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Assignment and Functional Roles of the *cyoABCDE* Gene Products Required for the *Escherichia coli bo*-Type Quinol Oxidase[1]

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Cytochrome *bo* from *Escherichia coli* belongs to the heme-copper terminal oxidase superfamily and functions as a redox-driven proton pump. In the present study, we examined the functional roles of the *cyoABCDE* genes, which encode cytochrome *bo*. We expressed the *cyoABCDE* genes in minicells using pTTQ18 derivatives and identified subunits II, I, III, and IV of the oxidase complex and heme O synthase as polypeptides with molecular weights of 33,500, 75,000, 20,500, 12,000, and 28,000, respectively. The expression level of heme O synthase (CyoE) was much lower than those of the oxidase subunits and seems to be controlled just tightly enough for the incorporation of heme O into the oxidase complex. To facilitate functional analysis of the gene products, we developed a single copy expression vector pHNF2, a derivative of the F-sex factor. Genetic complementation tests showed that deletions in each gene resulted in nonfunctional enzymes. Western blotting analysis indicated that the expression levels of subunits I and II were not affected by the deletions in the other *cyo* gene products. However, spectroscopic analyses of the mutant membranes revealed that all the deletions perturbed or eliminated the redox metal centers in subunit I. Present findings suggest that subunits II, III, and IV of the oxidase complex are required for the assembly of the metal centers in subunit I.

Key words: cytochrome *bo*, deletion analysis, minicell, single copy vector, terminal oxidase.

Cytochrome *bo* is one of two terminal ubiquinol oxidases in the aerobic respiratory chain of *Escherichia coli* (*1*) and belongs to the heme-copper terminal oxidase superfamily (*2*). The enzyme is predominantly expressed under highly aerated growth conditions and functions as a redox-driven proton pump, an alternative oxidase, cytochrome *bd*, is predominant under microaerobic conditions and catalyzes only scalar protolytic reactions (*1, 3*).

Cytochrome *bo* is encoded by the *cyoABCDE* operon (*4*) and has been purified as two-, four-, and five-subunit enzymes (*5-7*). Subunits I, II, and III of the oxidase complex have been assigned as CyoB, CyoA, and CyoC, respectively, by partial protein sequencing (*8*), maxicell protein-labeling experiments (*9*), and a combination of molecular biological and immunological studies (*10*). Subunit I binds all the redox metal centers, low-spin heme *b*, high-spin heme *o*, and Cu_B, and the latter two centers form the heme-copper binuclear center where dioxygen is reduced to water (Ref. *11* for a review). Subunit II was identified as the binding site(s) for azidoubiquinone and appears to have a quinol oxidation site (*13*). Subunit IV remains to be confirmed as CyoD, as demonstrated for the counterparts in other bacterial terminal oxidases (*14-16*). CyoE has been demonstrated to be a novel enzyme, heme O synthase, which supplies heme O specifically to the binuclear center of cytochrome *bo* (*17-19*). In contrast to subunit I (*11, 12*), functional roles of other subunits remain obscure in bacterial heme-copper terminal oxidases.

In the present study, we developed a single copy expression vector pHNF2 and demonstrated that it was useful for mutational analysis of intrinsic membrane proteins such as cytochrome *bo*. We identified all the gene products of the cytochrome *bo* (*cyoABCDE*) operon and found that subunits II, III, and IV are required for the assembly of the redox metal centers in subunit I. Based on these observations, we discuss the importance of interactions of subunit I with subunits II, III, and IV for the correct assembly of the metal-binding sites in subunit I.

EXPERIMENTAL PROCEDURES

Construction of Terminal Oxidase-Deficient Strains—The *cyo* coding region in the 6.4 kb *Sal*I fragment of pHN3795 (*10*) was replaced with the chloramphenicol-resistance gene cassette in pHP45Ω-Cm (*20*) to give pHN4001-Cm. ST4674 (*Δcyo::cat cyd*$^+$) was constructed from TH1559 (*recB21 recC22 sbc15*; Ref. *10*) by double homologous recombination with linearized pHN4001-Cm

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Abbreviation: IPTG, isopropyl-β-D-thiogalactopyranoside.

as described previously (*21*). A terminal oxidase-deficient mutant ST4683 (*Δcyo::cat Δcyd::kan*) was constructed from GO103 (*Δcyd::kan*; Ref. *22*) by transduction with P1 lysate prepared from ST4674 as described (*23*). ST4533 (*Δcyo::kan recA1*) was constructed from W3092 (*galK2*) by transduction with P1 lysate prepared from ST4501 (TH1559 *Δcyo::kan*; Ref. *10*), followed by Hfr conjugation with NK6659 (*recA1 recA56 srlA1300::Tn10*; Ref. *10*).

Construction of pTTQ18 Derivatives—Each *cyo* gene accompanied with its own ribosome-binding site was subcloned into pTTQ18 (*24*) (Fig. 1A). pTTQ-*cyoABCDE* was obtained by subcloning of the 5.1 kb *Sac*I-*Sph*I fragment of pHN12 (*10*). pTTQ-*cyoA* and -*cyoB* were constructed by subcloning of the 1.4 kb *Sac*I-*Pst*I fragment of pHN12 and the 2.4 kb *Eco*RI fragment of pHN13.5 (*25*), respectively. pTTQ-*cyoC* was made by ligation of the 0.8 kb *Hin*dIII-*Sph*I fragment of pMFO1-*Δ*DE1 (described below) with the *Bam*HI-*Eco*RI-*Sph*I-(*Hin*dIII) linker and the 4.3 kb *Bam*HI-*Sph*I fragment of pTTQ18. pTTQ-*cyoD* was constructed as follows. The 1.7 kb *Eco*RI-*Sph*I fragment of pHN3795 was subcloned into pUC19 to give pUC-*cyoDE*. The *Eco*RV and *Nru*I sites in the insert were joined by blunt-end ligation, then the 0.9 kb *Eco*RI-*Sph*I fragment of the resulting plasmid pUC-*cyoD* was subcloned into pTTQ18. pTTQ-*cyoE* was made by ligation of the 1.3 kb *Bgl*I-*Sph*I fragment of pUC-*cyoDE* with the *Bam*HI-stop-*Bgl*I linker and the *Bam*HI-*Sph*I fragment of pTTQ18.

Labeling of Gene Products in Minicells—Strain TH5101 (*minA minB*; T. Harayama of The University of Tokyo) harboring the pTTQ18 derivatives was grown aerobically in LB medium containing 0.5% glucose and 100 μg/ml ampicillin until the culture reached the late exponential phase of growth, then minicells were isolated by sucrose density gradient centrifugation (*26*). The minicells were suspended with 1 ml of minimal medium A (*27*) supplemented with 0.5% glucose and 1% methionine assay medium (Difco) at an optical density at 650 nm of 0.6, then incubated at 37°C for 30 min. The expression of the *cyo* genes, which is under the control of the *tac* promoter and the *lacI*^{q} gene on pTTQ18, was induced by addition of IPTG at a final concentration of 1 mM in the presence of 9.2 nM L-[^{35}S]-Met (42.8 TBq/mmol; DuPont New England Nuclear). Incubation was continued at 37°C for 1 h followed by a chase with non-radioactive L-Met at a final concentration of 1.3 mM for 10 min. The labeled minicells were collected by centrifugation at 10,000 × *g* for 10 min at 4°C. After incubation with 50 μl of the sample buffer at 37°C for 30 min, 2 to 5 μl of the solubilized proteins was subjected to urea-SDS-19% polyacrylamide gel electrophoresis (*28*). Amounts of the samples were normalized by the intensity of a band corresponding to LacI encoded by the vector. Gene products were visualized by fluorography using Amplify (Amersham) and their molecular weights were estimated based on Rainbow Molecular Weight Marker (Amersham).

Construction of Single Copy Expression Vectors—The unique *Eco*RI site in a mini-F plasmid pMF3 (12.6 kb; Ref. *26*), which carries the *f5* region of F-sex factor and the *bla* gene of *Staphylococcus aureus*, was eliminated by blunt-end ligation using mung bean nuclease (Fig. 2). The 2.1 kb *Bam*HI-*Hin*dIII fragment of a resulting plasmid, pHNF1, was replaced with a linker 1 to introduce the *Bam*HI, *Eco*RI, and *Sph*I sites but to eliminate the *Hin*dIII site. The 5.4 kb *Ase*I-*Sph*I fragment of pHN3795 carrying the entire *cyo* operon was subcloned into a resulting plasmid pHNF2 (10.5 kb) with a linker 2 containing the *Bam*HI and *Ase*I sites. The resultant plasmid was designated as pMFO1. Alternatively, the 7.7 kb *Bam*HI-*Sph*I fragment of pHN-

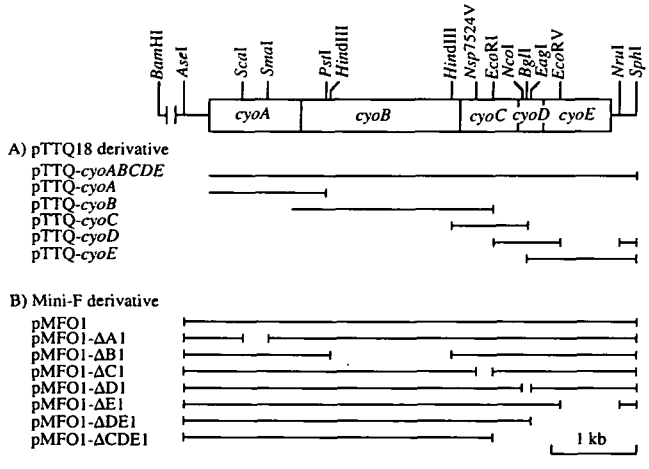

Fig. 1. **Physical maps of the pTTQ18 derivatives for expression of individual *cyo* genes and of the pMFO1 derivatives for genetic complementation tests.** The chromosomal DNAs cloned in pTTQ18 and pHNF2 are shown below the physical map of the *cyo* operon. The 5′-end of the inserts in pTTQ-*cyoABCDE* and -*cyoA* was generated by exonuclease digestion and is 59 bp upstream of the *cyoA* gene (*10, 25*). Similarly, the 5′-end in the insert of pTTQ-*cyoB* is 31 bp upstream of the *cyoB* gene. These 5′-ends were ligated with the *Sma*I site of pTTQ18.

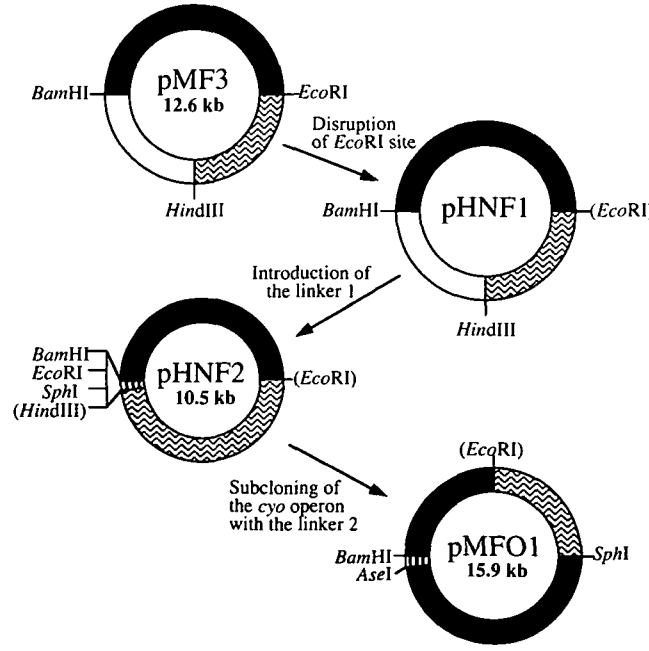

Fig. 2. **Construction of a single copy expression vector pMFO1 carrying the entire *cyoABCDE* operon.** See details in text. In pMF3, the *f5* region of F sex factor and the DNA segment of *Staphylococcus aureus* containing the *bla* gene are indicated by a shaded box and a wavy box, respectively. In pHN3795, the *E. coli* chromosomal DNA containing the *cyoABCDE* operon and the pBR322 DNA are indicated by a closed box and an open box, respectively. Parentheses indicate impaired restriction sites.

3795 was subcloned into pHNF2 to give pMFO2. Deletion series of pMFO1 were constructed as follows (Fig. 1B). pMFO1-⊿A1, -⊿B1, and -⊿E1 were constructed by eliminating the 0.42 kb *Sca*I-*Sma*I fragment [corresponding to Leu^{106}-Pro^{213} of CyoA; a total of 315 residues (*4*)], the 1.4 kb *Hin*dIII fragment (Phe^{146}-Ser^{629} of CyoB; 663 residues) and the 0.83 kb *Eco*RV-*Nru*I fragment of the *cyoE* gene (Ile^{64} to the C-terminus of CyoE; 296 residues), respectively, from pMFO1. pMFO1-⊿C1 and -⊿D1 were made by blunt-end ligation using T4 DNA polymerase to eliminate the 0.17 kb *Nsp*7524V-*Eco*RI fragment (Glu^{65}-Tyr^{118} of CyoC; 161 residues) and the 0.10 kb *Nco*I-*Eag*I fragment (Ser^{14}-Pro^{43} of CyoD; 109 residues), respectively. pMFO1-⊿DE and -⊿CDE were constructed by eliminating the 1.3 kb *Bgl*I-*Sph*I fragment (Pro^{43} of CyoD to the C-terminus of CyoE) and the 1.7 kb *Eco*RI-*Sph*I fragment (Glu^{119} of CyoC to the C-terminus of CyoE), respectively, using synthetic linkers.

Genetic Complementation Test—The terminal oxidase-deficient strain ST4683 (*⊿cyo ⊿cyd*), which cannot grow aerobically on non-fermentable carbon sources, was anaerobically transformed with the pMFO1 derivatives, then transformants were allowed to grow aerobically on minimal agar plates containing 15 μg/ml ampicillin in the presence of either 1% glucose or glycerol for two days.

Miscellaneous—DNA manipulations, plasmid direct sequencing, preparation of cytoplasmic membranes, spectroscopic measurements, assay of ubiquinol-1 oxidase, determination of heme, copper, and protein contents, Western blotting, HPLC analysis of heme composition were performed as described previously (*5, 7, 10*). Cytochrome *o* was determined from CO-binding difference spectra using a molar extinction coefficient of 206,000, the average value of six independent preparations (T. Mogi, unpublished results). Cytochrome *bo* was purified by DEAE-HPLC using octyl β-glucoside (Wako Pure Chemicals, Tokyo) in place of sucrose monolaurate (*7*). Ubiquinone-1 was a generous gift from M. Ohno, Eisai (Tokyo). All other chemicals were commercial products of analytical grade.

RESULTS

Assignment of the cyo Gene Products—Upon addition of IPTG to minicells isolated from TH5101/pTTQ-*cyoABCDE*, eight polypeptides were isotopically labeled with L-[^{35}S]Met (Fig. 3). Polypeptides with apparent M_r of 38,400, 28,000, and 31,000 in urea-SDS-19% PAGE were found in all constructions; they were assigned to LacI, β-lactamase and its precursor, respectively. Polypeptides with apparent M_r of 33,500, 75,000, 20,500, 12,000, and 28,000 were specifically overexpressed by pTTQ-*cyoA*, -*cyoB*, -*cyoC*, -*cyoD*, and -*cyoE*, respectively; they were assigned to CyoA, CyoB, CyoC, CyoD, and CyoE , respectively. The apparent M_r of CyoA, CyoB, CyoC, and CyoD were identical to those of subunits II, I, III, and IV of the four-subunit enzyme purified in octyl β-glucoside (*6*). Differences between the apparent M_r of subunit proteins determined by urea-SDS PAGE and their M_r deduced from the nucleotide sequence (35,000, 75,000, 23,000, 12,000, and 32,000, respectively) are attributable to post-translational modifications or to unusual electrophoretic mobility of hydrophobic membrane proteins in SDS-PAGE. Densitometric analysis of the *cyo* gene products followed by normalization with the content of Met residue (*i.e.*, CyoA : CyoB : CyoC : CyoD : CyoE = 16 : 33 : 11 : 11 : 12) indicated that the expression level of CyoE (heme O synthase) was about 1/30 of those of subunits of the oxidase complex (lane 2, Fig. 3). Specific incorporation of heme O into the binuclear center of cytochrome *bo* may be controlled not only by specific interactions of hemes with the high-spin heme binding site (*17*) but also by the translation of the *cyoE* gene.

Effects of Deletions in the cyo Gene Products on the Catalytic Activity of Cytochrome bo—To facilitate genetic analysis and stable expression of intrinsic membrane proteins, we developed the single copy expression vector pHNF2, a derivative of F-sex factor (Fig. 2). The *f5* region of the mini-F plasmid can maintain a copy number of 1 to 2 copies per cell, and its use therefore avoids multicopy suppression of the mutations or lethal effects upon overproduction of membrane proteins. Subsequently, we subcloned the entire *cyo* operon with the 0.55 kb 5′-noncoding regions into pHNF2 to give pMFO1. We found that pMFO1 complemented a defect of the aerobic growth of the terminal oxidase-deficient mutant ST4683 and expressed cytochrome *bo* with quinol oxidase activity comparable to those expressed by the chromosomal copy (Tables I and II).

To probe the functional role(s) of each *cyo* gene product, we examined the effects of deletions in the *cyo* genes on the catalytic activity and spectroscopic properties of cyto-

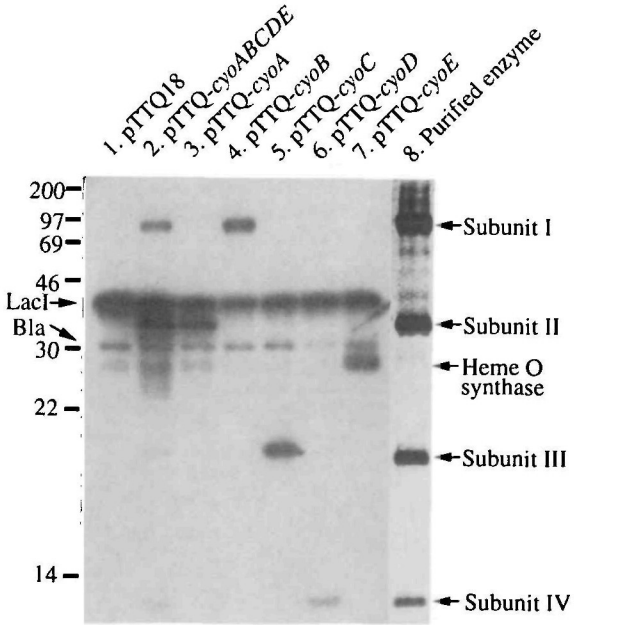

Fig. 3. **Urea-SDS PAGE analysis of the *cyo* gene products labeled in minicells.** Polypeptides encoded by recombinant plasmids were labeled with L-[^{35}S]Met in the isolated minicells carrying the pTTQ18 derivatives, then subjected to urea-SDS 19% PAGE followed by fluorography (lanes 1 to 7). Subunits of the purified cytochrome *bo* (lane 8) were visualized by silver staining (*6*). The corresponding *cyo* gene products and vector-encoded proteins (the LacI repressor and β-lactamase) are indicated by arrowheads. Molecular weight standards used are myosin (200,000), phosphorylase *b* (97,400), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), trypsin inhibitor (21,500), and lysozyme (14,300).

TABLE I. **Enzyme activity and spectroscopic properties of the deletion mutant oxidases.** The catalytic activity of the mutant oxidases *in vivo* was estimated from genetic complementation tests using ST4683 harboring the pMFO1 derivatives. The expression levels of subunits I and II in ST4533 harboring the pMFO1 derivatives were determined by Western blotting analysis of the mutant membranes using anti-subunit antisera. Contents of low-spin heme *b* (cytochrome $b_{563.5}$) were estimated by the absorbance of the 563.5 nm peak in the redox difference spectra at 77 K (Fig. 5B). Amounts of high-spin heme *o* (cytochrome *o*) were determined from the CO-binding difference spectra and the values presented in this table are the upper boundary of the estimates since there is significant spectral contribution from cytochrome *bd*. Copper contents were determined by atomic absorption spectroscopy (*7*). Heme composition was determined by reverse-phase HPLC analysis of acid-acetone extractable hemes (*17*).

Vector	*In vivo* activity	Subunit		Low-spin heme *b*	High-spin heme *o*	Cu	Heme B : O
		I	II		(nmol/mg protein)		
pHNF2 (Control)	−	−	−	−	<0.03	0.01	100 : 0
pMFO1 (Wild-type)	+	++	++	+++	0.40	0.34	72 : 28
pMFO1-⊿A1	−	++	−	++	0.14	<0.01	77 : 23
pMFO1-⊿B1	−	−	++	−	<0.03	<0.01	85 : 15
pMFO1-⊿C1	−	++	++	−	<0.03	<0.01	74 : 26
pMFO1-⊿D1	−	++	++	++	0.29	0.04	85 : 15
pMFO1-⊿E1	−	++	++	+	0.10	0.18	100 : 0

TABLE II. **Expression of the wild-type cytochrome *bo* by pMFO1.** ST4683/pMFO1 and GO103 were grown aerobically in LB-0.5% glucose medium, while ST4683 was cultured anaerobically under a stream of N_2 gas. Cytoplasmic membranes were isolated from the cells at the log phase of growth. The contents of cytochromes *b* and *o* were estimated from the heme content and the CO-binding difference spectra, respectively. Ubiquinol-1 oxidase activity was determined spectrophotometrically (*5*).

Strain	Cyt *b* (nmol/mg protein)	Cyt *o* (nmol/mg protein)	Ubiquinol-1 oxidase (U/mg protein)	Ubiquinol-1 oxidase (U/nmol Cyt *o*)
ST4683 (*⊿cyo ⊿cyd*)	0.24	ND[a]	0.54	−
ST4683/pMFO1 (*⊿cyo ⊿cyd/cyo*$^+$)	0.64	0.14	3.5	25
GO103 (*cyo*$^+$ *⊿cyd*)	0.77	0.24	5.3	22

[a] Not detected.

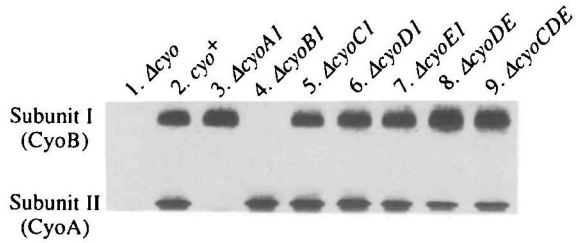

Fig. 4. **Western blotting analysis of the mutant cytoplasmic membranes with anti-subunit I and anti-subunit II antisera.** Cytoplasmic membranes were prepared from ST4533 harboring the pMFO1 derivatives. Ten micrograms of membrane proteins were loaded per lane on SDS 12.5% polyacrylamide gels.

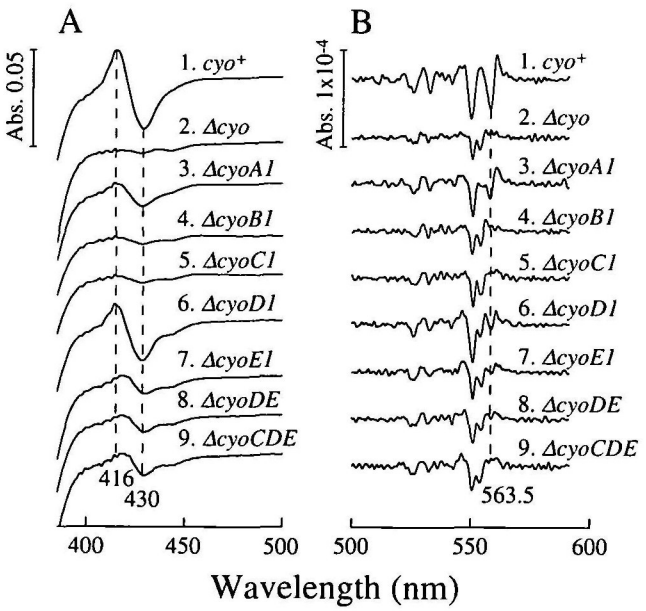

Fig. 5. **CO-reduced *minus* reduced difference spectra at room temperature (A) and second-order finite difference spectra of dithionite-reduced *minus* air-oxidized difference spectra at 77 K (B) of the mutant cytoplasmic membranes.** Spectra were recorded with a Shimadzu UV-3000 spectrophotometer as described previously (*10, 17*) at the protein concentrations of 1 and 3 mg/ml for (A) and (B) in 120 mM Tris-HCl (pH 7.4), respectively. Strain ST4533 with or without pMFO1 was used as a wild-type control and a negative control, respectively. In the case of the redox difference spectrum of ST4533/pMFO1-⊿B1, -⊿DE, and -⊿CDE the protein concentration was increased to 6 mg protein/ml to improve spectral quality.

chrome *bo*. In genetic complementation tests using the terminal oxidase-deficient mutant ST4683 harboring the pMFO1 derivatives, they were able to grow aerobically on minimal agar plates supplemented with glucose but not with glycerol (Table I), indicating that all the *cyo* genes are indispensable for the catalytic function of cytochrome *bo*. Western blotting analysis of the mutant membranes using anti-subunit I and anti-subunit II antisera showed that the amounts of subunits I and II were unaffected by the *cyo* deletions other than ⊿A1 and ⊿B1, which eliminated protein bands corresponding to subunits II and I, respectively (Fig. 4). This observation suggests that the absence of subunit-subunit interactions does not affect the localization or stability of subunits I and II.

Effects of Deletions in the cyo Gene Products on the Redox Metal Centers of Cytochrome bo—Cytoplasmic membranes were isolated from the cytochrome *bo*-deficient mutant harboring the pMFO1 derivatives and their spectroscopic properties were examined. In ST4533/pMFO1 (*cyo*$^+$), the amount of high-spin heme *o* (cytochrome *o*) was consistent with that of bound copper (*i.e.*, Cu_B) associated with the membranes (Table I). In the CO-binding difference spectrum at room temperature, the wild-type enzyme showed a peak at 416 nm and a trough at 430 nm (Fig. 5A), which are diagnostic for cytochrome *o* (*5, 17, 29*). In contrast, low-spin heme *b* (cytochrome $b_{563.5}$)

showed a split α peak at 555 and 563.5 nm in the second-order finite difference spectrum of the redox difference spectrum at 77 K (Fig. 5B) (*17, 29*). The latter peak is a diagnostic feature for cytochrome *bo* in the *E. coli* membranes. The membranes from ST4533 and ST4533/pMFO1-⊿B1 showed two troughs at 556 and 559 nm in the 77 K redox difference spectra, which are ascribable to cytochrome b_{556} of succinate dehydrogenase and cytochrome *bd*, which exhibits a split α peak at 556 and 559.5 nm (T. Mogi, unpublished results). Upon CO binding, these membranes showed two troughs at 428 and 443 nm in the Soret region, characteristic of cytochrome *bd* (*5*). These data are consistent with the absence of subunit I (Fig. 4).

The amplitude of the 563.5 nm peak was reduced by the *⊿cyoA1*, *⊿cyoD1*, and *⊿cyoE1* mutations and this peak was absent in the *⊿cyoC1* mutant membranes (Table I and Fig. 5). The *⊿cyoA1*, *⊿cyoC1*, *⊿cyoD1*, and *⊿cyoE1* mutations decreased the CO-binding activity to 35, <8, 70, and 25% of the wild-type level, respectively. The *⊿cyoC1* mutation did not affect the stability of subunit I (Fig. 4) but eliminated all three metal centers. The *⊿cyoD1* mutation resulted in a specific loss of Cu_B in cytochrome *bo*, as reported previously (*30*), while the *⊿cyoE1* mutation completely eliminated heme O from the cytoplasmic membranes (*17*). Since heme O was still present in the *⊿cyoB1* mutant membranes, the concentration of heme O in the cells does not seem to control heme O biosynthesis. In addition, the Soret peak was a few nm red-shifted in the CO-binding difference spectra of ST4533/pMFO1-⊿E1, -⊿DE, and -⊿CDE (Fig. 5A). This finding indicates that these mutations resulted in replacement of heme O with heme B at the high-spin heme binding site (*17*). The signal for cytochrome $b_{563.5}$ was still significant in the *⊿cyoDE* mutant, whereas all the deletions except ⊿CyoE eliminated completely or almost completely the copper-binding activity (Table I). These observations indicate that strict coordination chemistry is required for the Cu_B ligation by His^{284}, His^{333}, and His^{334} in subunit I (*31*).

DISCUSSION

Terminal oxidases of the aerobic respiratory chain are multi-subunit enzymes, and subunit I, which serves as a reaction center of the enzymes, has been extensively studied biochemically and molecular biologically (*10-12, 29*). However, functional roles of non-catalytic subunits remain obscure. In the present study, we examined possible roles of the gene products encoded by the *cyoABCDE* operon. Minicell protein labeling experiments assigned subunits II, I, III, and IV of the four-subunit enzyme (*6*) to be CyoA, CyoB, CyoC, and CyoD, respectively, with the apparent M_r of 33,500, 75,000, 20,500, and 12,000, respectively (Fig. 3). We found that the *cyoE* gene encodes heme O synthase with the apparent M_r of 28,000. The expression of CyoE by pTTQ-*cyoABCDE* was weaker than those of the oxidase subunits (Fig. 3). Previous studies showed that the anti-CyoE antiserum can detect CyoE in the cytoplasmic membranes when overexpressed by the pTTQ18 derivative, whereas CyoE was undetectable when the *cyo* operon was expressed by the chromosomal copy or single copy vector pMFO1 (*32*). The amount of heme O in the membranes was not affected by the absence of subunit I, therefore, free heme O molecules in the cells seem not to control heme O biosynthesis. These observations indicate that the expression level of CyoE is controlled translationally at a much lower level than that of the oxidase complex, even though both are encoded by the same *cyo* operon. Accordingly, a catalytic amount of CyoE is enough for the supply of heme O specifically to the binuclear center of cytochrome *bo*. A putative transcriptional terminator present between the *cyoB* and *cyoC* genes (*4*) may affect the expression level of CyoE. In addition, among deletions introduced in the *cyo* genes, only the *⊿cyoE1* mutation completely eliminated heme O from the cytoplasmic membranes, thereby resulting in the heme BB-type nonfunctional enzyme, which shows a red-shift of the Soret peak (*17, 32*). This fact suggests that the other *cyo* gene products are not required for heme O biosynthesis in *E. coli*.

The single copy expression vector developed in this study facilitated genetic analysis of the amino acid substitutions in cytochrome *bo* (*11, 17, 29, 30, 32*) and avoided the multicopy suppressor effect or harmful gene dosage effects previously observed (*10*). Genetic complementation tests demonstrated that all the *cyo* gene products are indispensable for the functional expression of cytochrome *bo*. A deletion in either CyoA or CyoB eliminated a polypeptide that crossreacted with the corresponding anti-subunit antiserum, but deletions in the other *cyo* gene products did not affect stability of subunits I and II (Fig. 4). Spectroscopic studies on the mutant cytoplasmic membranes revealed that the deletions in subunits II, III, and IV perturbed the redox metal centers (low-spin heme *b*, high-spin heme *o*, and Cu_B) in subunit I (Table I and Fig. 5). Deletions in CyoA, CyoC, and CyoD completely or markedly reduced the binding of copper ions (Cu_B) to subunit I (Table I). In addition, the *⊿cyoC1* mutation showed a profound effect on all three metal centers.

In aa_3-type cytochrome *c* oxidase of *Paracoccus denitrificans*, disruption of the *ctaC* gene encoding subunit II resulted in not only a complete loss of *a*-type cytochrome, subunit I and subunit II but also a substantial decrease of the oxidase activity (*33*). On the other hand, the deletion of the subunit III gene decreased the enzyme activity and the amount of cytochrome *a*, whose absorption maximum was 2-3 nm blue-shifted (*34*). Substitutions of the conserved acidic residues in subunit III reduced the amount of the fully assembled enzyme but did not affect the electron transfer activity and the proton translocation stoichiometry (*35*). Deletions in subunits IV, Va, Vb, VI, VII, and VIIa of yeast cytochrome *c* oxidase resulted in lack of the spectral features of *a*-type cytochrome and the enzyme activity (*36*). Recently, X-ray crystallographic studies on bacterial and mammalian cytochrome *c* oxidases revealed the presence of protein-protein interactions between subunit I and other subunits (*37, 38*). These observations indicate that the correct folding of the redox metal centers in subunit I requires the presence of subunit II and non-catalytic subunits. Bacterial oxidases with the almost same properties can be isolated as the two-subunit enzyme in the presence of stronger detergents such as Triton X-100 (*5, 39-42*). Thus, non-catalytic subunits including subunits III and IV of cytochrome *bo* are unlikely to participate in the catalytic functions. Similarly, release of non-catalytic subunits (*i.e.*, VIa, VIb, and VIIa) from the oxidase complex by Triton X-100 has been reported for eukaryotic

cytochrome *c* oxidases (*43, 44*).

We found recently from deletion and chemical cross-linking studies that subunit IV of cytochrome *bo* is present between subunits I and III (*30*), as found in cytochrome *c* oxidase from *P. denitrificans* (*33*). Subunit II of cytochrome *bo* seems to serve as the substrate (*i.e.*, quinol) oxidation site (*13*), which subsequently transfers electrons to low-spin heme *b* in subunit I. Therefore, subunit II of quinol oxidases must be present adjacent to subunit I in the oxidase complex. Accordingly, direct interactions of subunits II, III, and IV with subunit I appear to be required for the proper folding of the binding sites for the redox metal centers in subunit I. They may serve as a domain-specific molecular chaperone for subunit I during the assembly of the oxidase complex.

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