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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[1]

Akiko Uchida,* Teruo Kusano,* Tatsushi Mogi,† Yasuhiro Anraku,† and Nobuhito Sone*,[2]

*Department of Biochemical Engineering and Science, Kyushu Institute of Technology, 680-4 Kawazu, Iizuka, Fukuoka 820; and †Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113

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The C-terminal periplasmic domain of subunit II of the *Escherichia coli bo*-type ubiquinol oxidase was replaced with the counterpart of the thermophilic *Bacillus* caa_3-type cytochrome *c* oxidase containing the Cu_A-cytochrome *c* domain by means of gene engineering techniques. The chimeric terminal oxidase was expressed by a pBR322 derivative in a terminal oxidase-deficient mutant of *E. coli*, although the amount of the chimeric enzyme was smaller than that of the *Escherichia coli bo*-type ubiquinol oxidase expressed by the original cytochrome *bo*-expressing plasmid. The chimeric enzyme showed much higher TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) oxidase activity than the wild-type cytochrome *bo*, but lower activity than the thermophilic *Bacillus* caa_3-type cytochrome *c* oxidase. The chimeric subunit II was confirmed to bind to heme C. These results suggest that the Cu_A-cytochrome *c* domain grafted to this membrane anchor can facilitate electron transfer from reduced TMPD to low-spin protoheme *b* in subunit I.

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

The cytochrome *bo*-type ubiquinol oxidase from *Escherichia coli* and the cytochrome caa_3-type cytochrome *c* oxidase from thermophilic *Bacillus* PS3 are four-subunit terminal oxidases of the aerobic respiratory chain (*1-3*), and generate an electrochemical gradient of protons across the cytoplasmic membrane. The oxidases catalyze the four-electron reduction of dioxygen to water which is coupled to proton pumping reactions (*4, 5*). Subunits I, II, III, and IV of the oxidase complexes are encoded by the *cyoABCDE* operon (*6, 7*), and the *caaABCD* (*ctaCDEF*) operon (*3, 8*). The *cyoE* gene in the *cyo* operon and the *caaE* gene adjacent to the *caa* operon encode protoheme IX farnesyltransferase (heme O synthase), which supplies heme O essential for the high-spin heme binding site of cytochrome *bo* and for the biosynthesis of heme A (*9-11*). Subunits I, II, and III are homologous to the counterparts of a 13-subunit aa_3-type cytochrome *c* oxidase from bovine mitochondria (*6, 8*). Structural and spectroscopic studies indicated that they belong to a superfamily of heme-copper terminal oxidases (*12, 13*).

Subunit I of bacterial and mammalian enzymes binds to low-spin heme and a binuclear metal center, where high-spin heme and Cu_B are antiferromagnetically coupled, and functions as a reaction site for dioxygen reduction and proton pumping (*13-16*). Subunit I (CyoB) of the *E. coli* cytochrome *bo* binds to protoheme IX and heme O at the low-spin heme *a* site and high-spin heme a_3 site, respectively, of the counterpart (CaaB) of PS3 cytochrome caa_3, which ligates two heme A molecules. Subunit II (CyoA) of cytochrome *bo* contains a quinol oxidation site (*17*) in place of Cu_A, the binuclear copper center, of cytochrome *c* oxidase, which mediates electron transfer from reduced cytochrome *c* or TMPD to low-spin heme *a* in subunit I. Besides a low-affinity quinol-oxidation site (Q_L), a high-affinity quinone-binding site (Q_H) is involved in the two-electron transfer from quinols to low-spin protoheme, and can not only stabilize the ubisemiquinone radical but also accept two electrons as a bound form (*18-20*). On the other hand, subunit II (CaaA) of cytochrome caa_3 contains a cytochrome *c* domain that is fused to the C-terminus of the Cu_A domain (*8, 21*). The C-terminal hydrophilic domain of subunit II of both enzymes has the Greek key β-barrel structure, as found for blue-copper proteins (*22, 23*), which seems to be derived from the purple-copper center of nitrous oxide reductase in denitrifying bacteria (*24*). Saraste and colleagues succeeded in the restoration of a bimetallic purple-copper (*i.e.*, Cu_A) site and a monometallic blue-copper site in CyoA by introducing the putative metal

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[2] To whom correspondence should be addressed. Tel: +81-948-29-7813, Fax: +81-948-29-7801, E-mail: sone@bse.kyutech.ac.jp

Abbreviations: Caa, cytochrome caa_3; Cyo, cytochrome *bo*; Cyt, cytochrome; Q_1H_2, ubiquinol-1; Q_H, high-affinity quinone-binding site; Q_L, low-affinity quinol-oxidation site; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

ligands conserved in cytochrome *c* oxidases and nitrous oxide reductases and those in blue-copper proteins, respectively (*25, 26*). Introduction of the Cu_A site into CyoA resulted in a non-functional enzyme and perturbed the $g=3$ signal of low-spin protoheme, suggesting an interaction between the Cu_A domain and low-spin protoheme in subunit I (*25*). Tryptic digestion of cytochrome caa_3 cut CaaA into the N-terminal transmembrane domain and the C-terminal hydrophilic domain, but did not affect the cytochrome *c* oxidase activity (*21*). 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 (*27, 28*). Subunits III (CyoC) and IV (CyoD, CaaD) are not required for catalytic functions (*3, 29*), but are indispensable for binding of the metal centers (*30-32*).

In order to explore the physical interactions that ensure inter-subunit electron transfer, and to localize the substrate oxidation site, we designed a chimeric terminal oxidase of which almost all the C-terminal hydrophilic domain of CyoA was replaced with the counterpart of CaaA containing the Cu_A-cytochrome *c* domain. Expression of the chimeric oxidase suppressed a defect of the aerobic growth of a terminal oxidase-deficient *E. coli* mutant. The chimeric oxidase in the membranes showed that TMPD oxidase activity and the recovery of the catalytic activity were associated with folding of the cytochrome *c* domain.

EXPERIMENTAL PROCEDURES

Construction of a Chimeric Oxidase—Multicopy vector pHN3795-1 carries the *cyoABCDE* operon on pBR322 and can overproduce cytochrome *bo* 4-fold higher than the chromosomal copy (*33*). For replacement of the periplasmic domain of CyoA, *Eco*RV and *Sac*II sites were introduced in the *cyoA* gene on pHN3795-1 by oligonucleotide-directed site-specific mutagenesis using a Sculptor *in vitro* mutagenesis system (Amersham). Subsequently, the 705-bp *Eco*RV-*Sac*II fragment of the resultant plasmid was replaced by the 722-bp *Aat*II-*Sph*I fragment of the *caaABCD* operon (*8*) on pUCcaa-5, a derivative of pUC118 (T. Kusano, unpublished results). The resultant plasmid, pHNchi, encodes a chimeric subunit II, in which the Cu_A-cytochrome *c* domain (Asp^{119}-Glu^{356}) of CaaA is connected to Leu^{106} of the second transmembrane helix of CyoA through a gene-engineered dipeptide "Thr-Trp." It was used for anaerobic transformation of an *E. coli* terminal oxidase-deficient mutant, ST2592 (*Δcyo::cat Δcyd::kan*) (*34*), which lacks both *bo*- and *bd*-type ubiquinol oxidases, and is defective in aerobic growth on non-fermentable carbon sources. Transformants were obtained using 2×YT plates supplemented with 50 μg/ml ampicillin and 30 μg/ml chloramphenicol. DNA manipulations were carried out according to Sambrook *et al.* (*36*). Strain ST4676 (*Δcyo::cat cyd*$^+$) (*35*) was used as a reference strain which expresses *bd*-type quinol oxidase as a sole terminal oxidase.

Cell Culture and Measurement of Respiration—A 2 ml aliquot of an overnight culture was inoculated into 200 ml of minimal medium A (*37*) supplemented with 1% polypeptone, 0.5% yeast extract, 0.5% casamino acids, 1% glycerol, 20 μg/ml ampicillin, and 6 μg/ml chloramphenicol in a 1 litter flask. *E. coli* cells were grown aerobically at 37°C with shaking at 160 rpm. It should be noted that the preculture for strain ST2592/pHNchi was performed anaerobically in a test tube, with a tight plastic cap, containing 6 ml of the above medium, and then a large-scale culture was performed without shaking until the A_{650} reached about 0.5. The cells were harvested at the late log phase of growth by centrifugation, washed with 50 mM NaCl-containing 5 mM potassium phosphate buffer, pH 7.1, and then suspended in the same buffered saline. The respiration of cells was polarographically followed using a Yellow Springs No. 4005 oxygen electrode in a semi-closed vessel (3.0 ml) containing a reaction medium of 50 mM sodium phosphate buffer, pH 7.1.

Preparation of Membrane Vesicles—*E. coli* cells (2 g wet weight) were suspended in 10 ml of 20% sucrose containing 30 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.1-0.2 mg/ml egg white lysozyme. After 30 min incubation on ice, spheroplasts were recovered by centrifugation and resuspended in 2 ml of 20% sucrose containing 3 mM EDTA (pH 7.4). The spheroplasts were disrupted by sonication, and the crude membranes were recovered by ultracentrifugation at 144,000×*g* for 30 min.

Analytical Procedures—The TMPD and ubiquinol-1 (Q_1H_2) oxidase activities were spectrophotometrically measured in 20 mM sodium phosphate buffer (pH 6.0) containing 1 mM EDTA, using millimolar extinction coefficients of 10.5 at 562 nm (*21*), and 8.5 at 278.5-251.4 nm, respectively. The former activity was measured with a single-beam spectrophotometer (Beckman DU-70) at 25°C, and the latter activity with a double-beam spectrophotometer (Hitachi 556) at 35°C. The final concentration of TMPD was 0.25 mM. Q_1H_2 was prepared from ubiquinone-1 by reduction with a tiny granule of $Na_2S_2O_4$, excess $Na_2S_2O_4$ being decomposed by air, and the final substrate concentration was 0.2 mM. The contents of *b*-type and *c*-type cytochromes were calculated using the following millimolar extinction coefficients of redox difference spectra: 18.7 at 560-580 nm for the *E. coli* cytochrome *bo* (*9*), and 21.2 at 550-541 nm for cytochrome *c* in the PS3 cyto-chrome *c* oxidase (*5*), respectively, as described previously. Protein concentration determination, sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), heme staining for detection of heme C-attached proteins after SDS-PAGE, and Western blotting analysis for detection of subunit II of cytochrome caa_3 were carried out as described previously (*21*).

Materials—The wild-type cytochrome *bo* from *E. coli* (*38*) and cytochrome caa_3 from thermophilic *Bacillus* PS3 (*3*) were purified as described previously. Other materials were obtained as described previously (*21*).

RESULTS AND DISCUSSION

Expression of the Chimeric Oxidase in a Terminal Oxidase-Deficient Mutant—We constructed a chimeric ubiquinol oxidase, in which the C-terminal hydrophilic domain of CyoA from cytochrome *bo* was replaced with the Cu_A-cytochrome *c* domain of CaaA from PS3 cytochrome caa_3. The chimeric oxidase was expressed by pHNchi in a terminal oxidase-deficient mutant, ST2592 (*Δcyo Δcyd*), and was able to suppress the defect in the aerobic growth on non-fermentable carbon sources, although its effect was weaker than that of the wild-type plasmid, pHN3795-1

(*cyo*$^+$). Such cells showed a characteristic respiration pattern, which is different from that of cells expressing cytochrome *bo* or cytochrome *bd* as the terminal oxidase (Fig. 1). The endogenous respiration of ST2592/pHNchi was accelerated by the addition of TMPD (with ascorbate), but was severely inhibited by 0.2 mM KCN (line A), while ST2592/pHN3795-1 showed KCN-sensitive endogenous respiration without TMPD acceleration (line B), and ST4676 (*Δcyo cyd*$^+$) showed cyanide-resistant (at least at 0.2 mM) respiration without TMPD acceleration (line C). Western blotting analysis of the membranes with an anti-cytochrome caa_3 antiserum (*21*) confirmed the presence of the chimeric subunit as a 37 kDa band (lane F of Fig. 2). The band for the membranes of ST4676 (*Δcyo cyd*$^+$)/pHNchi was feasible (lane B), and ST2592/pHN3795-1 expressing the native cytochrome *bo* (Cyo) and ST4676 (*Δcyo cyd*$^+$) gave no band (lanes A and C, respectively).

The aerobic growth of ST2592/pHNchi was severely impaired and the generation time in the glycerol medium was about 7 h or more, *i.e.* over 8-fold longer than those of ST2592/pHN3795-1 and a cytochrome *bo*-deficient mutant, ST4676 (*Δcyo cyd*$^+$). It is also noteworthy that colony formation of ST2592/pHNchi took in almost 5 days under aerobic growth conditions in contrast to the 1.5 days for its anaerobic transformation. These results indicate that ST2592/pHNchi grows very slowly under aerobic conditions, and grows at a moderate velocity only after the cells have detoxified dissolved dioxygen within the colonies. It is thus likely that the Cu_A-cytochrome *c* domain introduced into CyoA has deleterious effects on its biosynthesis or the catalytic function of the chimeric oxidase.

Characterization of the Chimeric Oxidase in Membranes—We obtained redox difference spectra of the solubilized crude membranes at room temperature and examined spectroscopic properties of the chimeric cytochrome *bo* (Fig. 3). ST2592/pHN3795-1 gave a pronounced α peak at around 560 nm due to low-spin heme *b* of the wild-type enzyme, but *c*-type cytochrome was absent in the wild-type membranes (line A). ST2592/pHNchi exhibited an increased 550-nm peak attributable to the cytochrome *c* component of cytochrome caa_3 (line B), while ST4676 showed an α peak at around 560 nm again (line C). The amount of *c*-type cytochrome was a little less than those of *b*-type cytochromes, *i.e.* low-spin heme *b* of the chimeric oxidase and cytochrome b_{556} of succinate dehydrogenase, and may be comparable to that of the former. The spectrum of ST4676/pHNchi membranes was very similar to that of ST4676, indicating that the amount of cytochrome *c* was very low, if any.

Table I summarizes the cytochrome contents and oxidase

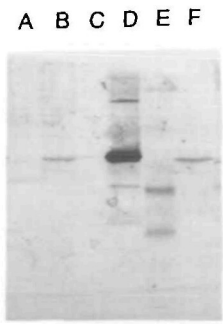


Fig. 2. **Western blotting analysis of crude membranes of transformants for the PS3 cytochrome c oxidase.** SDS-PAGE was carried out using 40 μg protein of membranes per lane. A, ST2592/pHN3795-1; B, ST4676/pHNchi; C, ST4676; D, PS3 cytochrome caa_3 (1.0 μg protein); E, pre-stained molecular mass standards (27 and 19 kDa); F, ST2592/pHNchi.

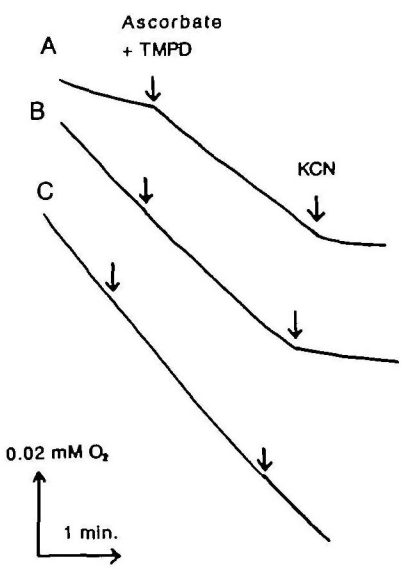


Fig. 1. **Effects of ascorbate-TMPD addition and cyanide-inhibition on whole cell respiration.** The oxygen concentration in the medium (50 mM NaCl containing 5 mM potassium phosphate buffer, pH 7.1) was polarographically measured. Cells (0.2–0.3 mg dry weight) were harvested at the log phase and washed with the above medium. The final concentrations of sodium ascorbate, TMPD, and KCN were 5.0, 0.25, and 0.20 mM, respectively. A, ST2592/pHNchi; B, ST2592/pHN3795-1; C, ST4676.

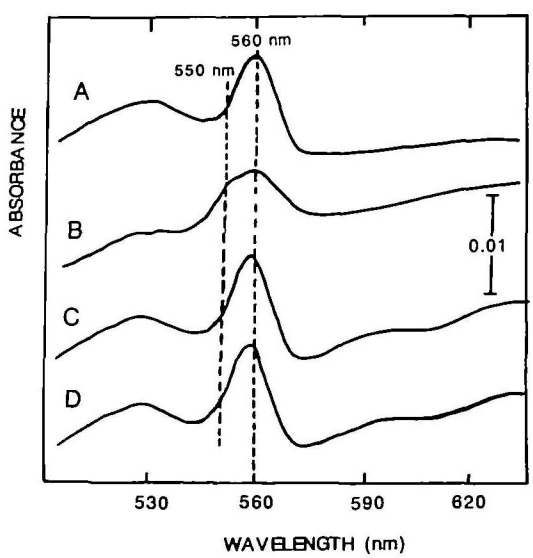


Fig. 3. **Redox difference spectra of crude membranes containing the chimeric oxidase.** The crude membranes were solubilized with 2% Triton X-100 containing 0.2 M Tris-HCl, pH 8.0, and then dithionite-reduced *minus* air-oxidized difference spectra were obtained at room temperature. A, ST2592/pHN3795-1 (1.0 mg protein/ml); B, ST2592/pHNchi (clone 5) (2.5 mg protein/ml); C, ST4676 (2.3 mg protein/ml); D, ST4676/pHNchi (2.7 mg protein/ml).

activities of one clone, ST2592/pHNchi (named clone 5), which showed relatively high-level expression of cytochrome *c*, as well as typical results for ST2592/pHN3795-1 and ST4676 expressing cytochromes *bo* and *bd*, respectively, as the sole terminal oxidase. The contents of both *c*-type and *b*-type cytochromes of ST2592/pHNchi (clone 5) were less than that of *b*-type cytochromes found in ST2592/pHN3795-1. However, the expressed chimeric oxidase seems to bind a stoichiometric amount of heme C at the C-terminus of subunit II, because the rest (about 0.04 nmol/mg protein) was due to the *b*-type cytochrome of succinate dehydrogenase. This expression of cytochrome *c* in ST2592/pHNchi was accompanied by a large increase in TMPD oxidase activity. Since this activity was quite sensitive to 0.2 mM KCN, like that of the wild-type cytochrome *bo* in ST2592/pHN3795-1, the activity cannot be attributed to an alternative *bd*-type ubiquinol oxidase encoded by the *app* operon (*39*). The activity of KCN-sensitive TMPD oxidation seems to be proportional to the amount of cytochrome *c* present in the transformant membranes. The presence of chimeric subunit II with Cu_A-cytochrome *c* with the expected molecular mass was also shown on Western blotting analyses after SDS-PAGE (Fig. 2). The turnover number of TMPD oxidase activity of the heme C-bound chimeric oxidase based on the amount of cytochrome *c* (0.07 nmol/mg protein) was 6.0 e^-/s, which is about 22% of that of the purified cytochrome caa_3 under the measurement conditions (*21*). The turnover number with Q_1H_2 was 20% of that of the purified cytochrome *bo* (Table I). Thus the chimeric cytochrome *bo* was shown to be functionally active as a cytochrome *c* oxidase, although the enzyme lost a part of its quinol oxidase activity. The low Q_1H_2 oxidase activity of the membranes from ST2592/pHNchi is consistent with its slow growth phenotype. The reason for the lower Q_1H_2 oxidase activity of the chimeric enzyme is not known at present, but it may be due to the absence of the low-affinity quinol-oxidation site (Q_L) of cytochrome *bo*. This site, with a K_m of 75 μM for Q_1H_2 (*19*), is probably not present in transmembrane helices I and II of the *E. coli* subunit II. Our recent studies on quinone analogue-resistant mutations in cytochrome *bo* (M. Sato-Watanabe, T. Mogi, H. Miyoshi, and Y. Anraku, unpublished results) also indicated that the site is not present in transmembrane helices I and II.

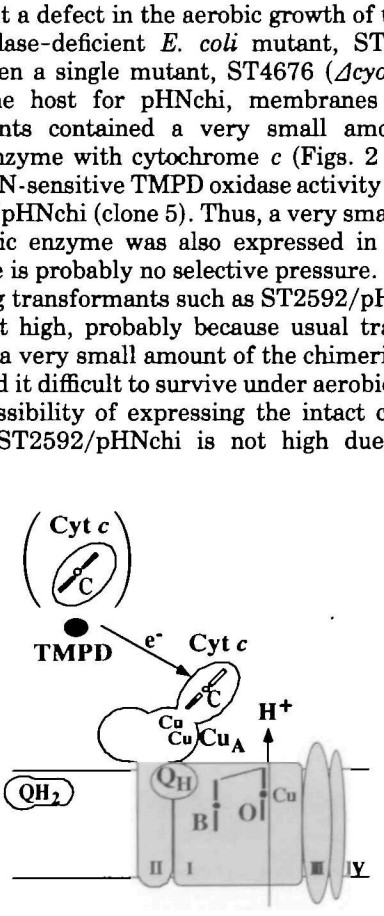
Conclusion and General Discussion—We succeeded in the partial conversion of ubiquinol oxidase to cytochrome *c* oxidase by replacement of the C-terminal soluble domain of subunit II (CyoA) from the *E. coli* cytochrome *bo* with the counterpart from the caa_3-type cytochrome *c* oxidase of thermophilic *Bacillus* PS3 (Fig. 4). The Cu_A-cytochrome *c* domain of cytochrome caa_3 fused to the membrane anchor of ubiquinol oxidase can confer the ability to mediate electron transfer from reduced TMPD to low-spin protoheme in subunit I. TMPD has successfully been used as a simple and efficient substrate for cytochrome *c* oxidase (*13, 21*). This inter-subunit electron transfer indicates the presence of a physical interaction of the Greek key β-barrel domain in subunit II with the redox metal center in subunit I even in the chimeric oxidase. However, the TMPD oxidase activity of the chimeric oxidase is not sufficiently high. Time-resolved electron transfer studies on the chimeric oxidase may provide a clue as to the mechanism underlying the inter-subunit electron transfer in heme-copper terminal oxidases. The residual Q_1H_2 oxidase activity of the chimeric oxidase seems to be attributable to the oxidation at the Q_H or Cu_A site. Therefore, analysis of bound Q_8 in the purified chimeric oxidase will provide a clue as to the location of the Q_H site in cytochrome *bo* for the molecular mechanism of intramolecular electron transfer at the substrate oxidation site of bacterial quinol oxidases.

We isolated ST2592/pHNchi (clone 5), which was able to complement a defect in the aerobic growth of the terminal quinol oxidase-deficient *E. coli* mutant, ST2592 (*Δcyo Δcyd*). When a single mutant, ST4676 (*Δcyo cyd*$^+$), was used as the host for pHNchi, membranes from most transformants contained a very small amount of the chimeric enzyme with cytochrome *c* (Figs. 2 and 3), and showed KCN-sensitive TMPD oxidase activity of about 1/4 of ST2592/pHNchi (clone 5). Thus, a very small amount of the chimeric enzyme was also expressed in ST4676, in which there is probably no selective pressure. The chances of obtaining transformants such as ST2592/pHNchi (clone 5) were not high, probably because usual transformants expressing a very small amount of the chimeric enzyme in ST2592 find it difficult to survive under aerobic conditions, and the possibility of expressing the intact chimeric enzyme for ST2592/pHNchi is not high due to protein

TABLE I. **Characterization of the chimeric oxidase in crude membranes.** The contents of cytochromes *c* and *b* were calculated from ascorbate (*plus* TMPD)-reduced *minus* oxidized, and $Na_2S_2O_4$-reduced *minus* oxidized difference spectra, respectively. Oxidase activities were measured as described under "MATERIALS AND METHODS."

Strain	Cytochrome content (nmol/mg)		Oxidase activity (nmol/nmol Cyt *b*/min)	
	Cyt *b*	Cyt *c*	TMPD	Q_1H_2
ST2592/pHN3795-1	0.57	<0.01	8.9(1.3)[a]	298
ST2592/pHNchi(clone 5)	0.11	0.07	228 (<1)	63
ST4676	0.23	<0.01	6.5(5.6)	211

[a]In the presence of 0.2 mM KCN.



Chimeric *bo*-type ubiquinol /cytochrome *c* oxidase

Fig. 4. **A model of the chimeric enzyme from *E. coli* cytochrome *bo* (Cyo), and PS3 cytochrome caa_3 (Caa).** The contributions from the *E. coli* cytochrome *bo* are dotted. Roman numerals are the numbers of the subunits. Chromophores, such as the binuclear oxygen-reducing site (O with Cu), the low-spin cytochrome site (B), the binuclear copper site (Cu_A), cytochrome *c* (Cyt *c*), and the high-affinity quinone-binding site (Q_H), are presented. Note that the low-affinity quinone-binding site (Q_L) has been removed.

degradation. On the other hand, by using this selective pressure of aerobic growth, we may select a transformant of ST2592 expressing a sufficient amount of the chimeric enzyme, as in the case of ST2592/pHN3797-1, if *E. coli* synthesizes enough heme C and transfers it to the apo-cytochrome *c*-moiety of the chimeric enzyme, which will give us a sufficient amount of the chimeric enzyme.

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