 0.1% SML, and (reduced *plus* inhibitor) *minus* reduced difference spectra were calculated.

and 422 nm, respectively (Fig. 2B, E). A broad α peak at 561 nm diagnostic for haem *b* (*38*, *39*, *41*, *49*) was changed to a sharp peak at 556 nm. The second-order finite difference spectra revealed the change in the intensity and peak position of the split α peak at 559 and 565 nm. In contrast, a haem *bb*-type variant of cytochrome *bo* isolated from the haem O synthase-defective mutant showed peaks at 427 and 560 nm in the fully reduced form and at 414 nm in the air-oxidized form (*26*, *27*). The intense 556 nm component in the haem *oo*-type variant indicates that interactions of the 2-vinyl group of haem B with the protein cause the distortion in the electronic transitions in the haem plane, yielding the splitting of the α peak (*42*). These spectra are essentially identical to those of cytochrome *bo* in the presence of 50 mM KCN, which could suppress redox changes of high-spin haem *o* at the binuclear centre (data not shown).

In cytochrome *bd*, the misincorporation of haem O reduced the amount of the haem b_{595}-*d* binuclear centre but did not affect the peak positions of high-spin haem b_{595} (440 and 596 nm peaks of the reduced form) and *d* (647 and 630 nm peak of the air-oxidized and fully reduced form, respectively) (Fig. 2C, F). In contrast, the Soret and α peak of low-spin haem b_{558} were blue shifted from 428 and 561 nm, respectively, to 423 and 558 nm, respectively. These observations indicate that haem b_{558} was substituted by haem O upon overproduction of haem O in *E. coli*.

Perturbation of the Quinol Oxidation Site by Misincorporated Haem O—Overproduction of haem O in *E. coli* resulted in the substitution of electron-accepting low-spin haem *b* by haem O in both cytochromes *bo* and *bd* (Figs 2 and 4). Thus, effects of the haem substitution on the nearby quinol oxidation site was probed with potent inhibitors for cytochromes *bo* and *bd*. In the wild-type cytochrome *bo*, binding of aurachin C1–10 to the reduced enzyme induced spectral shifts of low-spin haem *b* (Fig. 5A). Substitution of haem *b* by haem O reduced the amplitude of the Soret peak change to 40% of the control level. In the wild-type cytochrome *bd*, binding of aurachin D5–10 to the reduced enzyme induced red shifts of the Soret and α peaks (*10*, *14*), whereas the inhibitor binding to the reduced cytochrome *obd* resulted in blue shifts of these peaks. Thus, inhibitor-binding difference spectra suggest that the misincorporated haem O at the low-spin haem-binding site of cytochromes *bo* and *bd* perturbs the quinol oxidation site.

Effects of Misincorporated Haem O on Quinol Oxidase Activity—Oxidation of ubiquinol-1 by cytochromes *bo* and *bd* follows the Michaelis–Menten kinetics (*38*, *39*) and the modified *ping–pong bi–bi* mechanism (*10*, *47*), respectively. Kinetic parameters were determined with Eq. 1 for cytochrome *bo* and Eq. 2 for cytochrome *bd*.

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

$$v = \frac{V_{max}[S]^2}{K_m^2 + K_m[S] + [S]^2} \quad (2)$$

Both cytochromes *bo* and *oo* isolated from the control and haem O synthase-overexpression strain, respectively, showed the hyperbolic concentration-dependence of the oxidase activity (Fig. 6A). Conversion of cytochrome *bo* to

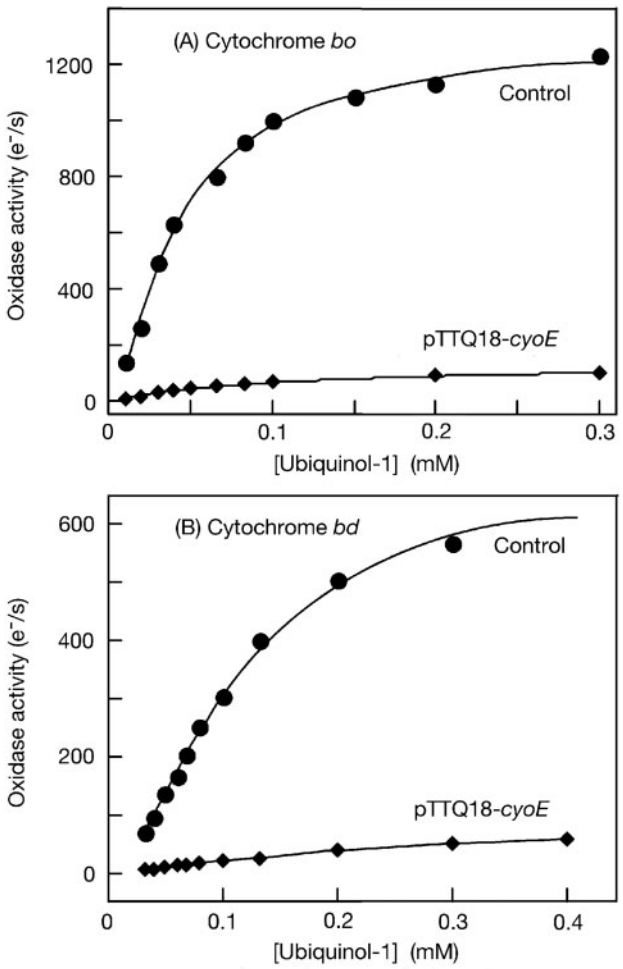

Fig. 6. **Dependence of ubiquinol oxidation by cytochrome *bo* (A) and *bd* (B) isolated from the control (closed circle) and haem O-overproducing strain (closed diamond).** Curve fitting was carried out with Kaleidagraph and Eq. 1 for cytochrome *bo* and Eq. 2 for cytochrome *bd* (*47*).

oo resulted in a 2.4-fold increase in the apparent K_m value for ubiquinol-1 (50 ± 4 to $121 \pm 11\,\mu M$) and a 10.6-fold decrease in the V_{max} value (1444 ± 35 to $136 \pm 6\,e^-/s$). In contrast, cytochromes *bbd* and *obd* showed the sigmoidal concentration dependence, and the conversion of haem type caused a 1.5-fold increase in the K_m value (87 ± 3 to $132 \pm 4\,\mu M$) and a 9.8-fold decrease in the V_{max} value (803 ± 20 to $82 \pm 2\,e^-/s$). Ascorbate/TMPD oxidase activity of cytochromes *oo* and *obd* was about one-fourth of the wild-type enzyme (data not shown), indicating that the major defect was perturbations at the quinol oxidation site. Thus, in the *E. coli* quinol oxidases, haem B is preferred as a low-spin haem for receiving electrons from quinols. A lowering of a mid-point redox potential of low-spin haem (*50*) as well as changes in interactions of low-spin haem with quinols or the protein would contribute the decrease in the oxidase activity.

DISCUSSION

Cytochrome bo—In the haem-copper terminal oxidase family, there are large variations in the haem-type of quinol oxidases [i.e. *bo*-type ubiquinol oxidase in *E. coli* (*42*, *48*), ba_3- or *bo*-type ubiquinol oxidase in *Acetobacter aceti* (*51*), ba_3-type ubiquinol oxidase in *Paracoccus denitrificans* (*52*), bb_3-type ubiquinol oxidase in *Bradyrhizobium japonicum* (*53*) and aa_3-type menaquinol oxidase in *B. subtilis* (*54*)]. Schröter *et al*. (*55*) demonstrated that the *E. coli* cytochrome *bo* can be expressed as a fully active cytochrome ba_3 in *P. denitrificans*. Cytochrome ba_3 shows a broad α peak at 564 nm and a new peak at 607 nm. Except for microaerophilic oxidases like bb_3-type ubiquinol oxidase and cbb_3-type cytochrome *c* oxidase, haem O and A, which have a hydroxyethyl farnesyl group at position 2 of the porphyrin ring (Fig. 1), are preferred at the dioxygen reduction site. It should be noted that replacement of haem O or A at the haem-copper binuclear centre with haem B results in a nonfunctional enzyme in *bo*-type ubiquinol oxidase from *E. coli* (*26*, *44*) and ba_3-type ubiquinol oxidase from *P. denitrificans* (*56*).

At the low-spin haem-binding site, haem B is preferred as an electron-accepting haem for quinols except for aa_3-type menaquinol oxidase from *B. subtilis*. The haem-copper quinol oxidase has evolved by gene duplication from caa_3-type cytochrome *c* oxidase in Gram-positive bacteria and has been distributed to Proteobacteria by lateral gene transfer (*57*). Accordingly, the *B. subtilis* aa_3-type menaquinol oxidase appears to still retain a higher affinity for haem A at the low-spin haem site. It is also possible that haem A is preferred for the oxidation of menaquinol or that haem O may not be accumulated in *B. subtilis* by direct transfer of haem O to haem A synthase in the CtaAB heterodimer (*58*). Here, we showed that low-spin haem *b* of the *E. coli* cytochrome *bo* was substituted by haem O upon transient overproduction of haem O synthase with the *tac*-promoter-based vector. As expected for the replacement of low-spin haem, which accepts electrons from the nearby quinol oxidation site, we found the changes in the enzyme kinetics on the quinol oxidation. Puustinen and colleagues (*41*, *42*) obtained the haem *oo*-type variant by the overexpression of cytochrome *bo* with a cosmid vector carrying the *cyo* operon and found similar spectroscopic perturbations of haems. However, they claimed that the *oo*-type oxidase was fully functional. As discussed previously (*29*), we have never observed such a variant when we expressed cytochrome *bo* by the chromosomal *cyo* operon, a single copy vector (*49*) or a multicopy vector (*59*), their expression vector may carry unidentified mutation(s) besides one reducing the expression level. Alternatively, the continuous high expression of haem O synthase and the haem O acceptor (cytochrome *bo*) by the multicopy vector (*41*, *42*) resulted in the phenotypic difference from our studies.

In the *E. coli* cytochrome *bo*, quinols are oxidized at the low-affinity quinol oxidation site (Q_L), which is assumed to be present at the interface between the periplasmic hydrophilic domain of subunit II and transmembrane helices I and II of subunit I (*31–33*). Then, within subunit I, electrons are transferred to the haem *o*-Cu_B binuclear centre through the high-affinity quinone-binding site (Q_H) and low-spin haem *b* (*34–37*). Changes in kinetic parameters for the ubiquinol-1 oxidation by cytochrome *oo* indicate the close proximity of the

Q_L site to haem *b*-binding site. Binding of haem O to the low-spin haem site would induce protein structural changes, which then affect the Q_L site directly or indirectly through the nearby Q_H site.

Cytochrome bd—In cytochrome *bd* (CydAB), quinols are oxidized at the *N*-terminal domain of periplasmic loop VI/VII of subunit I (*6–10*). Electrons are transferred to the haem b_{595}-*d* binuclear centre through low-spin haem b_{558} (*15–17*), which interacts with the quinol oxidation site (*14*). Under highly aerated growth conditions without glucose in *B. subtilis* (*60*) or during the N_2-dependent growth in *Gluconacetobacter diazotrophicus* (*61*), cytochrome *bd* was converted to cyanide-sensitive cytochrome *bb′*, which lost spectroscopic features of haems b_{595} and *d*. In the opportunistic pathogen *Pseudomonas aeruginosa* (*62*, *63*), the gastrointestine pathogen *Campylobacter jejuni* (*64*) and the photosynthetic bacterium *Rhodobacter sphaeroides* (*65*), cyanide-insensitive oxidase (CioAB) serves as one of terminal oxidases. Cyanide-insensitive oxidase is structurally related to cytochrome *bd* (*62*, *66*) but lacks spectroscopic features of haems b_{595} and *d* (*62–65*). The dioxygen-reducing haem *d* in cytochrome *bb′* and cyanide-insensitive oxidase may be substituted by haem B or may be present under unusual environments, which obscure electronic spectra. In this study, we replaced low-spin haem b_{558} by haem O through the overexpression of haem O synthase in *E. coli* and found that the resultant haem *obd*-type oxidase severely reduced the ubiquinol-1 oxidase activity. Changes in kinetic parameters and inhibitor-binding difference spectra support the perturbations at the quinol oxidation site by haem O bound to the low-spin haem-binding site.

In conclusion, by using the haem O synthase-overproducing system, we incorporated haem O into the low-spin haem-binding site of cytochromes *bo* and *bd* and demonstrated that haem B is required at the low-spin haem-binding site for the oxidation of quinols at the (low-affinity) quinol oxidation site in both enzymes. Steric hindrance at the position 2 of the porphyrin ring and/or change in the redox potential of the substituted haem (*50*) appears to affect the structure and function of terminal quinol oxidases. The low-spin haem-binding site in quinol oxidases appears to be more exposed to bulk water and or lipid phase to interact with quinols. If so, there is a potential to accommodate a bulky 2-hydroxyethylfarnesyl chain of haem O in the low-spin haem-binding site. Haem O can be easily overproduced in any *E. coli* strain including the *cyo* deletion mutant like BL21(Δcyo DE3) (*67*), and thus structural requirements of bound haems in other hemoproteins could be examined by the haem O synthase-overproducing system.

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CONFLICT OF INTEREST

None declared.

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