J. Biochem. 122, 422-429 (1997)

Substitutions of Charged Amino Acid Residues Conserved in Subunit I Perturb the Redox Metal Centers of the *Escherichia coli bo*-Type Ubiquinol Oxidase[1]

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Received for publication, April 8, 1997

Cytochrome *bo* is a four-subunit quinol oxidase in the aerobic respiratory chain of *Escherichia coli* and functions as a redox-coupled proton pump. 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 of the oxidase complex. This work focuses on the functional and structural roles of 14 charged amino acid residues that are conserved in subunit I of the heme-copper terminal oxidases. Substitutions of Lys^{55}, Tyr^{173}, Asp^{188}, Asp^{256}, Arg^{481}, and Arg^{482} by neutral amino acid residues did not affect the catalytic activity and spectroscopic properties of the cytoplasmic membranes. In contrast, genetic complementation tests indicated that replacements of Arg^{80}, Asp^{135}, Arg^{257}, Glu^{286}, Tyr^{288}, Lys^{362}, Asp^{407}, and Glu^{540} resulted in nonfunctional enzymes. The R80Q mutation caused loss of a diagnostic peak for low-spin heme *b* in the 77 K redox difference spectrum. The K362Q, D407N, and E540Q mutations affected the CO-binding by the heme-copper binuclear center. The D135N, R257Q, E286Q, and Y288F mutations specifically eliminated the Cu_B center from the oxidase complex, whereas the E286D mutant did not show significant perturbations on the redox metal centers even though it was still inactive. Based on these findings and recent crystallographic studies on cytochrome *c* oxidases, we discuss the possible roles of the conserved charged amino acid residues in subunit I of the heme-copper terminal oxidases.

Key words: cytochrome *bo*, proton pump, redox metal center, site-directed mutagenesis, terminal oxidase.

Cytochrome *bo* is a four-subunit quinol oxidase in the aerobic respiratory chain of *Escherichia coli* (*1, 2*) and generates an electrochemical proton gradient across the cytoplasmic membrane *via* not only scalar proteolytic reactions but also redox-coupled proton pumping (*3-5*). This enzyme is encoded by the *cyoABCDE* operon and belongs to the superfamily of heme-copper respiratory oxidases. The members of this family seem to share common mechanisms for dioxygen reduction and proton pumping (*6, 7*).

Subunit I binds all the redox metal centers, low-spin heme *b* (cytochrome $b_{563.5}$), high-spin heme *o* (cytochrome *o*), and Cu_B (*8, 9*). Heme *o* and Cu_B are antiferromagnetically coupled and form a heme-copper binuclear center, where reduction of molecular oxygen takes place (*10-12*). Electron transfer in subunit I seems to be mediated *via* a covalent bond system consisting of side-chains of heme ligands and the connecting peptide backbone "His^{421}-Phe^{420}-His^{419}" (*9, 13*). Substrates are oxidized at a low-affinity quinol-oxidation site (Q_L) in subunit II, then electrons are transferred to the binuclear center through a high-affinity quinone-binding site (Q_H) and heme *b* (*14-17*). Subunits III and IV are not involved in the catalytic functions (*18*) but are required for the assembly of the redox metal centers in subunit I (*19, 20*).

Previous site-directed mutagenesis studies on subunit I (the *cyoB* gene product) have identified that heme *b* is ligated by His^{106} in transmembrane helix II and His^{421} in helix X, heme *o* by His^{419} in X, and Cu_B by His^{284} in helix VI, His^{333} and His^{334} in helix VII (*21-26*). Recent X-ray crystallographic studies on cytochrome *c* oxidases (*27, 28*) confirmed the axial ligands of the metal centers, but His^{333} and His^{334} were actually found in loop VII-VIII (Trp^{331}-Gly^{341}) instead of helix VII in our structure models (*9, 21*) (Fig. 1). Among the conserved aromatic amino acid residues in subunit I, Trp^{280}, Tyr^{288}, Trp^{331}, and Phe^{348} were indispensable for the enzyme activity and are required for the assembly and/or the functions of the heme-copper binuclear center (*9*). Several pathways for proton transport in cytochrome *c* oxidases have been proposed on the basis of X-ray structures (*27, 29*). Mutagenesis studies suggested that Asp^{135} in loop II-III of subunit I participates in proton pumping by cytochrome *bo* (*30, 31*).

In the present study, we carried out site-directed mutagenesis studies on 14 charged amino acid residues

[1] This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas (08249106 and 08268216), for Scientific Research (A) (07558221) and (B) (08458202), and for Exploratory Research (08878097) from the Ministry of Education, Science, Sports and Culture of Japan. This is paper XXVI in the series "Structure-function studies on the *E. coli* cytochrome *bo* complex."

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Abbreviations: Q_H, a high-affinity quinone-binding site; Q_L, a low-affinity quinol-oxidation site; HPLC, high-performance liquid chromatography.

conserved in subunit I, and the effects of amino acid substitutions on the enzyme activity and the redox metal centers were carefully examined. Catalytic activities of the mutant enzymes were examined by genetic complementation tests using a single copy expression vector developed in our laboratory (*20*). It can avoid multicopy suppression of the mutational defects, which could be accompanied with 3- to 5-fold overproduction of the terminal oxidase by multicopy vectors (*32*), and also replacement of heme B with heme O at the low-spin heme-binding site due to overproduction of heme O synthase encoded by the same cytochrome *bo* operon (*33*). Based on the present observations and X-ray structures of cytochrome *c* oxidases, we discuss possible roles of the conserved charged amino acid residues in subunit I of the heme-copper terminal oxidases.

MATERIALS AND METHODS

Introduction of Amino Acid Substitutions into Subunit I—Fourteen charged amino acid residues conserved in subunit I (Fig. 1) were individually substituted by neutral residues by site-directed mutagenesis (*21*) using phagemid pCYOF9 (J. Minagawa, unpublished results). Six unique restriction sites gene-engineered in either the *cyoA* (*Nhe*I) or the *cyoB* (*Apa*I, *Xho*I, *Mlu*I, *Eco*81I, and *Hin*dIII) gene on pCYOF9 facilitated sequencing analysis and subcloning of the mutations (Fig. 2). Codons for Lys^{55} (AAA), Lys^{362} (AAG), Arg^{80}, Arg^{481} and Arg^{484} (CGT), Arg^{257} (CGC), Glu^{286} (GAA), and Glu^{540} (GAG) were changed to CAA (Gln), codons for Asp^{135} and Asp^{407} (GAC), Asp^{188} and Asp^{256} (GAT) to AAC (Asn), codons for Tyr^{173} (TAT) and Tyr^{288} (TAC) to TTC (Phe), and a codon for Glu^{286} to GAT (Asp). After confirmation of DNA sequences of mutant plasmids, unique restriction fragments carrying the mutations were subcloned into the wild-type pCYOF9 (Fig. 2). For expression of the *cyoB* mutant oxidases, the 2.6 kb *Nhe*I-*Eco*RI fragment of the resultant plasmids was replaced with the counterpart in single copy vector pMFO4 (*21*) to yield derivatives of pMFO9 (J. Minagawa, unpublished results). Since the native *cyoB* gene in pMFO4 contains two *Hin*dIII site in contrast to a single site in pMFO9 or pCYOF9, restriction site analysis of the pMFO9

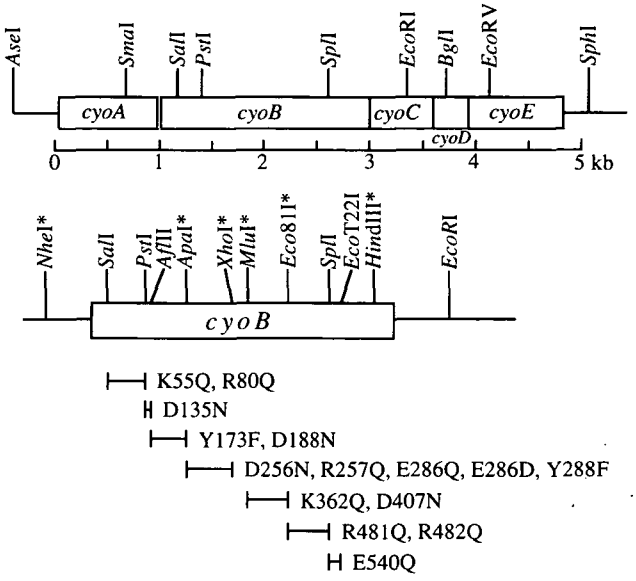

Fig. 2. **Physical map of the *cyo* operon in phagemid pCYOF9.** Coding regions of the *cyo* genes are shown by open rectangles. The gene-engineered *Nhe*I, *Apa*I, *Xho*I, *Mlu*I, and *Eco*81I sites are marked by asterisks. Restriction fragments used for sequencing analysis and subcloning are shown by horizontal lines.

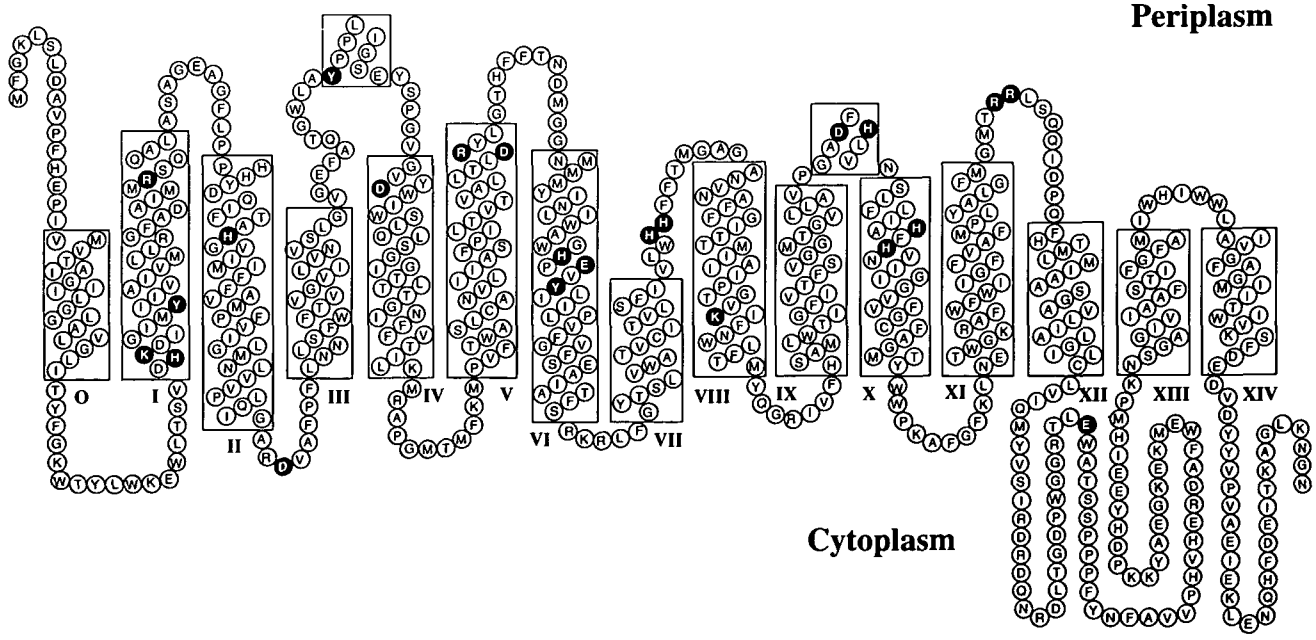

Fig. 1. **Secondary structure model of subunit I showing locations of conserved charged amino acid residues.** The previous model (*21*) was modified based on the X-ray structure of cytochrome *c* oxidase from *Paracoccus denitrificans* (*27*). Helices are indicated by rectangles. Locations of the charged amino acid residues examined in the present study and the previous studies (His^{54}, Tyr^{61}, His^{106}, His^{284}, Tyr^{288}, His^{333}, His^{334}, His^{411}, His^{419}, and His^{421}; Refs. *9* and *21*) are highlighted.

derivatives with *Hin*dIII ensures the subcloning of the mutations.

Genetic Complementation Test—Terminal oxidase-deficient strain ST2592 (*Δcyo Δcyd*; Ref. *21*) was anaerobically transformed with the pMFO9 derivatives, and the resultant colonies were allowed to grow aerobically at 37°C for three days on minimal-15 μg/ml ampicillin plates supplemented with either 0.4% glycerol or glucose as sole a carbon source (*21*). Strain ST2592 harboring pMFO9 or pHNF2 (*20*) was used as the wild-type control and a vector control, respectively.

Preparation of Cytoplasmic Membranes—Strain ST4676 (*Δcyo cyd*$^+$; H. Nakamura, unpublished results) harboring the pMFO9 derivatives was grown aerobically in rich medium supplemented with 15 μg/ml ampicillin, 50 μg/ml $FeSO_4 \cdot 7H_2O$, and 25 μg/ml $CuSO_4 \cdot 5H_2O$, then cytoplasmic membrane vesicles were isolated from cells at the mid-exponential phase of growth ($A_{650} = 0.35$) as described previously (*8, 21*).

Miscellaneous—DNA manipulations were carried out as described previously (*8, 21*). Measurements of optical spectra, determination of copper content and protein concentration, Western blotting analysis using the anti-subunit I antiserum, and analysis of acid acetone-soluble hemes by reverse-phase HPLC were performed as described previously (*8, 21*). Cytochrome *bo* was purified by anion-exchange HPLC using sucrose monolaurate (Mitsubishi-Kagaku Foods, Tokyo) as described (*12*). Heme content was calculated as the sum of hemes B and O using a molar extinction coefficient for heme B (*12*). Cytochrome *o* was determined from CO-binding difference spectra (*18*) using a molar extinction coefficient of 206,000, the average value of six independent preparations (T. Mogi, unpublished results). Proton pumping assay was carried out as described previously (*5*). Restriction endonucleases and other enzymes for DNA manipulations were purchased from Takara Shuzo (Kyoto) or New England BioLabs. Other chemicals are commercial products of analytical grade.

RESULTS

Effects of Mutations on the Catalytic Activity of Mutant Enzymes—A total of 23 charged amino acid residues are highly conserved in subunit I of the heme-copper terminal oxidases (Fig. 3). Our previous studies have shown that

TABLE I. **Properties of charged amino acid mutant oxidases.**

Strain	Aerobic growth	Subunit I	Cytochrome $b_{563.5}$	Cytochrome *o*[a]	Copper[a]
Wild-type	yes	++	++	100%	100%
K55Q	yes	++	++	73	100
R80Q	no	++	−	54	82
D135N	no	++	++	89	32
Y173F	yes	++	++	108	91
D188N	yes	++	++	81	100
D256N	yes	++	++	103	74
R257Q	no	++	++	81	15
E286Q	no	++	++	86	9
E286D	no	++	++	81	85
Y288F	no	++	++	78	12
K362Q	no	++	++	27	59
D407N	no	++	++	51	15
R481Q	yes	++	++	73	91
R482Q	yes	++	++	100	82
E540Q	no	++	++	57	26

[a]Amounts of cytochrome *o* and copper in the cytoplasmic membranes from strain ST4676/pMFO9 (wild-type control) were 0.43 and 0.47 nmol/mg protein, respectively, and those for strain ST4676/pHNF2 (vector control) were <0.06 and 0.13 nmol/mg protein, respectively. Specific contents were obtained by subtracting the values for the vector control and are expressed as % of the wild-type control.

	54	55	61	80	106	135	173	188	256	257	284	286	288	333	334	362	407	411	419	421	481	482	540
E. coli (*bo*)	H	K	Y	R	**H**	D	Y	D	D	R	**H**	E	Y	**H**	**H**	K	D	H	**H**	**H**	R	R	E
A. aceti (*ba*$_3$)	H	K	Y	R	**H**	D	Y	D	D	R	**H**	E	Y	**H**	**H**	K	D	H	**H**	**H**	R	R	E
P. denitrificans (*ba*$_3$)	H	K	Y	R	**H**	D	F	D	D	R	**H**	E	Y	**H**	**H**	K	D	H	**H**	**H**	R	R	E
B. subtilis (*aa*$_3$)	H	K	Y	R	**H**	D	Y	N	D	R	**H**	E	Y	**H**	**H**	K	D	H	**H**	**H**	R	R	E
Br. japonicum (*aa*$_3$)	H	K	Y	R	**H**	D	Y	D	D	R	**H**	E	Y	**H**	**H**	K	D	Q	**H**	**H**	R	R	E
P. denitrificans (*aa*$_3$)	H	K	Y	R	**H**	D	Y	A	D	R	**H**	E	Y	**H**	**H**	K	D	H	**H**	**H**	R	R	E
B. subtilis (*caa*$_3$)	H	K	Y	R	**H**	D	Y	D	D	R	**H**	E	Y	**H**	**H**	K	D	H	**H**	**H**	R	R	E
Yeast (*aa*$_3$)	A	K	Y	R	**H**	D	Y	D	D	R	**H**	E	Y	**H**	**H**	K	D	H	**H**	**H**	R	R	F
C. reihardtii (*aa*$_3$)	H	K	Y	R	**H**	D	Y	D	D	R	**H**	E	Y	**H**	**H**	K	D	H	**H**	**H**	R	R	E
Maize (*aa*$_3$)	H	K	Y	R	**H**	D	Y	D	D	R	**H**	E	Y	**H**	**H**	K	D	H	**H**	**H**	R	R	E
Soy bean (*aa*$_3$)	H	K	Y	R	**H**	D	Y	D	D	R	**H**	E	Y	**H**	**H**	K	D	H	**H**	**H**	R	R	E
Pa. aurelia (*aa*$_3$)	H	K	Y	R	**H**	D	I	D	D	R	**H**	E	Y	**H**	**H**	K	N	H	**H**	**H**	R	R	H
Sea urchin (*aa*$_3$)	H	K	Y	R	**H**	D	I	D	D	R	**H**	E	Y	**H**	**H**	K	N	H	**H**	**H**	R	R	H
Fruit fly (*aa*$_3$)	H	K	Y	R	**H**	D	Y	D	D	R	**H**	E	Y	**H**	**H**	K	D	H	**H**	**H**	R	R	E
Frog (*aa*$_3$)	H	K	Y	R	**H**	D	Y	D	D	R	**H**	E	Y	**H**	**H**	K	D	H	**H**	**H**	R	R	E
Bovine (*aa*$_3$)	H	K	Y	R	**H**	D	Y	D	D	R	**H**	E	Y	**H**	**H**	K	D	H	**H**	**H**	R	R	E
Th. thermophilus (*caa*$_3$)	H	K	Y	R	**H**	D	Y	D	E	R	**H**	T	Y	**H**	**H**	K	D	H	**H**	**H**	R	R	E
Th. thermophilus (*ba*$_3$)	E	K	Y	Q	**H**	E	Y	V	V	L	**H**	I	Y	**H**	**H**	T	D	H	**H**	**H**	R	R	E
H. salinarium (*aa*$_3$)	H	E	G	G	**H**	D	Y	M	D	R	**H**	E	Y	**H**	**H**	K	D	H	**H**	**H**	R	R	E
S. acidocaldarius (*ba*$_3$)	A	S	Y	R	**H**	D	Y	N	E	R	**H**	E	Y	**H**	**H**	K	D	N	**H**	**H**	R	R	-
S. acidocaldarius (*aa*$_3$)	W	P	V	M	**H**	K	Y	L	-	-	**H**	V	Y	**H**	**H**	T	N	H	**H**	**H**	R	R	-
P. denitrificans (CcoN)	G	V	W	A	**H**	-	G	E	V	Q	**H**	A	G	**H**	**H**	G	N	H	**H**	**H**	W	R	P
Rb.capsulatus (CcoN)	G	V	W	A	**H**	-	G	E	V	Q	**H**	A	G	**H**	**H**	G	N	H	**H**	**H**	W	R	P
Br. japonicum (FixN)	S	S	W	A	**H**	-	G	E	Y	V	**H**	A	G	**H**	**H**	G	N	H	**H**	**H**	W	R	Q
Ar. caulinodans (FixN)	G	T	W	A	**H**	-	G	E	Y	I	**H**	A	G	**H**	**H**	G	N	H	**H**	**H**	W	R	P
Ps. stutzeri (NorB)	S	Q	Y	G	**H**	S	T	K	S	R	**H**	W	E	**H**	**H**	M	N	H	**H**	**H**	L	Q	-
Ps. aeruginosa (NorB)	S	Q	Y	G	**H**	S	T	K	T	R	**H**	W	E	**H**	**H**	M	N	H	**H**	**H**	L	Q	-

Fig. 3. **Sequence alignment of the conserved charged amino acid residues in subunit I of the heme-copper respiratory oxidase superfamily and cytochrome *b* subunit of nitric oxide reductase.** Amino acid sequences aligned are: quinol oxidases [cytochrome *bo* from *E. coli*, cytochrome *ba*$_3$ from *Acetobacter aceti*, *Paracoccus denitrificans* (QoxB) and *Sulfolobus acidocaldarius* (SoxM), cytochrome *aa*$_3$ from *Bacillus subtilis* (QoxB), *Halobacterium salinarium*, and *S. acidocaldarius* (SoxB)], cytochrome *c* oxidases [cytochrome *aa*$_3$ from *Bradyrhizobium japonicum* (CoxA), *P. denitrificans* (CtaDII), yeast (*Saccharomyces cerevisiae*), *Chlamydomonas reinhardtii*, maize, soybean, *Paramecium aurelia*, sea urchin (*Paracentrotus lividus*), fruit fly (*Drosophila melanogaster*), frog (*Xenopus laevis*) and bovine, cytochrome *caa*$_3$ from *B. subtilis* (CtaD) and *Thermus thermophilus* (CaaB), cytochrome *ba*$_3$ from *T. thermophilus* (CbaA), cytochrome *cbb*$_3$ from *P. denitrificans* (CcoN), *Rhodobacter capsulatus* (CcoN), *Br. japonicum* and *Azorhizobium caulinodans* (FixN)], and nitric oxide reductase [cytochrome *b* (NorB) from *Pseudomonas stutzeri* and *Ps. aerginosa*) (*34-46*, references cited in Ref. *21*). The numbering refers to the *E. coli* sequence. The invariant histidines are indicated by boldface type.

His^{54} and Tyr^{61} in helix I, and His^{411} in helix IX-X are dispensable for the catalytic functions, while His^{106} in helix II, His^{284} in helix VI, and His^{333} and His^{334} in loop VII-VIII serve as the axial ligands of the redox metal centers (*9, 21, 25, 26*). A side-chain of Tyr^{288} in helix VI appears to extend to the heme-copper binuclear center (*9*).

In the present study, we replaced 14 charged amino acid residues with neutral residues and examined effects of each amino acid substitution on the catalytic activity of the mutant oxidases. Thus, Lys^{55} and Arg^{80} in helix I, Arg^{257} in helix V, Glu^{286} in helix VI, Lys^{362} in helix VIII, Arg^{481} and Arg^{482} in loop XI-X, and Glu^{540} in loop XIII-XIV were changed to Gln, Asp^{135} in loop II-III, Asp^{188} in helix IV, Asp^{256} in helix V and Asp^{407} in helix IX-X to Asn, and Tyr^{288} in helix VI and Tyr^{173} in helix III-IV to Phe (Fig. 1). Glu^{286} was also substituted by Asp.

The catalytic activity of the mutant enzymes was examined by genetic complementation test using the terminal oxidase-deficient strain ST2592 (*Δcyo Δcyd*) harboring the mutant pMFO9. We found that the K55Q, Y173F, D188N, D256N, R481Q, and R482Q mutants can grow aerobically on both minimal-glycerol and minimal-glucose plates (Table I), and their growth rates in liquid medium were the same as that of the wild-type (data not shown). In contrast, the R80Q, D135N, R257Q, E286Q, E286D, Y288F, K362Q, D407N, and E540Q mutants failed to grow aerobically on minimal-glycerol plates, even after prolonged incubation (Table I). Supplementation of copper ions to the growth medium or lowering of growth temperature did not affect the growth phenotype. Western blotting analysis of the cytoplasmic membranes isolated from ST4676 (*Δcyo cyd*$^+$) harboring the mutant pMFO9 showed that none of the mutations affected the stability of subunit I (Table I). Subsequently, spheroplasts isolated from the defective mutant cells, which had been grown in a jar fermentor under highly aerated conditions, were subjected to proton pumping assay. We found a H^+/e ratio of 1.6 to 2.0 for the strain expressing the wild-type cytochrome *bo* as a sole terminal oxidase, as reported previously (*5*). All the defective mutants showed the ratio of below 1, similar to the strain expressing cytochrome *bd* as a sole terminal oxidase (*5*). These observations indicated that substitutions of Arg^{80}, Asp^{135}, Arg^{257}, Glu^{286}, Tyr^{288}, Lys^{362}, Asp^{407}, and Glu^{540} eliminate or severely impair the catalytic activity of the mutant oxidases including proton pumping activity.

Effects of Mutations on Binuclear Metal Center—The mutant oxidases were expressed in ST4676 (*Δcyo cyd*$^+$) harboring the pMFO9 derivatives, and the effects of the amino acid substitutions on the redox metal centers were examined in the cytoplasmic membranes. High-spin heme *o* of the wild-type enzyme shows a peak at 416 nm and a trough at 430 nm in the reduced, CO-bound *minus* reduced difference spectra at room temperature (*18, 21*) and can be quantitated as cytochrome *o* (Figs. 4 and 5 and Table I). The amount of Cu_B was estimated from the copper content of the membranes determined by atomic absorption spectroscopy (Table I). We found that all the defective mutations affected the heme-copper binuclear center. The R80Q, K362Q, D407N, and E540Q mutations reduced the CO-binding activity of high-spin heme to about 30-60% of the wild-type level. The amount of Cu_B in the D135N, R257Q, E286Q, Y288F, D407N, and E540Q mutations was reduced to about 10-30% of the control level. It should be noted that the E286D mutation can restore the copper binding to the mutant enzyme although the mutant strain was still unable

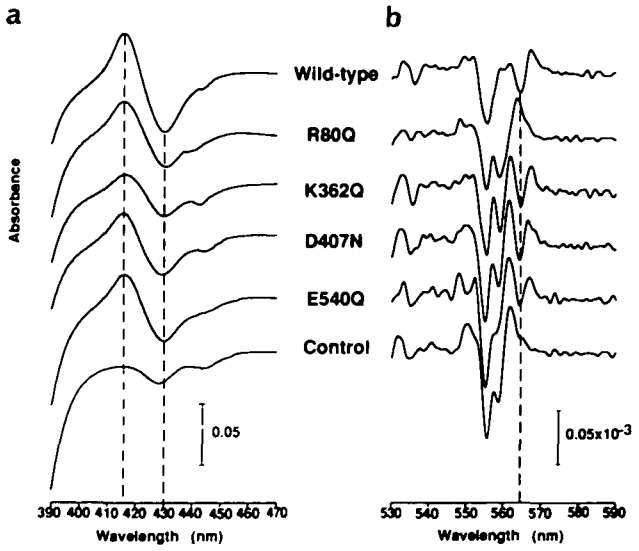

Fig. 4. **CO-binding difference spectra and redox difference spectra of the R80Q, K362Q, D407N, and E540Q mutant membranes.** Dithionite-reduced, CO-bound *minus* dithionite-reduced difference spectra of the Soret region (a) were recorded at room temperature with a Shimadzu UV-3000 spectrophotometer at a protein concentration of 1 mg/ml. Redox difference spectra of the α region (b) were recorded at 77 K and at a protein concentration of 3 mg/ml. Second-order finite difference spectra were obtained as described previously (*21*). A spectral band width was 1 nm and a scanning rate was 50 nm/min. The light path length was 10 and 2 mm for CO-binding difference spectra and redox difference spectra, respectively. Cytoplasmic membranes isolated from ST4676/pMFO9 and ST4676/pHNF2 were used as wild-type and negative control, respectively.

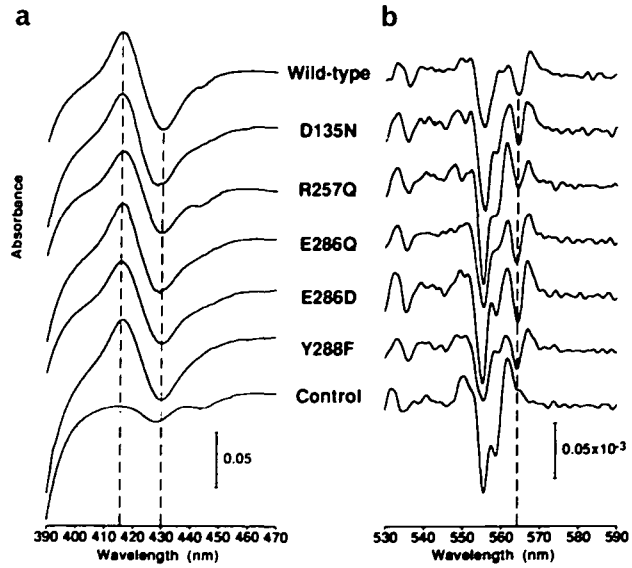

Fig. 5. **CO-binding difference spectra and redox difference spectra of the D135N, R257Q, E286Q, E286D, Y288F, and E540Q mutant membranes.** Details are the same as described in the legend to Fig. 4.

to grow aerobically.

We purified the E286Q and Y288F mutant oxidases and found that their heme B to heme O ratio was altered from 1.01 : 0.99 of the wild-type to 1.18 : 0.82 and 1.84 : 0.16, respectively. Ubiquinol-1 oxidase and CO-binding activities were reduced to 2 and 77%, respectively, in the E286Q mutant and <0.1 and 18%, respectively, in the Y288F mutant. Phenotypes of the E286Q and Y288F mutant oxidases are similar to those of the Cu_B ligand mutant H333A (*21, 32*) and the heme O synthase mutants (*47, 48*), respectively. Therefore, a negative charge at position 286 seems to be essential for the Cu_B binding, and Tyr^{288} seems to be essential for specific incorporation of heme O into the binuclear center. Spectroscopic studies on the mutant oxidases suggest that loss or severe reduction of the catalytic activity in the defective mutant oxidases is related to perturbations at the heme-copper binuclear metal center.

Effects of Mutations on Low-Spin Heme—Dithionite-reduced *minus* air-oxidized difference spectra of the mutant membranes were recorded at 77 K and their second-order finite difference spectra were obtained as described previously (*21*) (Figs. 4 and 5). The peak at 563.5 nm is a diagnostic feature for low-spin heme *b* of the wild-type cytochrome *bo* in the *E. coli* membranes (*18, 21, 22*). Notably, only the R80Q mutation completely eliminated the 563.5 nm peak. Spectral features of the α-peak region were similar to those of the vector control strain ST4676/pHNF2 and are attributable to cytochrome *bd*, an alternative quinol oxidase, and to cytochrome b_{556} of succinate dehydrogenase. Effects of the R80Q mutation on the low-spin and high-spin hemes were similar to those found for the Ala mutants of His^{106} and His^{421}, the axial ligands of low-spin heme *b* (*21*).

DISCUSSION

The *E. coli* cytochrome *bo* is one of the most extensively studied oxidases in the heme-copper terminal oxidase superfamily since it is amenable to molecular biological studies. Previous studies on subunit I have shown six invariant histidines as the axial ligands for three redox metal centers (*21-26*) as well as critical residues for proton translocation and the binuclear center (*9, 30, 31, 49-52*). Recent X-ray crystallographic studies on cytochrome *c* oxidases established molecular structures of the metal centers in subunits I and II and further suggested possible pathways for proton translocation (*27-29*). In bacteriorhodopsin (*53-56*) and photosynthetic reaction center (*57, 58*), charged amino acid residues are known to mediate proton translocation across the membrane. Here we report site-directed mutagenesis studies on 14 conserved charged residues in subunit I (Fig. 1) to examine whether they have crucial roles in redox-driven proton pumping, binding of the metal centers, or interactions with other subunits.

Replacement of the target residues with neutral residues (*i.e.*, Asp to Asn; Glu, Lys, and Arg to Gln; and Tyr to Phe) was carried out using phagemid pCYOF9 where five unique restriction sites have been gene-engineered in the subunit I gene to facilitate sequencing analysis and subcloning of the mutations (Fig. 2). For genetic complementation test, mutant oxidases were expressed in the terminal oxidase-deletion strain ST2592 (*Δcyo Δcyd*) by using single copy vector pMFO9, which can express cytochrome *bo* at a level similar to that expressed by the chromosomal copy. Thus, we can avoid multicopy suppressor effects that often accompany overexpression by multicopy vectors. We found that Lys^{55} in helix I, Tyr^{173} in periplasmic helix III-IV, Asp^{188} in helix IV, Asp^{256} in helix V, Arg^{481} and Arg^{482} in loop XI-X (see Fig. 1) are dispensable for the catalytic functions (Table I), as previously shown for His^{54} and Tyr^{61} in helix I and His^{411} in periplasmic helix IX-X (*9, 21*). In contrast, substitutions of Arg^{80} in helix I, Asp^{135} in loop II-III, Arg^{257} in helix V, Glu^{286} and Tyr^{288} in helix VI, Asp^{407} in helix IX-X, and Glu^{540} in loop XII-XIII resulted in nonfunctional enzymes (Table I). Spectroscopic analyses of the mutant membranes revealed that defects are associated with perturbations of the redox metal centers (Figs. 4 and 5). As shown for W280L (Mogi *et al.*, unpublished results), the generation time of the mutants is well correlated with the *in vitro* quinol oxidase activity and the catalytic activity of the defective mutant oxidases appears to be eliminated or severely impaired.

Previously, Gennis and colleagues expressed the D135N, D188N, D256N, E286Q, E286A, Y288F, K362Q, and D407N mutant oxidases by multicopy vectors and found that all the mutations except E286A, Y288F, and K362Q can complement a defect of the aerobic growth of RG129 (*cyo cyd recA*) on nonfermentable carbon source and do not affect the redox metal centers (*30, 49, 51*). In this study, Y288F severely reduced ubiquinol oxidase activity (*i.e.*, $<0.1\%$) and Cu_B binding (12%) and perturbed the high-spin heme environment (*49, 50*) because of replacement of heme O with protoheme IX (this study), suggesting that Tyr^{288} is essential for binding of both heme O and Cu_B. X-ray crystallographic studies on cytochrome *c* oxidases demonstrated the direct interactions of a OH group of Tyr^{288} with the N(ε) of His^{284} in the same helix and a OH group of a hydroxyethyl group of high-spin heme (*27, 28*). Thus, Tyr^{288} may be also functionally important in mediating electron transfer from high-spin heme to Cu_B through the peptide backbone in helix VI ("HPEVY") (*28*). K362Q resulted in a nonfunctional enzyme and perturbed CO-binding (*51, 52*; this study). The side-chain of Lys^{362} in helix VIII is buried completely in a hydrophobic environment or H-bonded to a fixed water molecule which is placed at Ser^{299} in helix VI (*27, 29*). Disruption of an indirect interhelix interaction by K362Q may disrupt interactions of Tyr^{288} with His^{284} and high-spin heme. Our present observations are consistent only with their data for D188N, D256N, Y288F, and K362Q.

Asp^{135} is conserved in the proton-pumping oxidases other than cytochrome cbb_3 (FixN-type cytochrome *c* oxidase) (Fig. 3) and is proposed to form the entry site of the proton channel (*27, 29*). Thomas *et al.* claimed that Asp^{135} is essential for proton pumping since D135N retained 45% of the enzyme activity but lost proton pumping activity without affecting the redox metal centers (*30*). However, our D135N did not complement the growth defect of ST2592 (*Δcyo Δcyd*) and reduced Cu_B binding (Table I). The discrepancy may be partly related to a difference in the plasmids used for expression of the mutant enzyme.

A side-chain of Glu^{286} is present at the opposite side of His^{284} and Tyr^{288} in helix VI and is proposed to be a part of the proton pump channel (*27*). Gennis's group found that E286Q retained 69% of the quinol oxidase activity and

showed spectroscopic properties similar to those of the wild-type enzyme, whereas E286A reduced the enzyme activity to 6% and perturbed the Cu_B environment (*30, 49*). Notably, the same authors reported for the homologous cytochrome *c* oxidase of *Rhodobacter sphaeroides* that E286Q completely inactivated the enzyme and reduced CO-binding (*59, 60*). We found that E286Q in cytochrome *bo* perturbed the environment of the high-spin heme binding site. This mutation also largely reduced enzyme activity and Cu_B binding to 2 and 9% of the wild-type control, respectively. Since E286D can restore Cu_B binding, a negative charge at position 286 seems to be crucial for a local structure at the peptide segment Gly^{283}-His^{284}-Pro^{285}-Glu^{286}-Val^{287}-Tyr^{288}, where a helical structure is distorted by two helix breakers (*27, 29*). Such structural features may also be required for the oxygen channel, in which Val^{287} plays an important role (*61*). Our data on E286Q are consistent with the phenotypes of E286A in cytochrome *bo* (*30, 49, 51*) and E286Q in cytochrome *c* oxidase (*59, 60*) but do not support the earlier observations on E286Q in cytochrome *bo* (*30*).

Asp^{407} is in a negatively charged cluster at the interface of subunit I and subunit II, just above the heme-copper binuclear center, of cytochrome *c* oxidases (*27, 29*) and is proposed to be the proton exit site in a pumping pathway, as well as a possible ligand to Mg (*27*). Thomas *et al.* reported that D407N reduced the quinol oxidase activity to 31% of the wild-type level and claimed that D407N does not affect the metal centers (*30*). On the contrary, we found that D407N largely perturbed both CO binding and Cu_B binding (Table I). Recently, Ferguson-Miller and colleagues reported that D407N and D407A in cytochrome *c* oxidase from *R. sphaeroides* did not affect the catalytic activity, proton pumping, CO-binding activity, and Mn^{2+}/Mg^{2+} binding (*62*). Eubacterial quinol oxidases lack two ligands for Mg^{2+} (*i.e.*, Asp^{412} in subunit I and Glu^{209} in subunit II) (*6, 34-36*) and the purified cytochrome *bo* binds neither Mg^{2+} nor Mn^{2+} (<3% of Cu; T. Mogi, unpublished results). Discrepancies in the mutant phenotypes between this study and Wenjun *et al.* (*62*) can be attributed to a structural or functional difference of helix IX-X between quinol oxidases and cytochrome *c* oxidases (see Fig. 1).

H-Bond with Heme Propionate Groups—Crystallographic studies of cytochrome *c* oxidases suggest that H-bonds are formed between Arg^{482} and one of the propionate groups of low-spin heme, Asp^{407} and His^{411} in helix IX-X and one of the propionate groups of high-spin heme, and Arg^{481} in loop XI-XII and another propionate group of high-spin heme (*27-29*). Arg^{80} was suggested to form H-bond with the formyl group of low-spin heme. R80Q eliminated completely low-spin heme signal and D407N perturbed high-spin heme whereas substitutions of Arg^{481} and Arg^{482} did not affect the redox metal centers. Thus, we conclude that Arg^{80} and Asp^{407} are required for binding of low-spin heme *b* and high-spin heme *o* of cytochrome *bo*, respectively.

Finally, we found also that R257Q and E540Q perturbed the binuclear center. Arg^{257} and Glu^{540} as well as Asp^{135} are present in hydrophilic loops or at the boundary of transmembrane helix and are distal to the binuclear center, therefore, these mutations may affect correct folding of a bundle of the transmembrane helices in subunit I.

In conclusion, we found that Arg^{80} and Asp^{407} may form H-bonds with propionate groups of low-spin heme *b* and high-spin heme *o*, respectively. Tyr^{288} was shown to be essential for binding of both high-spin heme and Cu_B, as revealed by crystallographic studies on cytochrome *c* oxidases (*27, 28*). A negative charge provided by Glu^{286} to the polypeptide segment "GHPEVY," which is highly conserved in the heme-copper terminal oxidases, is crucial for Cu_B binding and the catalytic activity. Asp^{135}, Arg^{257}, and Glu^{540} appear to be required for the folding of subunit I and may also be involved in the entry or exit site of the proton channel. Time-resolved studies using pH indicator dyes and Fourier-transform infrared spectroscopy will provide further clues about the functional role(s) of 8 charged amino acid residues identified in the present study in redox-coupled proton pumping by the heme-copper terminal oxidases.

We would like to thank M. Ohno (Eisai Co. Ltd., Tsukuba) for ubiquinone-1.

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