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Defining the Structural Domain of Subunit II of the Heme-Copper Terminal Oxidase Using Chimeric Enzymes Constructed from the *Escherichia coli bo*-Type Ubiquinol Oxidase and the Thermophilic *Bacillus* caa_3-Type Cytochrome *c* Oxidase[1]

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To probe the location of the quinol oxidation site and physical interactions for inter-subunit electron transfer, we constructed and characterized two chimeric oxidases in which subunit II (CyoA) of cytochrome *bo*-type ubiquinol oxidase from *Escherichia coli* was replaced with the counterpart (CaaA) of caa_3-type cytochrome *c* oxidase from thermophilic *Bacillus* PS3. In pHNchi5, the C-terminal hydrophilic domain except a connecting region as to transmembrane helix II of CyoA was replaced with the counterpart of CaaA, which carries the Cu_A site and cytochrome *c* domain. The resultant chimeric oxidase was detected immunochemically and spectroscopically, and the turnover numbers for Q_1H_2 (ubiquinol-1) and TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) oxidation were 28 and 8.5 s^{-1}, respectively. In pHNchi6, the chimeric oxidase was designed to carry a minimal region of the cupredoxin fold containing all the Cu_A ligands, and showed enzymatic activities of 65 and 5.1 s^{-1}, and an expression level better than that of pHNchi5. Kinetic analyses proved that the apparent lower turnover of the chimeric enzyme by pHNchi6 was due to the higher K_m of the enzyme for Q_1H_2 (220 μM) than that of cytochrome *bo* (48 μM), while in the enzyme by pHNchi5, both substrate-binding and internal electron transfer were purturbed. These results suggest that the connecting region and the C-terminal α_1-α_2-β_{11}-α_3 domain of CyoA are involved in the quinol oxidation and/or physical interactions for inter-subunit electron transfer, supporting our previous proposal [Sato-Watanabe, M., Mogi, T., Miyoshi, H., and Anraku, Y. (1998) *Biochemistry* 37, 12744-12752]. The close relationship of *E. coli* quinol oxidases to cytochrome *c* oxidase of Gram-positive bacteria like *Bacillus* was also indicated.

Key words: chimera, Cu_A, cytochrome *c* oxidase, heme-copper terminal oxidase, subunit II, ubiquinol oxidase.

Cytochrome *bo*-type ubiquinol oxidase from *Escherichia coli* and cytochrome caa_3-type cytochrome *c* oxidase from thermophilic *Bacillus* PS3 are closely-related members of the heme-copper terminal oxidase super-family (*1-3*). Despite differences in electron donors and binding hemes, they catalyze the same dioxygen reduction chemistry and function as a redox-driven proton pump.

Both enzymes are composed of four subunits, CyoABCD (*4, 5*) and CaaABCD (*6, 7*), respectively, and subunits I and II form the catalytic core (*8-10*). In cytochrome *bo*, subunit II (CyoA) contains a low-affinity quinol-oxidation site (Q_L) in the C-terminal hydrophilic domain (*11-15*), and electrons are sequentially transferred from quinols at the Q_L site to low-spin heme *b* in subunit I (CyoB) through a high-affinity quinone-binding site (Q_H), which functions as a transient electron reservoir and an electron gate (*16-19*). Subsequently, electrons are transferred to the high-spin heme *o*-Cu_B binuclear center in subunit I, and used for the four-electron reduction of dioxygen (*1, 2*). In cytochrome caa_3, subunit II (CaaA) oxidizes reduced cytochrome *c* or TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) at Cu_A, a binuclear purple-copper center, which mediates electron transfer to the high-spin heme a_3-Cu_B binuclear center through low-spin heme *a* in subunit I (CaaB). In *Bacillus*, the cytochrome *c* domain is fused to the C-terminus of subunit II (*3, 6, 7*).

The C-terminal hydrophilic domain of subunit II of both quinol and cytochrome *c* oxidases contains the Greek key

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Abbreviations: Q_1H_2, ubiquinol-1; Q_H, a high-affinity quinone-binding site; Q_L, a low-affinity quinol-oxidation site; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

β-barrel structure (cupredoxin fold) of blue-copper proteins (*20, 21*). The amino acid residues liganding the bimetallic Cu_A center were first examined through gene-engineering by introducing the putative metal ligands conserved in cytochrome *c* oxidases and nitrous oxide reductases, and those in blue-copper proteins into CyoA lacking any bound copper (*21-23*). Photoaffinity cross-linking studies involving azido-ubiquinone (*12, 13*), and random and site-directed mutagenesis studies on cytochrome *bo* (*14, 15*) indicated that the Cu_A end of the cupredoxin fold and the quinol oxidase-specific (Qox) domain provides the Q_L site and is involved in electron transfer to the metal centers in subunit I. Tryptic digestion of cytochrome caa_3 produced a cut of CaaA into the N-terminal transmembrane domain and the C- terminal hydrophilic domain, but did not affect the cytochrome *c* oxidase activity (*24*). The tight physical interaction between the Cu_A domain and subunit I seems essential for inter-subunit electron transfer, as revealed by X-ray crystallographic studies on cytochrome aa_3 (*25, 26*).

In this study, we designed and characterized two chimeric oxidases in which the C-terminal hydrophilic domain of CyoA was replaced with the counterpart of CaaA containing either the Cu_A-cytochrome *c* domain or a minimal fragment containing all the Cu_A ligands (Cu_A ligand domain; Lys195-Met207), and identified the structural domains of subunit II for quinol oxidation. In the former study, we designed a chimeric terminal oxidase in which all the C-terminal hydrophilic domain of CyoA was replaced with the counterpart of CaaA containing the Cu_A-cytochrome *c* domain (*27*). The defect in the aerobic growth of the terminal oxidase-deficient *E. coli* mutant was suppressed in rare cases, and analyses of one case showed that a membrane fraction of the transformant had both quinol and TMPD oxidase activities, but at much reduced levels.

MATERIALS AND METHODS

Construction of Plasmids for Chimeric Oxidases—For replacement of the periplasmic domain of CyoA, *Tth*HB8I and *Age*I sites were introduced into the *cyoA* gene on the cytochrome *bo*-overexpression vector, pHN3795-1 (*cyoABCDE*+Ap^r) (*28*), by oligonucleotide-directed site-specific mutagenesis using a Sculptor *in vitro* mutagenesis system (Amersham) (Fig. 1). pHNchi5 was constructed by replacement of the *Tth*HB8I-*Sac*II fragment of pHN3795-1 with a PCR-amplified DNA encoding the Cu_A-cytochrome *c* domain of CaaA, and pHNchi6 was made by replacement of the *MnI*I-*Age*I fragment of pHN3795-1 with a PCR-amplified DNA encoding the Cu_A ligand domain of CaaA (Fig. 1). pHNchi2-1, in which the N- and C-termini of the Cu_A-cytochrome *c* domain of CaaA were fused to the C-terminus of CyoA and N-terminus of CyoB, respectively, was similarly constructed from pHNchi (*27*) by deleting the stop codon of CyoA (Fig. 1). DNA manipulations were carried out according to Sambrook *et al.* (*29*).

The *E. coli* mutant, ST2592 (*Δcyo::cat Δcyd::kan*) (*30*), which lacks both *bo*- and *bd*-type ubiquinol oxidases, and is defective in aerobic growth on non-fermentable carbon sources, was used for transformation with the pHN3795-1 derivatives. Transformants were obtained using 2×TY agar plates supplemented with 50 μg/ml ampicillin and 30 μg/ml chloramphenicol.

Cell Culture, and Preparation of Crude Membranes and Purified Enzymes—Two-milliliter aliquots of overnight cultures were inoculated into 100 ml of minimal medium A (*31*) supplemented with 1% polypeptone, 0.5% yeast extract, 0.5% casamino acids, 1% glycerol, 20 μg/ml ampicillin, and 6 μg/ml chloramphenicol in 1 liter flasks. ST2592 harboring pHNchi5, pHNchi6, pHNchi2-1 or pHN3795-1, and ST4676 (*Δcyo::cat cyd+*) (*32*) were aerobically grown at 37°C with shaking at 160 rpm, and crude membranes were prepared as described previously (*27*). The wild-type cytochrome *bo* from *E. coli* (*33*) and cytochrome caa_3 from thermophilic *Bacillus* PS3 (*3*) were purified as described previously.

Analytical Procedures—TMPD and Q_1H_2 oxidase activities were measured in 20 mM sodium phosphate buffer (pH 6.0) containing 1 mM EDTA, using millimolar extinction coefficients of 10.5 at 562 nm and 8.5 at 278.5-252.4 nm, respectively, as described previously (*27*), except for the following measurement. For turnover measurement, Q_1H_2 oxidase activity (Fig. 4) was measured in 50 mM Tris-HCl, pH 7.1, containing 0.1% sucrose monolaurate, using a millimolar extinction coefficient of 12.3 at 275 nm at 25°C (*34*). Absorption spectra were measured with a Beckman DU-70 spectrophotometer, and the contents of cytochrome *bo*, cytochrome *bd*, and heme *d* were calculated using millimolar extinction coefficients of redox difference spectra; 27.6 at 562-600 nm (Mogi, unpublished results), 21.0 at 561-580 nm, and 27.9 at 628-651 nm (*35*), respectively. The cytochrome *bo* contents in the presence of other *b*-type cytochromes were determined from CO-difference spectra using a millimolar extinction coefficient of CO-difference spectra of 206 at 416-430 nm. Determination of protein concentrations, heme staining for detection of heme C-attached proteins after SDS-PAGE, and Western blotting analyses with rabbit antiserum against cytochrome *bo* or cytochrome caa_3 were carried out as cited previously (*27*). Other materials were obtained as described previously (*27*).

RESULTS AND DISCUSSION

Expression of Chimeric Oxidases—To probe the location of the Q_L site and a pathway for inter-subunit electron transfer, we constructed plasmids that produce chimeric ubiquinol oxidases in which parts of the C-terminal hydrophilic domain of subunit II (CyoA) in cytochrome *bo*-type ubiquinol oxidase from *E. coli* were replaced with the Cu_A-cytochrome *c* (pHNchi5) or Cu_A (pHNchi6) domain of CaaA, *i.e.* subunit II, of cytochrome caa_3-type cytochrome *c* oxidase from thermophilic *Bacillus* PS3 (Fig. 1). pHNchi5 and pHNchi6 suppressed a defect in the aerobic growth of a terminal oxidase-deficient mutant, ST2592 (*Δcyo Δcyd*), on a minimal-1% glycerol agar plate, whereas pHNchi2-1, the subunit II-I fusion with the Cu_A-cytochrome *c* domain of CaaA (Fig. 1), failed to grow aerobically on the same agar plate. This indicates that the expression level and/or the enzymatic activity of the chimeric oxidase encoded by pHNchi2-1 are not enough for aerobic growth under oxygen stress.

Expression of the chimeric enzymes was examined by Western blot analysis of crude membranes using anti-cytochrome caa_3 serum (Fig. 2). Only the 39 kDa band of the ST2592/pHNchi5 membranes (lane A) cross-reacted

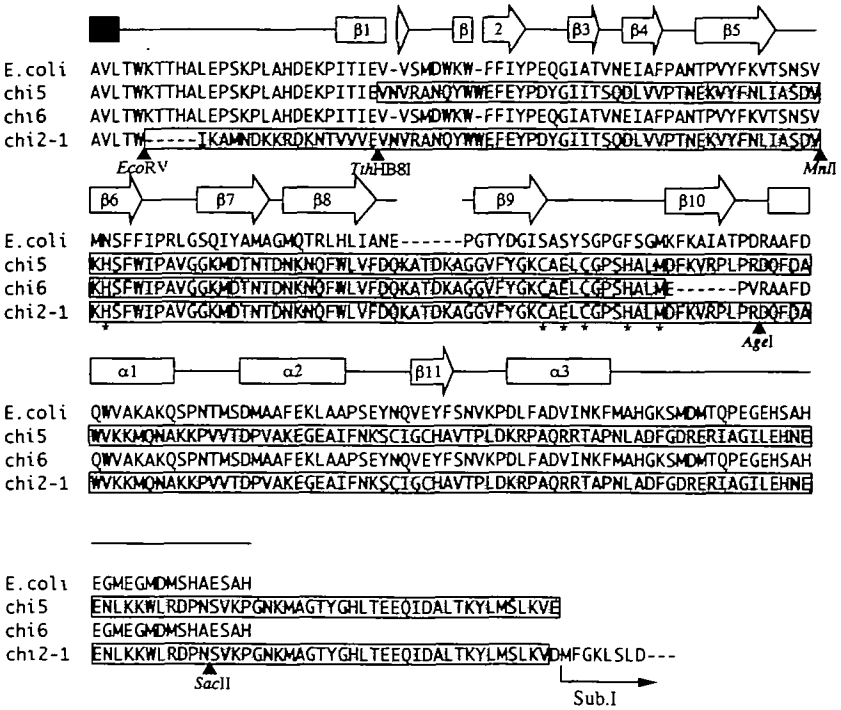

Fig. 1. **Designs for construction of chimeric subunit II of cytochrome *bo*-type ubiquinol oxidase.** Alignment of the primary sequences for the hydrophilic region of subunit II from the *E. coli* cytochrome *bo* and the thermophilic *Bacillus* PS3 cytochrome caa_3 (shadowed) is shown with the secondary structures of cytochrome *bo* revealed by X-ray crystallography (*23*) and the C-terminal portion of a putative transmembrane helix II (closed box). The Cu_A ligands are indicated by stars (*22, 23*). Five restriction sites used for the construction of the chimeric subunit II are also indicated.

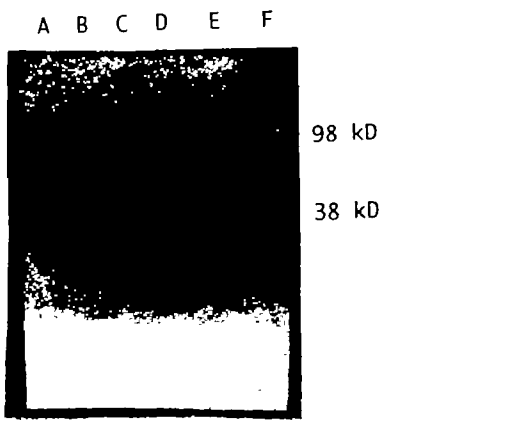

Fig. 2. **Western blot analysis of crude membranes isolated from transformants using the anti-cytochrome caa_3 antiserum.** SDS-PAGE was carried out using 50 μg protein of the membranes or 1 μg of the purified enzymes per lane. A, ST2592/pHNchi5; B, ST2592/pHNchi6; C, ST2592/pHN3795-1; D, ST4676; E, cytochrome caa_3; F, cytochrome *bo*.

TABLE I. **Efficiency of transformation and growth, and characterization of crude membranes.** The cytochrome *b* content was calculated from the $Na_2S_2O_4$-reduced *minus* air-oxidized difference spectra. TMPD and quinol oxidase activities were measured spectroscopically in 20 mM sodium phosphate buffer (pH 6.0) containing EDTA with 0.25 mM TMPD or 0.20 mM Q_1H_2 as the substrate. The numbers of membrane preparations examined were eight for ST2592/pHN3795-1, ST2592/pHNchi5, and ST2592/pHNchi6.

Strain	Ratio of colonies ($+O_2/-O_2$)	Cytochrome *b* content (nmol/mg)	Oxidase activity	
			TMPD (nmol/min/mg)	Q_1H_2 (nmol/min/mg)
ST2592/pHN3795-1	1.00	0.49±0.06	10±5	104±11
ST2592/pHNchi5	0.91	0.20±0.05	19±7	13±6
ST2592/pHNchi6	0.94	0.34±0.07	31±9	57±15
ST2592/pHNchi2-1	0	–	–	–

with the anti-cytochrome caa_3 serum, besides the 38 kDa band (CaaA) of the purified cytochrome caa_3 (lane E). The absence of a cross-reaction with the ST2592/pHNchi6 membranes suggests that the antigenic group(s) is localized in the C-terminal third of the cupredoxin fold or the cytochrome *c* domain of CaaA.

Growth Phenotypes of ST2592 Transformants—In our previous studies (*27*), the number of aerobic transformants of ST2592 with pHNchi, in which the putative C-terminal hydrophilic domain of CyoA was completely replaced with the Cu_A-cytochrome *c* domain of CaaA, was unexpectedly low. Thus, we examined the ratio of aerobic *vs.* anaerobic transformants with the new constructs, and found that the values for pHNchi5 and pHNchi6, 0.91 and 0.94, respectively, were slightly smaller than the 1.0 of the wild-type control with pHN3795-1 (Table I). The doubling times of ST2592/pHNchi5 and ST2592/pHNchi6 are about 2-fold and 1.5-fold greater than that of ST2592/pHN3795-1, respectively, indicating that the expression level and/or specific activity of the chimeric enzymes determine the aerobic growth rate. On the contrary, ST2592 without transformation or ST2592 transformed with pHNchi2-1 (ST2592/pHNchi2-1) gave few colonies (*i.e.*, <0.001) under aerobic growth conditions. Unlike the subunit II-I-III fusion of the native subunits (*36*), the subunit II-I fusion with the Cu_A-cytochrome *c* domain of CaaA seems to result in a nonfunctional enzyme.

Spectroscopic Properties of Chimeric Oxidases—Redox difference spectra of the solubilized membranes from ST2592/pHNchi5 and ST2592/pHNchi6 showed a broad α peak at 560 nm (data not shown), characteristics of low-spin heme *b* of wild-type cytochrome *bo*, as shown for ST2592/pHN3795-1 (*27, 30*). Although the contributions of cytochrome b_{556} of succinate dehydrogenase and cyto-

chrome *bd*-II are not negligibly low, the contents of total *b*-type cytochromes were estimated from the redox difference spectra as an indicator of the expression levels of the chimeric oxidases, and were found to be reduced to 41 and 69% of that of ST2592/pHN3795-1 in ST2592/pHNchi5 and /pHNchi6, respectively (Table I). It is also noteworthy that unlike ST2592/pHNchi (*27*), we found no shoulder at around 550 nm in the redox difference spectrum and no heme-staining on SDS-PAGE gels (data not shown), indicating the absence of *c*-type cytochrome.

High-spin heme *o* of the chimeric oxidases was examined by means of CO-binding spectra of the solubilized membranes (Fig. 3). ST2592/pHN3795-1, ST2592/pHNchi5, and ST2592/pHNchi6 gave essentially the same CO-binding spectra with a peak at 416 nm and a trough at 430 nm, indicating that there is no effect on high-spin heme *o* in subunit I. The expression levels of cytochrome *bo* estimated as the heme *o* content were 0.15, 0.06, and 0.12 nmol/mg protein, respectively.

Enzymatic Activities of Chimeric Oxidases—The Q_1H_2 oxidase activity of the crude membranes from ST2592/pHNchi5 or /pHNchi6 was only 13 and 55%, respectively, of that of ST2592/pHN3795-1 (Table I). On the contrary, TMPD oxidase activity was very low even in the membranes from ST2592/pHNchi5 and ST2592/pHNchi6 in comparison with that of the *Bacillus* caa_3-type enzyme, but still not negligible in comparison to that of ST2592/pHN3795-1 (Table I). The specific activities of crude membranes should be related to the expression levels of the chimeric enzymes and turnover numbers of the chimeric enzymes. The amounts of chimeric enzymes can directly be estimated by means of CO-difference spectra as the CO-reacting heme *o* content (Fig. 3).

The turnover numbers of chimeric oxidases were compared with those of cytochromes *bo* and *bd* (Fig. 4), where the sensitivity to 0.2 mM NaCN is also shown. Cytochrome *bo* is known to be two-orders of magnitude more sensitive to cyanide than cytochrome *bd* (*37*), which might be induced under selective pressure during aerobic growth as the alternative *bd*-type quinol oxidase (cytochrome *bd*-II; AppBC) (*38, 39*). In this experiment, cells (ST2592/pHNchi5 and ST2592/pHNchi6) were grown from single colonies which gave typical results in Table I, respectively, to obtain mean values as in the table. The turnover numbers of ST2592/pHNchi5 and ST2592/pHNchi6 were about 28 and 65% compared to ST2592/pHN3795-1 with 0.20 mM Q_1H_2, and the activities were mostly inhibited by 0.2 mM cyanide. The chimeric oxidase, especially that from ST2592/pHNchi6, seem to retain a part of the Q_L site and the electron transfer pathway to heme *b* in subunit I. In contrast to ST4676 expressing only cytochrome *bd*, at least half of the TMPD oxidase activity of ST2592/pHNchi5 and ST2592/pHNchi6 was cyanide-sensitive as in the case of the wild-type cytochrome *bo* of ST2592/pHN3795. The turnover numbers for the cyanide-sensitive TMPD oxidation by the chimeric oxidases were 3- to 5-fold higher than that of the wild-type cytochrome *bo* of ST2592/pHN3795-1, but below 5% of that of the purified cytochrome caa_3 (*24*), indicating that the TMPD oxidation at Cu_A and/or subsequent electron transfer to low-spin heme *b* are unfavorable in the chimeric enzymes. Similar results were obtained with yeast cytochrome *c* as the substrate, but the turnover numbers of chimeric enzymes were about 1/5 of that in the case of TMPD oxidation.

The quinol oxidase activity of the chimeric oxidases was further analyzed kinetically. For ST2592/pHNchi6, the V_{max} value, 140 s^{-1}, was almost identical to that of the

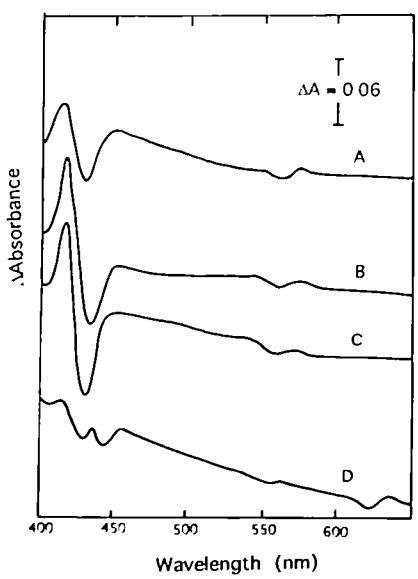

Fig. 3. **CO-difference spectra of crude membranes containing the chimeric oxidase.** The membranes were solubilized with 2% Triton X-100 containing 0.2 M Tris-HCl, pH 8.0, and CO-reduced minus reduced difference spectra were taken at room temperature. A, ST2592/pHNchi5 (5.6 mg protein/ml); B, ST2592/pHNchi6 (6.0 mg protein/ml); C, ST2592/pHN3795-1 (5.1 mg protein/ml); D, ST4676 (5.7 mg protein/ml).

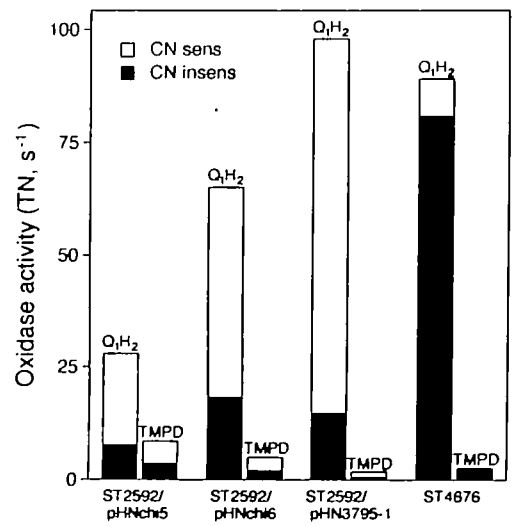

Fig. 4. **Cyanide sensitivity of the Q_1H_2 and TMPD oxidase activities of crude membranes isolated from ST2592/pHNchi5, ST2592/pHNchi6, ST2592/pHN3795-1, and ST4676.** For the activity, the cyanide-insensitive portions are shadowed. The TMPD and Q_1H_2 oxidase activities, measured at 562 nm in 20 mM potassium phosphate (pH 6.0) containing 1 mM EDTA, and at 275 nm in 50 mM Tris-HCl buffer (pH 7.1) containing 0.1% sucrose monolaurate, respectively, are normalized as to the cytochrome *o* content. The final concentrations of cyanide, TMPD, and Q_1H_2 were 0.2, 0.25, and 0.2 mM, respectively.

wild-type control (*i.e.*, ST2592/pHN3795-1), whereas the K_m value for Q_1H_2 increased to 220 μM from the control value, 48 μM, indicating a structural change at the Q_L site. In contrast, both the K_m and V_{max} values for ST2592/pHNchi5 changed, to 340 μM and 63 s^{-1}, respectively, indicating that the chimeric oxidase is perturbed not only in sustrate-binding but also in internal electron transfer.

Conclusion and General Discussion—In our previous study (*27*), all the C-terminal periplasmic domain of CyoA including the connecting region (*i.e.*, Lys109-Glu121; see Fig. 1) between transmembrane helix II and the cupredoxin fold was replaced with the counterpart of CaaA containing the Cu_A-cytochrome *c* domain. ST2592 transformants with pHNchi were generally unable to grow aerobically, but we could isolate a few aerobic clones. One of them showed appreciable TMPD oxidase activity and heme *c*-binding to the chimeric subunit II, indicating electron transfer from reduced TMPD to low-spin heme *b* through Cu_A (*27*). Conversion of this chimeric enzyme to the subunit II-I fusion protein (*i.e.*, pHNchi2-1) did not alter the phenotypes (Table I).

Of our new constructs, pHNchi5 was designed to include the connecting region of CyoA compared to pHNchi (*27*), whereas pHNchi6 was constructed to introduce only the Cu_A ligand domain of CaaA (Fig. 1). These attempts were successful, and we were able to demonstrate the aerobic growth and cyanide-sensitive Q_1H_2 oxidase activities of the chimeric oxidases (Table I and Fig. 4). The kinetic analysis of the Q_1H_2 oxidation by the chimeric oxidases revealed the importance of both the connecting region and the α_1-α_2-β_{11}-α_3 domain at the C-terminus (Qox domain in Ref. *15*) of CyoA. We found recently that five (Gln233His, Met248Ile, Ser258Asn, Phe281Ser, and His284Pro) of seven ubiquinone-related inhibitor-resistant mutations are localized in the Qox domain (*15*). It should be noted that the K_m mutation at Trp136 was found next to the connecting region (*14*). Thus, the connecting region and the Qox domain may be a part of the Q_L site or provide physical inter-subunit interactions that favor electron transfer from the Q_L site to heme *b*. These results provide further support for our proposal that the Cu_A end of the cupredoxin fold and the quinol oxidase specific (Qox) domain provides the Q_L site and is involved in electron transfer to the metal centers in subunit I (*15*). Since the bound ubiquinone at the Q_H site is essential for the quinol oxidation at the Q_L site (*16, 18, 19*), the Q_H site may be located in transmembrane helices of subunit II and/or subunit I.

On the basis of the results of phylogenetic analyses of subunits I and II of the heme-copper terminal oxidases, similarities in operon structures and arrangements of transmembrane helices, it was proposed that eubacterial SoxM-type quinol oxidases including *E. coli* cytochrome *bo* have evolved from cytochrome *c* oxidase of Gram-positive bacteria like *Bacillus* (*40*). The close similarity in the primary sequences of subunit II suggests that such changes should not be so drastic. Cytochrome *c* oxidase of the present thermophilic *Bacillus* may have a protein structure that is still convertible to that of quinol oxidase. The poor TMPD and cytochrome *c* oxidase activities of the chimeric oxidases may be related to the amount of the fully and functionally assembled Cu_A, or perturbation of the TMPD oxidation at Cu_A and/or subsequent electron transfer to heme *b* in these constructs. Such possibilities can be examined by detailed analyses of the purified chimeric oxidases.

In conclusion, through the construction and characterization of chimeric oxidases, we again realized the importance of the connecting region and the quinol oxidase domain of CyoA in the oxidation of ubiquinols. Defining the functional residues of the Q_L site of quinol oxidases will reveal not only a unique molecular mechanism for quinone redox chemistry in bacterial quinol oxidases but also provide a clue as to molecular evolution of eubacterial quinol oxidases from the SoxM-type cytochrome *c* oxidase.

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REFERENCES

1. Mogi, T., Tsubaki, M., Hori, H., Miyoshi, H., Nakamura, H., and Anraku, Y. (1998) Two terminal quinol oxidase families in *Escherichia coli*: Variations on molecular machinery for dioxygen reduction. *J. Biochem. Mol. Biol. Biophys.* **2**, 79-110
2. García-Horsman, J.A., Barquera, B., Rumbley, M.J., and Gennis, R.B. (1995) The superfamily of heme-copper respiratory oxidases. *J. Bacteriol.* **176**, 5587-5600
3. Sone, N. and Yanagita, Y. (1982) A cytochrome aa_3-type terminal oxidase of a thermophilic bacterium. Purification, properties and proton pumping. *Biochim. Biophys. Acta* **682**, 216-226
4. Chepuri, V., Lemieux, L., Au, D.C.-T., and Gennis, R.B. (1990) The sequence of the *cyo* operon indicates substantial structural similarities between the cytochrome *o* ubiquinol oxidase of *Escherichia coli* and the aa_3-type family of cytochrome *c* oxidases. *J. Biol. Chem.* **265**, 11185-11192
5. Nakamura, H., Yamato, I., Anraku, Y., Lemieux, L., and Gennis, R.B. (1990) Expression of *cyoA* and *cyoB* demonstrates that the CO-binding heme component of the *Escherichia coli* cytochrome *o* complex is in subunit I. *J. Biol. Chem.* **265**, 11193-11197
6. Sone, N., Shimada, S., Ohmori, T., Souma, Y., Gonda, M., and Ishizuka, M. (1990) A fourth subunit is present in cytochrome *c* oxidase from the thermophilic bacterium PS3. *FEBS Lett.* **262**, 249-252
7. Ishizuka, M., Machida, K., Shimada, S., Mogi, A., Tsuchiya, T., Ohmori, T., Souma, Y., Gonda, M., and Sone, N. (1990) Nucleotide sequence of the gene coding for four subunits of cytochrome *c* oxidase from the thermophilic bacterium PS3. *J. Biochem.* **108**, 866-873
8. Kita, K., Konishi, K., and Anraku, Y. (1984) Terminal oxidases of *Escherichia coli* aerobic respiratory chain I. Purification and properties of cytochrome b_{562}-*o* complex from cells in the early exponential phase of aerobic growth. *J. Biol. Chem.* **259**, 3368-3374
9. Haltia, T., Saraste, M., and Wikström, M. (1991) Subunit III of cytochrome *c* oxidase is not involved in proton translocation: A site-directed mutagenesis study. *EMBO J.* **10**, 2015-2021
10. Ludwig, B. and Schatz, G. (1980) A two-subunit cytochrome *c* oxidase (cytochrome aa_3) from *Paracoccus denitrificans*. *Proc. Natl. Acad. Sci. USA* **77**, 196-200
11. Sato-Watanabe, M., Mogi, T., Miyoshi, H., Iwamura, H., Matsushita, K., Adachi, O., and Anraku, Y. (1994) Structure-function studies on the ubiquinol oxidation site of the cytochrome *bo* complex from *Escherichia coli* using *p*-benzoquinones and substituted phenols. *J. Biol. Chem.* **269**, 28899-28907
12. Welter, R., Gu, L.-Q., Yu, L., Yu, C.-A., Rumbley, J., and Gennis, R.B. (1994) Identification of the ubiquinone-binding site in the cytochrome bo_3-ubiquinol oxidase of *Escherichia coli*. *J. Biol. Chem.* **269**, 28834-28838
13. Tsatsos, P.H., Raynolds, K., Nickels, E.F., He, D.-Y., Yu, C.-A., and Gennis, R.B. (1998) Using matrix assisted laser desorption ionization mass spectroscopy to map the quinol binding site of cytochrome bo_3 from *Escherichia coli*. *Biochemistry* **37**, 9884-9888

14. Ma, J., Puustinen, A., Wikström, M., and Gennis, R.B. (1998) Tryptophan-136 in subunit II of cytochrome bo_3 from *Escherichia coli* may participate in the binding of ubiquinol. *Biochemistry* **37**, 11806-11811
15. Sato-Watanabe, M., Mogi, T., Miyoshi, H., and Anraku, Y. (1998) Isolation and characterizations of quinone analogue-resistant mutants of *bo*-type ubiquinol oxidase from *Escherichia coli*. *Biochemistry* **37**, 12744-12752
16. Sato-Watanabe, M., Mogi, T., Ogura, T., Kitagawa, T., Miyoshi, H., Iwamura, H., and Anraku, Y. (1994) Identification of a novel quinone-binding site in the cytochrome *bo* complex from *Escherichia coli*. *J. Biol. Chem.* **269**, 28908-28912
17. Sato-Watanabe, M., Itoh, S., Mogi, T., Matsuura, K., Miyoshi, H., and Anraku, Y. (1995) Stabilization of a semiquinone radical at the high affinity quinone binding site of the *Escherichia coli bo*-type ubiquinol oxidase. *FEBS Lett.* **374**, 265-269
18. Sato-Watanabe, M., Mogi, T., Miyoshi, H., and Anraku, Y. (1998) Characterization and functional role of the Q_H site of *bo*-type quinol oxidase from *Escherichia coli*. *Biochemistry* **37**, 5356-5361
19. Mogi, T., Sato-Watanabe, M., Miyoshi, H., and Orii, Y. (1999) Role of a bound ubiquinone on reactions of the *Escherichia coli* cytochrome *bo* with ubiquinol and dioxygen. *FEBS Lett.*, in press
20. Witting, P., Källebring, B., and Malmström, B.G. (1994) The cupredoxin fold is found in the soluble Cu_A and CyoA domains of two terminal oxidases. *FEBS Lett.* **349**, 286-288
21. Kelly, M., Lappalainen, P., Talbo, R., Haltia, T., van der Oost, J., and Saraste, M. (1994) Two cysteines, two histidines, and one methionine are ligands of a binuclear purple copper center. *J. Biol. Chem.* **268**, 16781-16787
22. van der Oost, J., Lappalainen, P., Lemieux, L., Rumbley, J., Gennis, R.B., Aasa, R., Pascher, T., Malmström, B., and Saraste, M. (1992) Restoration of a lost metal-binding site: Construction of two different copper sites into a subunit of the *E. coli* cytochrome *o* quinol oxidase complex. *EMBO J.* **11**, 3209-3217
23. Wilmanns, M., Lappalainen, P., Kelly, M., Sauer-Eriksson, E., and Saraste, M. (1995) Crystal structure of the membrane-exposed domain from a respiratory quinol oxidase complex with an engineered dinuclear copper center. *Proc. Natl. Acad. Sci. USA* **92**, 11955-11959
24. Tashiro, H. and Sone, N (1995) Preparation and characterization of the hydrophilic Cu_A-cytochrome domain of subunit II of cytochrome *c* oxidase from the thermophilic *Bacillus* PS3. *J. Biochem.* **117**, 521-526
25. Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) Structure at 2.8 Å resolution of cytochrome *c* oxidase from *Paracoccus denitrificans*. *Nature* **376**, 660-669
26. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1996) The whole structure of the 13-subunit oxidized cytochrome *c* oxidase at 2.8 Å. *Science* **272**, 1136-1144
27. Uchida, A., Kusano, T., Mogi, T., Anraku, Y., and Sone, N. (1997) Expression of the *Escherichia coli bo*-type ubiquinol oxidase with a chimeric subunit II having the Cu_A-cytochrome *c* domain from the thermophilic *Bacillus* caa_3-type cytochrome *c* oxidase. *J. Biochem.* **122**, 1004-1009
28. Mogi, T., Hirano, T., Nakamura, H., Anraku, Y., and Orii, Y. (1995) Cu_B promotes both binding and reduction of dioxygen at the heme-copper binuclear center in the *Escherichia coli bo*-type ubiquinol oxidase. *FEBS Lett.* **370**, 259-263
29. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, second edition, Cold Spring Harbor Press, Cold Spring Harbor, NY
30. Minagawa, J., Mogi, T., Gennis, R.B., and Anraku, Y. (1992) Identification of heme and copper ligands in subunit I of the cytochrome *bo* complex in *Escherichia coli*. *J. Biol. Chem.* **267**, 2096-2104
31. Davis, B.D. and Mingioloi, E.S. (1959) Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. *J. Bacteriol.* **60**, 17-28
32. Kawasaki, M., Mogi, T., and Anraku, Y. (1997) Substitutions of charged amino acid residues conserved in subunit I perturb the redox metal centers of the *Escherichia coli bo*-type ubiquinol oxidase. *J. Biochem.* **122**, 422-429
33. Tsubaki, M., Mogi, T., Anraku, Y., and Hori, H. (1993) Structure of heme-copper binuclear center of the cytochrome *bo* complex of *Escherichia coli*: EPR and Fourier-transform infrared spectroscopic studies. *Biochemistry* **32**, 6065-6072
34. Sakamoto, K., Miyoshi, H., Takegami, K., Mogi, T., Anraku, Y., and Iwamura, H. (1996) Probing substrate binding site of the *Escherichia coli* quinol oxidases using synthetic ubiquinol analogues. *J. Biol. Chem.* **271**, 29897-29902
35. Tsubaki, M., Hori, H., Mogi, T., and Anraku, Y. (1995) Cyanide-binding site of *bd*-type ubiquinol oxidase from *Escherichia coli*. *J. Biol. Chem.* **270**, 28565-28569
36. Ma, J., Lemieux, L., and Gennis, R.B. (1993) Genetic fusion of subunits I, II, III of the cytochrome *bo* ubiquinol oxidase from *Escherichia coli* results in a fully assembled and active enzyme. *Biochemistry* **32**, 7692-7697
37. Kita, K., Konishi, K., and Anraku, Y. (1984) Terminal oxidases of *Escherichia coli* aerobic respiratory chain. II. Purification and properties of cytochrome b_{558}-*d* complex from cells grown with limited oxygen and evidence of branched electron-carrying systems. *J. Biol. Chem.* **259**, 3375-3381
38. Dassa, J., Fsihi, H., Marck, C., Dion, M., Kieffer-Bontemps, M., and Boquet, P.L. (1991) A new oxygen-regulated operon in *Escherichia coli* comprises the genes for a putative third cytochrome oxidase and for pH 2.5 acid phosphatase (*appA*). *Mol. Gen. Genet.* **229**, 341-352
39. Sturr, M.G., Krulwich, T.A., and Hicks, D.B. (1996) Purification of a cytochrome *bd* terminal oxidase encoded by the *Escherichia coli app* locus from a *Δcyo Δcyd* strain complimented by genes from *Bacillus firmus* OF4. *J. Bacteriol.* **176**, 1742-1749
40. Castresana, J., Lübben, M., Saraste, M., and Higgins, D.G. (1994) Evolution of cytochrome oxidase, an enzyme older than atmospheric oxygen. *EMBO J.* **13**, 2516-2525