Discovery and reaction mechanism of the heme O synthase in *Escherichia coli*

大腸菌ヘムO合成酵素の発見と反応機構の研究

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Discovery and reaction mechanism of the heme O synthase in *Escherichia coli*

by

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Dedicated to

Jean-Nicolas-Arthur Rimbaud

and

Jaco Francis Pastorius III

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Abbreviations

kb	kilo base pairs
SDS	sodium dodecyl sulfate
IPTG	isopropyl-1-thio-β-D-galactopyranoside
HPLC	high performance liquid chromatography
FPP	farnesyl pyrophosphate
GPP	geranyl pyrophosphate
GGPP	geranylgeranyl pyrophosphate
amp	ampicillin
Cm	chloramphenicol
Km	kanamycin
tet	tetracycline
SDS-PAGE	SDS polyacrylamide gel electrophoresis
PMS	phenazine methosulfate
PMSF	phenylmethanesulfonyl fluoride
SM-1200	sucrose monolaurate-1200
ORF	open reading frame

Abstract of the dissertation

The cytochrome *bo* complex is the heme-copper terminal oxidase in the aerobic respiratory chain of *Escherichia coli* and functions as a redox-coupled proton pump. It contains three redox centers, the low spin heme B, the high spin heme O, and Cu_B, in subunit I and catalyzes the 2-electron oxidation of ubiquinol-8 and the 4-electron reduction of molecular oxygen. Heme O has been recently found in the cytochrome *bo* complex and assigned to be a protoheme IX derivative in which the vinyl group at pyrrole ring A is substituted by a 17-carbon hydroxyethylfarnesyl group. Heme O is related to heme A because of the hydroxyethylfarnesyl group, but it lacks the formyl group at pyrrole ring D as in protoheme IX. Despite of extensive genetic studies on the pathway and regulation of heme biosynthesis, only a little is known about heme A biosynthesis. Those unknown synthases of heme O and heme A are likely the key enzymes participating in assembly and expression of the oxidase complexes.

The genes (*cyoABCDE*) for the cytochrome *bo* complex have been cloned and sequenced. Subunits I-III of the *bo* complex have been assigned to be the products of the *cyoB, cyoA,* and *cyoC* genes, respectively, and are structurally related to the counterparts of the *aa*3-type cytochrome *c* oxidases in mitochondria and some aerobic bacteria. Subunit IV is likely to be a product of the *cyoD* gene, as demonstrated in some bacterial oxidases. On the contrary, the functional role of the *cyoE* gene is unknown. Since the *cyoE* gene homologues have been found not only in the genes for bacterial *aa*3- and *caa*3-type oxidases, but also in the yeast nuclear gene, the CyoE protein may play the essential role in the oxidase functions. To identify the functional role(s) of the *cyoE* gene, I constructed 40 alanine replacement and 6 deletion mutants of CyoE and, as a result, 29 oxidase-defective mutants were obtained. I found that cytoplasmic membranes of all the defective *cyoE* mutants showed abnormal red-shifted CO-binding spectra for the high-spin heme species. Then, I demonstrated that these observed spectroscopic alterations were likely due to a complete loss of heme O from the *bo* complex based on the results of heme analysis of partially purified *bo* complexes and cytoplasmic membranes of the *cyoE* deficient mutants. Furthermore, I showed that a deletion of the *cyo* operon from *E. coli* resulted in no production of heme O whereas over-expression of the *cyoE* gene had this deletion mutant accumulated heme O in the cytoplasmic membranes. Finally, I developed the *in vitro* heme O synthetic system and discovered that CyoE over-produced cytoplasmic

membranes effectively converted heme B to heme O in the presence of farnesyl diphosphate, divalent metal cations such as Mg^{2+} or Ca^{2+} and a reductant dithionite. I also found that the expression of a CyoE homologue, the CaaE protein of thermophilic *Bacillus* PS3, resulted in an increase of thermotorelant heme O synthetic activity in the cytoplasmic membranes in *Escherichia coli*. All the results definitively indicated that CyoE and CaaE proteins are a novel enzyme, heme O synthase that supplies heme O and heme A essential for functional expression of the terminal oxidases. My study clarifies the functional role of the CyoE protein family to the heme-copper terminal oxidase superfamily and postulates the biosynthetic route of prenylated hemes such as hemes O and A that had been covered for long time. Hence my study provides the first biochemical observations concerning the presence of the successive heme biosynthetic pathway from heme B.

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Chapter 1. General Introduction

Background

History of tetrapyrroles

Hemes are the iron-porphyrin compounds and play the central roles in biochemical oxidative reactions. More than 200 enzymes are known as hemoproteins, such as oxidoreductases, electron carriers and oxygen carriers. Tetrapyrrole macromolecules such as hemes, chlorophylls and vitamin B_{12} are derivatives of uroporphyrinogen III that is the first closed intermediates in heme B biosynthetic pathway (**Fig. I-1**). Variations of tetrapyrroles seem to reflect the results of adaptation in the evolution of life. It is generally accepted that aerobic respiration was organized by the production of oxygen, and the production of oxygen was formed by primitive photosynthesis, and the primitive photosynthesis was performed in primitive photosynthetic organisms whose life was perhaps guaranteed by porphyrins. Indeed, metalloporphyrins were identified in bituminous coal (Bonnett and Czechowski 1984). The emergence of life on primordial earth supported by the porphyrin-like compounds synthesized by biological systems and/or abiotic synthesis.

Because of the typical red color of blood and dark red urea of porphyria, heme B (or hemin, an oxidized form of heme B) attracted person's attention at first in the past. So the study of porphyrins had begun with heme B since heme B was first acid-extracted from blood in 1837 by Lecanu (Moore *et al.* 1990). Study of porphyria contributed largely to the study of heme B because the name "porphyria" meant "the lustrous purple-red crystalline porphyrins" in the first place until Garrod defined as "inborn errors of metabolism" in 1923. The correct structure of heme B was postulated by Küster in 1912 (Küster 1912) and proved by Fischer and Zeile in 1929 (**Fig. I-2**). The complex but attractive structure of heme B stimulated more the students' attention, as Claude Rimington said, "It is arguably true that the tetrapyrrole system is Nature's most remarkable creation".

In 1949, Lemberg and Legge suggested that an order of heme B biosynthesis was from monopyrrole to tetrapyrrole. This scheme and the more brilliant work in heme B biosynthesis were done by Shemin's and Neuberger's groups through 1940s-1950s. They showed glycine and succinyl-CoA as a precursor of heme B in human and animals by use of the classical technique of isotopic labeling. The expected next intermediate, 5-aminolevulinate (ALA) was proved as the first committed intermediate in heme B biosynthetic pathway and 2 molecules of ALA were turned to monopyrrole compound, porphobilinogen (PBG) in 1955 (Schmid and Schemin 1955, Gibson *et al.* 1955). PBG was accepted by its structural resemblance to hemes since PBG, first isolated from urea of acute intermittent porphyria (Wastall 1952), had been determined by X-ray crystallography (Kennard 1953). PBG was indicated as a precursor of porphyrins in 1953 (Falk *et al.* 1953). And so, early part of heme B biosynthesis was determined but later part was unknown yet. Attempts of dark red porphyrins extracted from urea of porphyria as a substrate for heme biosynthesis were, however, all failed until Bogorad suggested that chemically reduced and non-colored porphyrinogens were the real intermediate of heme biosynthetic pathway in 1955 (Bogorad 1955). Then, the times were shifted to analyses of each biosynthetic enzymes in the heme B biosynthetic pathway.

Biosynthetic enzymes in heme B biosynthesis

Almost all the enzymes for the heme B biosynthetic pathway were purified and well characterized biochemically by now (Jordan 1990, Dailey 1990, Jordan 1994). Gene analyses indicated that heme B biosynthesis is catalyzed by well conserved enzymes among all cells studied. Two routes were identified to synthesize ALA. The formation of ALA from glycine and succinyl-CoA determined by Shemin and Neuberger is now called the C-4 pathway and is catalyzed by ALA synthase that was purified in various cells since 1971. The energy source of the entire heme B biosynthetic pathway with the C-4 pathway is derived from succinyl-CoA formed in the citric acid cycle and, of course, ALA synthase is shown to localize in mitochondria of eukaryotes and the cytoplasm in eubacteria. On the other hand, Beale and Castelfranco found that L-glutamate is effectively converted to tetrapyrroles in higher plants in 1974 (Beale and Castelfranco 1974). Kannangara *et al.* showed the involvement of $tRNA^{glu}$ in this reaction (Huang *et al.* 1984, Schön *et al.* 1986) and the C-5 pathway was identified as successive three enzyme reactions by glutamate-tRNA ligase, glutamate-tRNA dehydrogenase and glutamate 1-semialdehyde aminotransferase. Glutamate 1-semialdehyde is supposed as the possible intermediate (Houen *et al.* 1984). Jordan *et al.*, however, suggested that its cyclic form, 2-hydroxy, 3-aminotetrahydropyran (HAT), is the true precursor for ALA (Jordan *et al.* 1990). The C-5 pathway involves the reduction of a carboxylic acid, so it requires energy to progress heme B formation from ATP (in step by glu-tRNA ligase) and NADPH (in step by glu-tRNA dehydratase). The C-5 pathway, distributed in chloroplast of higher plants, most eubacteria (including *Escherichia coli* and *Bacillus species*) and archaebacteria, is more common than the C-4 pathway in the biosphere. All the C-5 pathway enzymes are cytoplasmic enzymes in eubacteria. Distribution of the C-4 and C-5 pathways among cells correlates well with Woese's phylogenic tree (Avissar *et al.* 1989). This indicates that the C-5 pathway was the ancient process, whereas the C-4 pathway evolved later. Some photosynthetic organisms as well as, perhaps, higher plants and *Euglena gracilis* may, however, operate both pathways.

Biosynthesis from ALA to coproporphyrinogen III is catalyzed by four cytoplasmic or soluble enzymes, ALA dehydrogenase, PBG deaminase (PBGD), uroporphyrinogen III synthase (Uro III synthase) and uroporphyrinogen III decarboxylase (Uro D). All the enzymes were purified from various species and investigated well. Recently, *Escherichia coli* PBGD that catalyses the stepwise polymerization of four molecules of PBG was defined by X-ray analysis for the first time among the pathway enzymes in 1992 (Louie *et al.* 1992). All these enzymes were identified to localize in the cytoplasm.

In the next steps, again, two routes are postulated in biosynthesis from coproporphyrinogen III to protoporphyrin IX. The enzymes, coproporphyrinogen III oxidase (CPO) and protoporphyrinogen IX oxidase (PPO), that synthesize the intermediates, protoporphyrinogen IX and protoporphyrin IX, respectively, require different electron acceptors. Oxygen-dependent type (aerobic type) requires oxygen as an electron acceptor and is well characterized in mammals (Camadro *et al.*, 1995). Both oxygen-dependent enzymes are identified to localize in mitochondria. CPO binds weakly to the peripheral inner membranes in the intermembrane space whereas PPO binds tightly to the inner membranes. So the heme biosynthesis route gets back to mitochondria again and the final

product, ferroprotoheme IX, is synthesized with ferrochelatase in mitochondria (Ferreira *et al.* 1988). In facultative anaerobic bacteria such as *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium* and *Rhodobacter spheroides*, the anaerobic pathway is postulated with aerobic type enzymes although the functional enzymes have not been purified yet. Genes for both types of CPO were cloned and sequenced as described latter.

The last step, ferrous iron insertion into protoporphyrin IX catalyzed by ferrochelatase, is well characterized. It locates in the inner membrane of mitochondria but in the cytoplasm or peripheral cytoplasmic membranes in eubacteria. Recently, [2Fe-2S] cluster was identified at the C-terminal region of mammal ferrochelatase (Ferreira *et al.* 1994). Interestingly, ferrochelatases in bacteria and yeast *S. cereviciae* lose the corresponding region.

Biosyntheses of siroheme and vitamin B_{12} are separated from uroporphyrinogen III (**Fig. I-1**). Recently, enzymes for the vitamin B_{12} pathway in *Pseudomonas denitrificans* were identified (Battersby 1994). Biosynthesis of chlorophyll starts at protoporphyrin IX (**Fig. I-1**). Isolated chloroplasts contain all the pathway enzymes for heme and chlorophyll (Fuesler *et al.* 1984) although identification of genes and proteins of the pathway enzymes were largely undone. Biosynthesis of heme D, a chlorin derivative of heme B, is still unknown. In *Bacillus subtilis*, heme D may derive from heme B (Hansson and von Wachenfeldt 1993).

Heme biosynthesis in *Escherichia coli*

There are two reasons for slow progress of this field in *Escherichia coli*. One is that hemin and porphyrins are impermeable to the organism (Sâsârman *et al.* 1968a) and the other is the *hem* genes are scattered on chromosome except *heme CD* locus in *Escherichia coli* . Permeability of tetrapyrroles is the key point to study the biosynthesis. On the contrary, the gram positive bacterium *Bacillus subtilis* can permeate tetrapyrroles and the gene cluster of *hem* genes located in two loci for heme B biosynthetic enzymes (*hemAXCDBL* and *hemEGY*) (Petricek *et al.* 1990, Hansson *et al.* 1991, Hansson and Hederstedt 1992, Hansson and Hederstedt 1994). The gene for aerobic type PPO is identified only in *Bacillus subtilis hemG* (previously identified as *hemY*) (Dailey *et al.* 1994) but the gene for anaerobic type PPO was not clarified in all lives.

Escherichia coli operates the C-5 pathway and both aerobic and anaerobic CPO and PPO synthesize heme B (**Fig. I-1**). These genes were mapped as *gltX* (52 min.; Russell *et al.* 1971) for glu-tRNA ligase, *hemA* (27 min.; Sâsârman *et al.* 1968b) for glu-tRNA dehydrogenase, *hemL* (4 min.; Powell *et al.* 1973) for aminotransferase, *hemB* (8 min.; Sâsârman *et al.* 1968a) for PBG synthase, *hemCD* (85 min.; McConville *et al.* 1979, Chartrand *et al.* 1979) for PBGD and Uro III synthase, *hemE* (90 min.; Sâsârman *et al.* 1975) for Uro D, *hemF* (17 min.; aerobic type; Cox and Charles 1973), *hemG* (86 min.; Sâsârman *et al.* 1979) for PPO and *hemH* (11 min.; Cox and Charles 1973) for ferrocheratase. Sequences of the genes were reported: the *gltX* (Breton *et al.* 1986), the *hemA* (Li *et al.* 1989, Verkamp *et al.* 1989, Drolet *et al.* 1989), the *hemL* (Verkamp *et al.* 1992), the *hemB* (Echelard *et al.* 1988), the *hemC* (Thomas *et al.* 1986), the *hemD* (Sâsârman*et al.* 1987), the *hemF* (Troup *et al.* 1994), the *hemN*(Plunkett *et al.* 1993), the *hemH* genes (cloned by the *visA* gene) (Miyamoto *et al.* 1991). The *hemN* gene was first sequenced as *o459* by the *E. coli* genome project

of the region from 87.2 to 89.2 min. (Plunkett *et al.* 1993) and determined by similarities with the *hemN* gene (about 90% identity in amino acids) in the corresponding region of *Salmonella typhimurium* (Xu and Elliott 1994).

Heme A and heme O biosyntheses

Hemes A and O are specifically found in the terminal oxidases in eukaryotic mitochondria and eubacteria. Structurally, heme O has a 2-hydroxyethylfarnesyl group in place of the vinyl group at position 2 of protoheme IX, and heme A has a formyl group in place of the methyl group at position 8 of heme O (**Fig. I-2**). Heme A was first isolated from bovine heart muscles in 1951 (Warburg and Gewitz 1951) and its correct structure was determined by Caughey in 1975 (Caughey *et al.* 1975). Very recently, Wikström's group found that the cytochrome *bo* complex of *Escherichia coli* has a novel heme A-like molecule, heme O, (Puustinen and Wikström 1991) and its structure was determined (Wu *et al.* 1992). The cytochrome *bo* complex has one molecule each of heme B and heme O in the wild-type strain but has two molecules of heme O when the *cyo* gene was over-expressed (Puustinen *et al.* 1992). After its discovery in *Escherichia coli*, heme O was found in other bacterial species. Replacement of heme A to heme O at the high-spin heme site took place, when cultured in air-limited conditions, in *caa*- and *ba*-type oxidases in thermophilic *bacillus* PS3 (Sone and Fujiwara 1991) and *Acetobacter aceti* (Matsushita *et al.* 1992), respectively. Cytochrome *bo* oxidases were identified in *Vibrio alginolyticus* (Miyoshi-Akiyama *et al.* 1993) and other cytochromes *o* were identified in various bacterial species such as *Acetobacter methanolicus*, *Azotobacter vinelandii*, *Bacillus* sp. strain YN1200, *Bacillus stearothermophilus*, *Gluconobacter suboxidans*, *Pseudomonas aeroginosa*, *Rhodospirllum rubrum*, and *Vitreoscilla* species, but less-well characterized by now (García-Horsman *et al.* 1994). So hemes O and A are widespread tetrapyrroles distributed among the heme-copper terminal oxidase superfamily.

There were, however, only little studies reported for heme A biosynthesis (Labbe-Bois and Labbe 1990). In 1961, Tait and Gibson showed that the first two steps in the conversion of protoporphyrin to bacteriochlorophyll were [protoporphyrin]→[magnesium protoporphyrin]→[magnesium protoporphyrin monomethyl ester] and that magnesium ion was inserted into protoporphyrin IX before the modification of the tetrapyrrole rings took place (Tait and Gibson 1961). In 1962, Porra and Jones showed that pig-liver extract could not form heme A from porphyrin *a* and indicated that porphyrin *a* was not involved in the biosynthesis of heme A (Porra and Jones 1963). This study suggested that the precursor of heme A was protoheme IX and the modification of tetrapyrrole rings took place after ferrous iron insertion into protoporphyrin IX like chlorophyll biosynthesis. According to this observation, in 1966, Lynen *et al.* proposed the formation of heme A from heme B (Seyffert *et al.* 1966) (**Fig. I-3**). Although the alkyl chain at position 2 of heme A was thought to be fully reduced in that time (and so the model was incorrect), the concept of their model was foresighted. The Lynen's model was that protoheme IX was alkylated by farnesyl-pyrophosphate (or another allyl-pyrophosphate) at the vinyl group in position 2 (and in subsequent reactions the side chain was reduced; this was apparently wrong) and the methyl group in position 8 is oxidized to the formyl group resulting in the formation of heme A. The Lynen's model

proposed two important and critical points; one is that protoheme IX is a precursor of heme A, and the other is that farnesyl moiety of heme A derive from farnesyl pyrophosphate. The former was supported by successive observations. In 1967, Sinclair *et al.* showed that *Staphylococcus aureus* heme-required mutant could synthesize heme A by adding exogenous protohemin IX (Sinclair *et al.* 1967). This line of evidence was further supported in *Saccharomyces cerevisiae* (Gollub *et al.* 1977) and *Bacillus subtilis* (Hansson and von Wachenfeldt 1993), as mutants of the two were blocked at the ferrochelatase step in heme B biosynthetic pathway. These evidence proved the Sinclair model, however, which were later criticized by Keyhani and Keyhani who proposed that porphyrin *a* was the true intermediate of heme A biosynthesis. Keyhani and Keyhani showed that accumulation of porphyrin *a* was observed in yeast mitochondria when the functional cytochrome *c* oxidase was damaged by mutations (Keyhani and Keyhani 1982) or growing in copper-deficient conditions (Keyhani and Keyhani 1980). Generally, the Lynen's model was accepted but no direct demonstration has been obtained yet. On the other hand, the latter of Lynen's model was supported by two findings. In 1978, Keyhani and Keyhani showed that [^{3}H]mevalonic acid, a precursor of farnesyl pyrophosphate, is incorporated into heme A in intact *Saccharomyces cerevisiae* (Keyhani and Keyhani 1978), and in 1986, Sinclair *et al.* definitively showed the *in vitro* incorporation of [^{14}C]mevalonic acid into heme A by cultured chicken liver cells (Weinstein *et al.* 1986). But the identification of biosynthetic enzymes for heme A was unsuccessful so that the Lynen's model had not been proved. The study of heme A biosynthesis seemed to be hampered until the discovery of heme O by Wikström's group. The discovery of heme O is a milestone in this field, and strengthens the Lynen's model, which suggested me that heme A biosynthesis can be catalyzed in a consecutive pathway, protoheme IX→heme O→heme A. This idea was the real start point of my study.

Heme prosthetic group in archaebacteria

Until recently, the heme groups of archaebacteria were believed to be the same as those of eukaryotes and eubacteria, i. e., hemes B, A and O. In 1994, Lübben and Morand investigated the heme groups in membrane fractions of the thermoacidophile *Sulfolobus acidocaldarius*, *Desulfurolobus ambivalens* and *Thermoplasma acidophilum*, the halophilic archaeon *Halobacterium salinarium*, and the extreme thermophile *Pyrobaculum aerophilium* , and found novel prenylated heme species instead of hemes A and O (Lübben and Morand 1994). The structures of these novel prenylated hemes were presumed by electrospray ionization mass spectrometry and reverse phase HPLC. Hemes A_S, O_T, OP_1 and OP_2 were found to contain modified prenyl side chains of hemes A and O. Namely, heme A_S has a hydroxyethylgeranylgeranyl group in position 2 of heme A. Heme O_T, heme OP_1, and heme OP_2 have a hydroxyethenylfarnesyl, a hydroxyethenylgeranylgeranyl group, and a hydroxyethylgeranylgeranyl group in position 2 of heme O, respectively (**Fig. I-2**). Although heme A_S was also found in the thermophile eubacterium *Thermus thermophilus* membrane fraction, these novel heme species were unique in archaebacteria. The *sox* operon of thermoacidophilic archaebacterium *Sulfolobus acidocaldarius* appears to encode an intact subunit I and a hydrophobic region of subunit II, which each is conserved as in a protein homologous to cytochrome *b* of cytochrome *c* reductases (Lübben *et al.* 1992). After the separation of *achaea* from

bacteria, they evolved as an ancestral oxidase independently (Castresana *et al.* 1994), resulted in different oxidase organizations and unique heme species.

The superfamily of heme-copper terminal oxidases

Terminal oxidases such as the cytochrome *bo* complex in *Escherichia coli* function as the terminal electron acceptors in the aerobic electron transport systems. They reduce molecular oxygen to water and pump out protons across the membranes by withdrawing a reducing power via ubiquinols or cytochrome *c*. The electrochemical potential difference of protons ($\Delta\mu_{H+}$) thus generated is converted to ATP by ATP synthase. The respiratory chain enzymes are located in the inner membrane of mitochondria in eukaryotes and in the cytoplasmic membrane in prokaryotes. Electron transfer chains are branched to several terminal oxidases according to growth environments and differences in electron acceptors and electron donors (Anraku and Gennis 1987, Anraku 1988).

The heme-copper terminal oxidase superfamily shares a conserved subunit I with a high degree of amino acid sequence identity and has the prosthetic groups consisting of two hemes (hemes B, A and O, and hemes A and O variants) and a copper atom in subunit I, which serve as the reaction center (**Fig. I-4**). Recent phylogenic analysis has suggested that oxidases of this superfamily would evolve from denitrification enzymes (Saraste and Castresana 1994). They postulated that an ancestral and monophyletic oxidase (uroxidase) existed before the split of archaea and bacteria, and that eubacterial quinol oxidase evolved from cytochrome *c* oxidase in Gram-positive bacteria by simplifying the subunit domains in oxidases (Castresana *et al.* 1994). Therefore, the cytochrome *bo* complex in *Escherichia coli* is the most promising model enzyme system among this superfamily. Furthermore, *E. coli* is known to have the simplest aerobic respiratory chain among eubacteria. In *Escherichia coli*, two terminal oxidases, the cytochrome *bd* and *bo* complexes, are found in the aerobic respiratory chain. Both are quinol oxidases and *Escherichia coli* does not contain a cytochrome *c*-dependent respiratory branch. They are expressed separately depending on growth conditions, i. e., the cytochrome *bo* complex is expressed when oxygen tension is high (Minagawa *et al.* 1990) and the cytochrome *bd* complex is predominant when oxygen tension is low (Kita *et al.*, 1984, Georgiou *et al.* 1988). The former oxidase has been described previously as the cytochrome *o*, a CO- binding pigment, by Castor and Chance (Castor and Chance 1959).

The cytochrome *bo* complex was purified as 2 (Kita, *et al.* 1984), 4 (Matsushita *et al.* 1984) and 5 subunit-enzymes (Minghetti *et al.* 1992, Tsubaki *et al.*, 1993) although the function of the 4th and 5th subunits have not been characterized yet. The 4th subunit was suggested to be subunit IV of the cytochrome *bo* complex by mini-cell experiment (Nakamura 1990). The cytochrome *bo* complex contains a hexa-coordinated low spin-heme, a penta-coordinated high-spin heme, and a copper ion (the Cu_B center) (Kita *et al.* 1984, Nakamura *et al.* 1990, Uno *et al.* 1985, Hata *et al.* 1985), and the latter two prosthetic groups form a binuclear metal center where the reduction of molecular oxygen to water (Minghetti and Gennis 1988) takes place (Tsubaki *et al.* 1993, Salerno *et al.* 1990, Hill *et al.* 1992). This redox reaction couples with H^+ pumping outwardly across the cytoplasmic membrane (Puustinen *et al.* 1989). Recent site-directed mutagenesis studies on subunit I of the cytochrome *bo* complex in *Escherichia coli* (Minagawa *et al.* 1992, Lemieux *et al.* 1992, Tsubaki *et al.*, 1994, Uno *et*

al., 1994) and of the *aa*3-type cytochrome *c* oxidase in *Rhodobacter sphaeroides* (Shapleigh *et al.* 1992) have demonstrated that His-106 and His-421 are the axial ligands of the low-spin heme, His-419 is the proximal ligand of the high-spin heme, and His-333 and His 334 are the ligands of the Cu_B center. Although hemes present in the cytochrome *bo* complex have been considered as heme B for long time (Anraku 1988, Anraku and Gennis 1987, Kita *et al.* 1984), Wikström' group found a novel heme, heme O, in the oxidase in 1991 (Puustinen *et al.* 1991).

The *cyoABCDE* operon that encodes the cytochrome *bo* complex was cloned (Au and Gennis 1987, Nakamura *et al.* 1990) and sequenced (Chepuri *et al.* 1990). The amino acid sequence identity of subunit I between cytochrome *o* and other cytochrome oxidases indicates that they are the members of the heme-copper oxidase superfamily. The *cyoA, B* and *C* genes encode subunit II, I and III, respectively, of the cytochrome *bo* complex by peptide sequence (Minghetti *et al.* 1992), and the *cyoD* gene encodes subunit IVB as demonstrated in the *caa*3-type cytochrome *c* oxidase in thermophilic *Bacillus* PS3 (Sone *et al.* 1990) and the *ba*3-type quinol oxidase in *Acetobacter aceti* (Fukaya *et al.* 1993). On the contrary, the last ORF, the *cyoE* gene, was not known for its function (**Fig. I-5**). The genes encoding CyoE protein homologues were found not only in bacterial oxidase gene clusters but also in genes involved in *Saccharomyces cereviciae* and human chromosomes. The bacterial genes include the *ctaB* gene in the gene clusters of the *caa*3-type cytochrome *c* oxidases in *Bacillus subtilis* (Saraste *et al.* 1991) and alkaliphilic *Bacillus firmus* OF4 (Quirk *et al.* 1993), the *caaE* gene in the gene cluster of the *caa*3-type cytochrome *c* oxidase in thermophilic *Bacillus* PS3 (Ishizuka *et al.* 1990), the *ctaB* genes in the gene clusters of the *aa*3-type cytochrome *c* oxidases in *Paracoccus denitrificans* (Raitio *et al.* 1987) and *Rhodobacter sphaeroides* (Cao *et al.* 1992), and the *COX10* gene in yeast (Nobrega *et al.* 1990) and human (Glerum and Tzagoloff 1994) (**Fig. I-6**). The *COX10* gene was isolated as an essential chromosomal gene for functional expression of subunit I of the *aa*3-type oxidase in mitochondria (Nobrega *et al.* 1990). Deletions of the *cyoE* and *ctaB* genes in *Escherichia coli* (Nakamura 1990) and *Paracoccus denitrificans* (Steinrücke *et al.* 1991), respectively, caused inactivation of the oxidases. These CyoE protein family has not been so far co-purified with any oxidase complex and seemed to function as an assembly factor of the terminal oxidase complexes. The biochemical study of the CyoE protein in *Escherichia coli* has not been done and its function is not known yet.

Scope of this thesis work

In this study, I aimed at finding and elucidating function of the CyoE protein. To this end, I made systematic site-directed mutagenesis studies, in which conserved amino acid residues among the CyoE family and charged residues in CyoE were replaced by alanine and the loops exposed to the cytoplasm were deleted by site-directed mutagenesis, and analyzed the prosthetic groups of the binuclear center in defective CyoE proteins (Chapter III). The results of biochemical, spectroscopic and genetic analyses of the defective cytochrome *bo* complexes led me the discovery of heme O synthase (Chapter IV, V and VI). In the chapter V, I present the biochemical properties and reaction mechanism of this novel enzyme, the protoheme IX-farnesyl transferase.

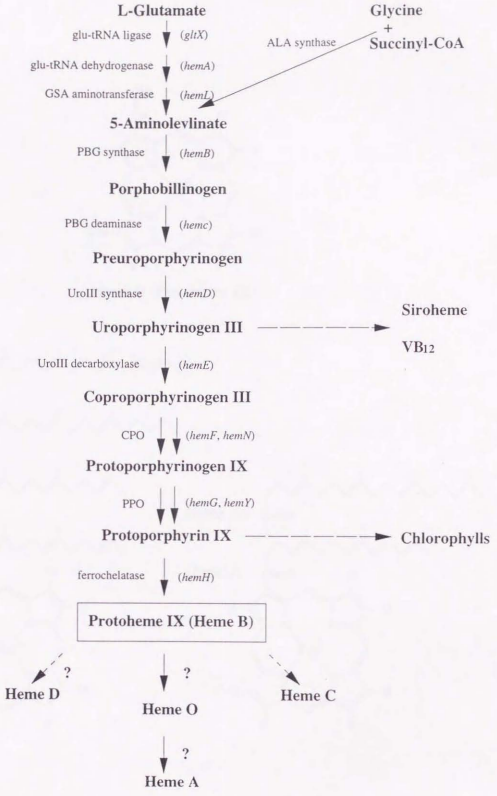
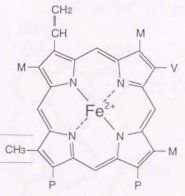


Fig. I-1. Biosynthesis of tetrapyrroles
Intermediates and enzymes in protoheme IX biosynthesis are indicated. The corresponding genes for protoheme IX biosynthetic enzymes in *Escherichia coli* are also indicated.



Heme B (Protoheme IX)

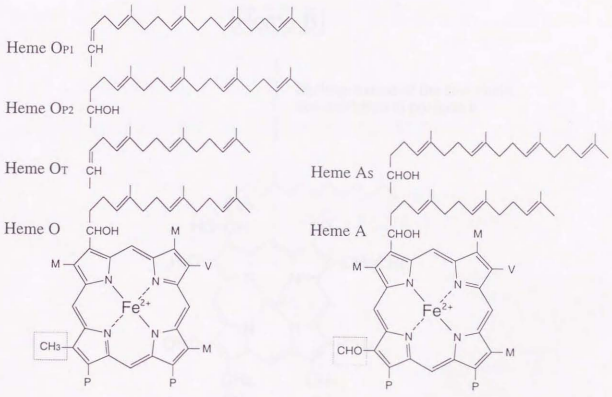
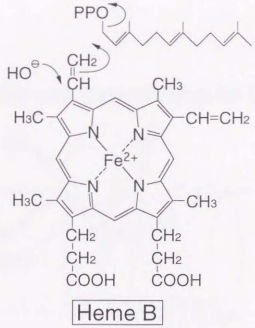
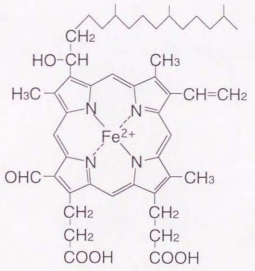


Fig. I-2 Structures of hemes found in the heme-copper oxidase superfamily



Heme B

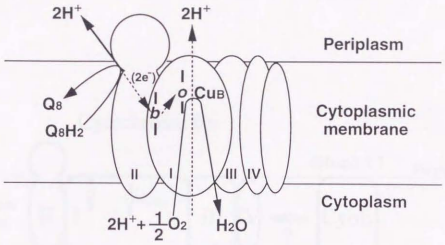
Hydrogenation of the side chain
and oxidation in position 8



Cytohemin

Fig. I-3 Hypothetical pathway for the biosynthesis of cytohemin (Lynen's model in 1966)
Cited from Seyffert *et al.*, 1966. Cytohemin is one of the wrong structures of heme A. The correct structure of heme A was determined in 1975 (Caughet *et al.*, 1975).

A. *Escherichia coli* Cytochrome *bo* Complex



B. Mitochondrial Cytochrome *aa*$_3$ Complex

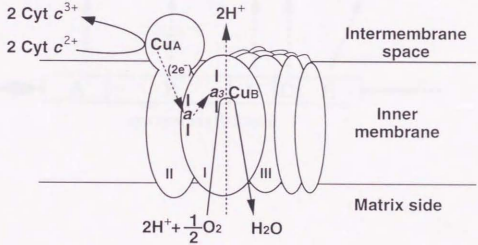


Fig. I-4 The cytochrome *bo* complex belongs to the heme-copper oxidase superfamily

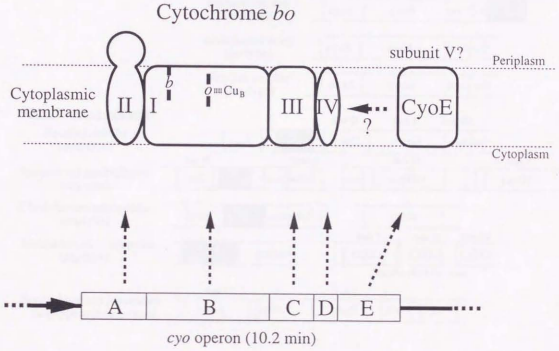


Fig. I-5 Assembly model of the cytochrome *bo* complex

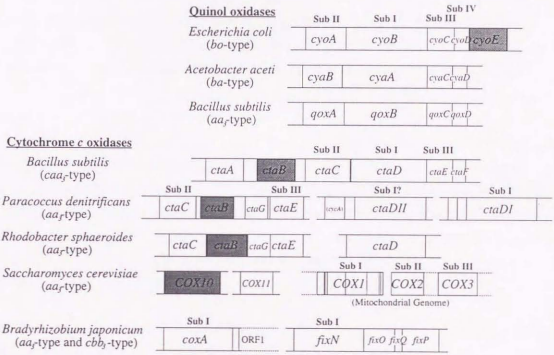


Fig. I-6 Genes for the heme-copper terminal oxidase superfamily
Genetic loci are shown for quinol oxidases in *E. coli*, *A. aceti* and *B. subtilis*; and cytochrome *c* oxidases in *B. subtilis*, *P. denitrificans*, *R. sphaeroides*, *Saccharomyces cerevisiae*, and *Bradyrhizobium japonicum*. Sub I, Sub II, Sub III and Sub IV shown above the gene loci indicate subunits of the heme-copper respiratory oxidases. The genes for CyoE and its homologues are indicated by shadow.

Chapter II. Experimental Procedures

Media

E. coli cells were grown in LB (Luria-Bertani) medium [1% (w/v), Bacto-tryptone (Daigo eiyo), 0.5% (w/v) Bacto-yeast extract (Daigo eiyo), 1% NaCl; pH 7.5] and on LB agar plate [LB medium containing 1.5% agar]. Preparation of plasmids and manipulation of plasmid DNAs were according to usual methods (Sambrook *et al.* 1989). For preparation of cytoplasmic membranes, cells were grown in a rich medium [(w/v) of 0.67% Bacto-yeast extract (DIFCO), 1.33% Bacto-casamino acids, technical (DIFCO), 0.13% sodium citrate, 0.27% $(NH_4)_2SO_4$, 0.9% K_2HPO_4, 1% glycerol] and harvested at OD_{650}=0.6-0.8. Ten μg/ml of $FeSO_4$ and 5 μg/ml of $CuSO_4$ were added to the rich medium only when the cytochrome *bo* complex was further purified. DM minimal medium (Davis and Mingioli 1959) containing 0.4% of glucose or glycerol as a carbon source was used for complementation tests of aerobic growth. Anaerobic growth of cells were in LB medium containing 40 mM of sodium nitrate and 0.4% of glucose using a GasPak system (BBL Microbiology System, Cockeysville, MD). Ampicillin was added at 100 μg/ml for multicopy plasmid, 50 μg/ml for pBR322 and its derivatives, 40 μg/ml for pTTQ18 and its derivatives and 15 μg/ml for mini-F plasmids.

Bacterial strains

For routine purposes for plasmid preparation, *E. coli* strain SCS1 was used (Sambrook *et al.* 1989). Strain TG1 was used for superinfection of helper phage M13KO7 when single-stranded DNAs of pCYOF6 derivatives were prepared. The *cyo cyd* double deletion mutant ST2592 (W3110 Δ*cyo*::Cm^r Δ*cyd*::Km^r *srlA*::Tn10 *recA*) was used for aerobic complementation test (Minagawa *et al.* 1992). Strain ST4676 (W3110 Δ*cyo*::Cm^r) was used for preparation of cytoplasmic membranes. JM109/pREP4 (QIAGEN inc., CA, USA) was used when His tagged CyoE protein was produced.

Plasmids

Multicopy phageimid **pCYOF6** (Saiki *et al.* 1993b), which carries the 3'-half of the *cyo* operon corresponding the half of the *cyoC* gene and the entire *cyoDE* genes, was used for site-directed mutagenesis. Multicopy plasmid **pCYO6** (Saiki *et al.* 1993b), which eliminates the phage f1-*ori* region from pCYOF6, was used for confirmation of the mutations by sequencing analysis. pCYOF6 and pCYO6 were made by subcloning the 1.7 kb *Eco* RI-*Sph* I fragment of pHN12 (Nakamura *et al.* 1990) into pCYOF1 and pCYO1, respectively.

pCYO111 is replaced with the multi-cloning site of pCYO1 (constructed by Dr. Mogi, unpublished) by another multi-cloning site (*Pvu* II-*Nhe* I-*Xho* I-*Mlu* I-*Spl* I-*Pma* CI-*Sph* I) and constructed as follows; the 0.35 kb *Bam* HI-*Dra* I fragment (the promoter region of pHN3795-1; Nakamura *et al.* 1990) was ligated to the 2.2 kb *Bam* HI-*Sph* I fragment of pCYO1 with annealed synthetic oligonucleotides (5'-AATCAGCTGCTAGCTCGAGACGCGTACGCACGTGCATG-3' and 5'-CACGTGCGTACGCGTCTCGAGCTAGCAGCTGATT-3').

pCYO61 has a deletion of the *cyoCD* genes from pCYO6 and has the *Spl* I site adjacent to the Shine-Dalgarno sequence of the *cyoE* gene. The construction was as follows; the 0.87 kb of *Dde* I-*Kpn* I fragment (containing the *cyoE* gene) of pCYO6 was ligated to the 2.5 kb of *Eco* RI-*Kpn* I

fragment of pCYO6 with the annealed synthetic *Spl* I oligonucleotides (5'-AATTCGTACG-3' and 5'-TTACGTACG-3'). **pCYO62** has an unique *Pvu* II site at the 3'-terminal end of the *cyoE* gene and the construction was as follows; a *Pvu* II site was introduced at the 3'-end of the *cyoE* gene of pCYO61 via site-directed mutagenesis, which replaced a codon for Ala-293, GCT, with GCA using a ΔE2-*Pvu* II primer (3'-TGCGACGAC**CGT**CGACACACACC-5'). **pCYO63** has a deletion of the non-essential DNA region after the transcriptional termination of the *cyo* operon from pCYO62. The construction was as follows; the 0.98 kb *Eco* RI-*Ssp* I fragment (containing the *cyoE* gene) of pCYO63 was ligated to the 2.2 kb *Eco* RI-*Eco* RV fragment of pCYO1. **pCYO64** is introduced with an unique *Pvu* II site between the *cyoD* and the *cyoE* gene of pCYO63 and was constructed as follows; the *Dde* I site next to the stop codon "TAA" of the *cyoD* gene was blunt-ended by T4 DNA polymerase and subcloned into the *Pma* CI site of pCYO111. **pCYO65** has a unique *Pst* I site in the 5'-terminal region of the *cyoE* gene by site-directed mutagenesis using an oligonucleotide, 5'-TTTCGTTAC**CT**GCAGGTATTG-3', corresponding to Gln^{5} to Lys^{11} of the CyoE.

The 0.4 kb of *Dde* I-*Dde* I DNA fragment including the entire region of the *cyoD* gene was blunted by T4 DNA polymerase and inserted into the *Pma* CI site of pCYO111. **pCYO12'** has a right oriented insertion and **pCYO12** has a converted insertion (**Fig. II-1**). Then, the 0.2 kb *Bgl* I-*Spl* I fragment (the C-half of the *cyoD* gene) of pCYO11 was ligated to the 3.5 kb *Bgl* I-*Spl* I fragment of pCYO63. **pCYO13** has an unique *Spl* I site between the *cyoD* stop codon "TAA" and the *Pma* CI site and constructed as follows; the 0.2 kb *Bgl* I-*Spl* I fragment of pCYO12 was ligated to the 1.7 kb *Bgl* I-*Bgl* I fragment of pCYO6 and the 1.0 kb *Bgl* I-*Spl* I fragment of pCYO111. **pCYO14** is introduced with the transcriptional termination of the *cyo* operon into pCYO13 and constructed as follows; the 0.2 kb *Pma* CI-*Bgl* I fragment (the C-half of the *cyoD* gene) of pCYO14 was ligated to the 0.9 kb *Pvu* II-*Bgl* I fragment of pCYO63 and the 1.7 kb *Bgl* I-*Bgl* I fragment of pCYO6 (**Fig. II-1**).

pCYO6-*caaE* is replaced with the *cyoE* gene by the *caaE* gene of the caa_3-type cytochrome *c* oxidase of thermotolerant *Bacillus* PS3. The construction was as follows. To create the unique *Pst* I site at the 5'-terminal of the *caaE* gene, a 0.26 kb *Pst* I-*Bam* HI fragment was amplified by PCR using VENT DNA polymerase (New England BioLabs) and lamda clone λE2 DNA carrying the entire *caa* gene cluster as a template. Oligonucleotides used for PCR were 5'-AGAGAGTTA**CTGCAG**GTTGTGAAAATCGGAATCGTC-3' corresponding Arg^{27} to Val^{38} and 5'-GCGGATCCATCCCGGTGACGGTCGG-3' corresponding to Pro^{111} to Pro^{103} of the CaaE. In the PCR product, codons for Ser^{30}-Ser^{31} were changed from "TCGTCT" to "CTGCAG". For subcloning of the 3'-terminal half of the *caaE* gene, pCO2-1 which carries the 1.75 kb *Bam* HI-*Sph* I fragment of λE2 was digested with *Ban* II followed by blunt-ending with T4 DNA polymerase treatment. Then, the 0.7 kb *Bam* HI-*Ban* II (blunt-ended) was isolated and introduced into the *Bam* HI-*Pvu* II site if pCYO62 before the transcriptional terminator of the *cyo* operon. The resultant plasmid was named **pCYO62-CO2-1**. Finally, the 0.24 kb *Pst* I-*Bam* HI fragment, the 0.8 kb *Bam* HI-*Bgl* II fragment of pCYO62-CO2-1, and the 2.6 kb *Bgl* II-*Pst* I fragment of pCYO65 were ligated together to obtain pCYO6-*caaE*.

For the expression of the *cyo* operon, the derivatives of the single copy mini-F plasmid, pMFO1 (Nakamura 1990) and its derivatives were used. **pMFO21** has an additional *Spl* I site between the *cyoD* and the *cyoE* genes of pMFO1 (Saiki *et al.* 1993b) and was constructed to introduce the 1.6 kb *Eco* RI-*Sph* I fragment of pCYO64 into the *Eco* RI-*Sph* I fragment of pMFO1. **pMFO21-ΔE2** was constructed by replacement of the 1.7 kb *Eco* RI-*Sph* I fragment of pMFO1 to the counterpart (0.6 kb) in pCYO14.

For the expression of the intact *cyoE* gene, pTTQ18 (Stark 1987) and its derivatives were used. **pTTQ18-*cyoE* (pHN31)** was constructed by Nakamura (Nakamura 1990). In order to avoid a possible effect of the 5'-upstream region of the *cyoE* gene, **pTTQ18-*cyoE*-2** was constructed, where only the entire *cyoE* coding region and its Shine-Dalgarno sequence have been cloned downstream of the *tac* promoter. The *Spl* I site in pCYO63 was blunt-ended by T4 DNA polymerase, and then the 1.0 kb (*Spl* I)-*Sph* I fragment containing the entire *cyoE* gene was isolated and ligated with the 4.5 kb (*Eco* RI)-*Sph* I fragment of pTTQ18.

For the expression of the His-tagged CyoE protein, the 1.1 kb *Pst* I-*Sph* I *cyoE* gene DNA fragment from pCYO65 was subcloned into the pQE10 (QIAexpress vector) using linker DNAs. The sequences of linker DNAs were 5'-GATCCAGTTGGGCCCGTTGGGCCAGTTGGGCCCATGATGTTTAAGCAATACCTGCA-3' and 5'-GGTATTGCTTAAACATCATGGGCCCAACTGGCCCAACGGGCCCAACTG-3', which compensated the DNA region corresponding the N terminus of CyoE of pCYO65. The resultant plasmid, **pQE10-EPst-Col**, was designed to introduce the Pro-Val-Gly-Pro-Val-Gly-Pro-Val-Gly-Pro sequence between the His tagged sequence and the CyoE protein.

Site-directed mutagenesis

Site-directed mutagenesis was carried out by the method of Tayler *et al.* (Tayler *et al.* 1985), using mutagenesis primers in the range of 21-38 nucleotides (**Table II-1**), pCYOF6, and an oligonucleotide-directed *in vitro* mutagenesis system (Amersham Co.). Oligonucleotides used as a primer for site-directed mutagenesis, linker ligation, and sequencing primer were synthesized with a model 381A DNA synthesizer and 394 DNA/RNA Synthesizer (Applied Biosystems Inc.). The unique restriction fragments containing the mutations were replaced with the counterparts in the wild-type pCYO6 (**Fig. II-3**). Loss of the *f1-ori* in pCYOF6 was as an index of replacement (**Fig. II-2**). Mutations were confirmed by direct plasmid sequencing (Sanger *et al.* 1977) using Sequenase version 2.0 (U. S. Biochemical Corp.).

Expression of mutant cyo operon

The *Eco* RI-*Sph* I fragment of mutant pCYO6 was introduced into the corresponding sites of pMFO21. The resultant mini-F plasmids were confirmed by a loss of the gene-engineered *Spl* I site at a junction between the *cyoD* and *cyoE* genes in pMFO21 and are thus the derivatives of pMFO1, which contains two *Spl* I sites in the *cyoB* gene and pHNF2 (**Fig. II-2**).

Genetic complementation test of aerobic growth

Escherichia coli has two terminal oxidases (the cytochrome *bo* and the cytochrome *bd* complexes encoded by the *cyo* and *cyd* operons, respectively) in the aerobic respiratory chain. One of which is sufficient for aerobic growth on non-fermentable carbon source and, of course, loss of both oxidase genes results in growing only depending on fermentable carbon source or anaerobic conditions (Au *et al.* 1985). Expression of the *cyo* operon by mini-F plasmid is the most natural way to complement the aerobic growth of the *cyo cyd* double mutant on non-fermentable carbon source since over-expression of the *cyo* operon caused spontaneous mutations (Nakamura 1990). The mutant pMFO1 derivatives were introduced into the *cyo cyd* double deletion mutant strain ST2592 followed by growing on LB plates containing 15 μg/ml ampicillin, 40 mM sodium nitrate and 0.4 % glucose under anaerobic conditions in a sealed jar (Gas-Pack Anaerobic System; BBL Microbiology System, Cockeysville, MD). The ability to grow under aerobic conditions were examined on DM minimal medium-plates containing 15 μg/ml and 0.4% of glucose or glycerol at 37°C for 5 days. If necessary, growth at 23, 30 and 42°C was examined within 2 weeks (**Fig. II-2**).

Preparation of cytoplasmic membranes

The *cyo* deletion strain ST4676 harboring mini-F plasmids, the pMFO1 derivatives, was cultured in a rich medium containing 15 μg/ml of ampicillin very aerobically with 2-liter Sakaguchi flasks. Cells were harvested at OD_{650}=0.6-0.8 (measured by a Coleman Junior IIA Linear Absorbance spectrophotometer) when the cytochrome *bd* was still minor in activity (**Fig. II-2**). Cytoplasmic membranes were prepared by the method of Yamato *et al.* (Yamato *et al.* 1975) with slight modifications. All the steps were done at 4°C. Collected cells (7,000×*g*, 10 min.) from 1 liter culture were washed once in 30 mM Tris-Cl pH8.0 and precipitated by 12,000×*g* for 5 min. The precipitant was suspended in 10 ml of 30 mM Tris-Cl pH8.0, and 20% sucrose. Then, 9 mM (f. c.) EDTA (pH8.0) and 1 mg/ml (f. c.) of lysozyme were added and the mixture was incubated on ice for two hours. The cells treated with lysozyme were disrupted by two passages through a French press at 1200 kg/cm^2 (Type 5501M; Ohtake Works, Tokyo) and were centrifuged at 16,000×*g* for 10 min. to precipitate the undisrupted cells. The lysate was diluted with 2 volume of DW and was centrifuged at 180,000×*g* for 1.5 hours. The precipitated crude membranes were homogenized in 5 ml of 3 mM EDTA (pH8.0) by a Dounce teflon glass homogenizer (Type SM-3; Omega Electric, Tokyo) and the membranes laid on top of 16 ml of 44% (w/w) sucrose, and 3 mM EDTA (pH8.0) followed by centrifugation at 120,000×*g* for 10 hours. A brown reddish zone of cytoplasmic membrane vesicles was collected, diluted with 3 mM EDTA (pH8.0) and centrifuged at 180,000×*g* for 1.5 hours. The precipitants were homogenized in 10% sucrose and 3 mM EDTA (pH8.0) by homogenizer. The cytoplasmic membrane vesicles were stored at -80°C. Right-side-out and inside-out cytoplasmic membrane vesicles were prepared according to procedures of Kaback (Kaback 1971) and Futai (Futai 1974), respectively.

Preparation of antiserum against the LacZ-CyoE chimera protein

pUR278-*cyoE* was the plasmid for an overproduction of the LacZ-CyoE chimera protein. The 1.2 kb *Bam* HI-*Bgl* II fragment of pCYO61 containing the entire *cyoE* gene was subcloned into

the *Bam* HI site of pUR278 (Ruther and Müller 1983) to construct the in-frame *lacZ-cyoE* fusion gene. Strain JM109 harboring pUR278-*cyoE* was grown in a rich medium, and the expression of the chimera protein was induced for 2 hours after addition of IPTG to a final concentration of 1 mM at OD_{650}=0.3. Cytoplasmic membranes were prepared from 10 liter culture of cells. Assay of β-galactosidase activity in the cytoplasmic membrane was determined by Miller' method (Miller 1972). The chimera protein with a molecular mass of about 120 kDa was separated from all the other cytoplasmic membranes proteins using 12.5% SDS-polyacrylamide gel electrophoresis. The chimera protein band was cut out and the protein eluted from the gel electrophoretically. The eluted protein was extensively dialyzed against distilled water at 4°C and lyophilized (Nakano *et al.* 1988). The purified chimera protein was suspended in PBS (20 mM sodium phosphate pH7.4, and 150 mM NaCl) and subjected for immunizing rabbits. Primary injection was done with 1 mg of the purified chimera protein in complete Freund's adjuvant and then boosts of 0.1-0.3 mg in incomplete Freund's adjuvant were given at 1- or 2-weeks intervals. The titer of the anti-LacZ/CyoE chimera polypeptide antiserum was examined by Western blotting analysis using the CyoE overproduced cytoplasmic membranes and reached to the maximal level after eight boosts.

UV-vis spectroscopic analysis

Measurements of the dithionite-reduced *minus* air-oxidized difference spectra at 77K, and the CO plus reduced *minus* reduced difference spectra at room temperature were performed with a Shimadzu UV-3000 spectrophotometer (Shimadzu Corp., Kyoto) as described (Kita *et al.* 1984, Minagawa *et al.* 1992). Digital outputs were recorded in a PC-286VS computer (Epson Co., Kyoto) using a program provided by Dr. Matsuura (Tokyo Metropolitan University), transferred to a Macintosh IICX computer (Apple Computer Inc., Cupertino, CA), and were processed using a software Igor (WaveMetrics, Lake Oswego, OR) as described (Minagawa 1992). The amount of cytochrome *o* was calculated from the CO-binding difference spectra at a wavelength pair of 416-430 nm using a value for the molecular extinction coefficient of 254,000 cm^{-1} (Tsubaki *et al.* 1993).

Copper analysis

Copper content in cytoplasmic membranes and purified oxidase samples were determined by atomic absorption analysis using a Shimadzu AA-640 atomic absorption spectrophotometer (Shimadzu Co., Kyoto). The output signals were calibrated by running standards of Cu (Waco Pure Chemical Ind. Ltd., Osaka) ranging from 0.02-0.4 ppm (Minagawa 1992).

Purification of the cytochrome bo complex

ST4676 harboring pMF01 derivatives were grown in 10 liter of a rich medium containing 10 μg/ml $FeSO_4$ and 5 μg/ml $CuSO_4$ by using a Magnaferm Fermenter (New Brunswick Scientific Co., New Jersey) with high aeration at 12 liter/min. and agitation at 800 rpm (Minagawa *et al.* 1992). Cells were harvested at OD_{650}=0.6-0.8. Cytoplasmic membranes were prepared from 60 liter culture cells. Conditions of solubilization were as follows: 50 mM Tris-Cl pH7.4, 10 mM $MgCl_2$, 1 mM PMSF, 1 mg/ml cytoplasmic membrane vesicles, and 1% sucrose monolaurate 1200 (SM-1200;

Mitsubishi-Kasei Food Corp., Tokyo) at 4°C for 1 hours. Solubilized proteins were separated by centrifugation at 180,000×*g* for 1 hour. The supernatant was filtered through a 0.45 μm cellulose acetate filter (W25-5; Tosoh Corp., Tokyo) and subjected to a preparative DEAE-5PW column (21.5 mm ID×15 cm; Tosoh Co., Tokyo) using a LC-9A HPLC system (Shimadzu Co., Kyoto) at a flow rate of 5 ml/min. Running buffer was 50 mM Tris-Cl pH7.4, 0.1 mM PMSF and 0.1% SM-1200. The cytochrome *bo* complex was eluted by a NaCl gradient from 0 to 1 M. The peak fractions were collected, diluted 2-fold with running buffer, and reloaded on the same column (Mogi and Anraku 1990, Minagawa 1992). The partially purified cytochrome *bo* complex fractions were concentrated by Centprep 30 (Amicon American Division W. R. Grace & Co.-Conn. Beverly, MA), yielding about 10-15 mg of protein from 60 liter culture.

Heme analysis

Heme contents in cytoplasmic membranes and the purified cytochrome *bo* complex samples were determined by pyridine hemochrome spectra (Berry and Trumpower 1987). Heme composition was determined by reverse phase HPLC as described (Puustinen and Wikström 1991). Samples (200 μl) containing 0.5 mg and 3 mg protein of the partially purified sample and cytoplasmic membrane vesicles, respectively, were suspended in 450 μl of freshly prepared acetone-HCl (9:1; v/v) and vortexed vigorously. After centrifugation (by 12,000 rpm for 2 min. at 4°C), the supernatant was transferred to 500 μl of dry diethyl ether and vortexed vigorously. After centrifugation, an upper ether phase was collected and washed two times in 500 μl distilled water. An upper ether phase was collected and dried up under stream of nitrogen gas. Hemes were dissolved in 15 μl of running buffer (EtOH:AcOH:DW=70:17:7 (v/v)), and 7.5 μl of samples were subjected to an Altex Ultrasphere ODS column (4.6 mm I.D.×25 cm) equilibrated with running buffer using a model LC-9A HPLC system (Shimadzu Co., Kyoto) at a flow rate of 0.5 ml/min. Separated heme fractions were detected by absorption at 398-402 nm. Heme samples were stored at -80°C.

Measurements of EPR Spectra

Spectra were taken at 15K using an air-oxidized oxidase solution (about 100 μM heme) in 50 mM Tris-Cl pH7.4, and 0.1% SM-1200. EPR measurements were carried out at X-band (9.23 GHz) microwave frequency with a home-built EPR spectrometer (Faculty of Engineering Science, Osaka University) with 100 kHz field modulation by using a Varian X-band cavity. An oxford ESR-900 flow cryostat (from 4K to 80K) was used at 15K for the measurements. The microwave frequency was calibrated with a microwave frequency counter (Takeda Riken Co., Ltd., model TR5212). The magnetic field strength was determined by a nuclear magnetic resonance of protons in water. Accuracy of the g-value was approximately ± 0.01 mT.

Quinol oxidase assay

Ubiquinol-1 oxidase activity was determined spectrophotometrically with a Shimadzu UV-3000 spectrophotometer (Shimadzu Co., Kyoto) (Kita *et al.* 1984). Measurements were done at 25°C in 50 mM Tris-Cl pH 7.4, 0.1% SM-1200, and 160 μM ubiquinol-1 as a substrate. The

activity was calculated using 15,000 at 278 nm as a molar extinction coefficient of ubiquinone-1. Ubiquinone-1 was a generous gift from Dr. M. Ohono (Eisai Co. Ltd., Tsukuba).

Synthesis of farnesyl diphosphate

Farnesyl diphosphate (FPP) was synthesized according to the method of Davisson *et al.* (Davisson *et al.* 1985). As a starting material, *trans, trans*-farnesyl-bromide (Aldrich Chemical Co., WI, USA) was used instead of farnesol. Four g (18 mmol) of disodium dihydrogen pyrophosphate (Aldrich) in 40 ml of deionized water was applied to 94 ml volume of Dowex AG 50W-8X cation exchange resin (analytical grade, 50-100 mesh, hydrogen form; BioRad) in a column (2 cm I.D.×23 cm) equilibrated with 2 volume of deionized water. The acidic eluent at a flow rate of 1-2 ml/min. was collected (about 70 ml) and was immediately neutralized to pH7.3 with 40% *tetra-n*-butylammonium hydroxide solution (Aldrich) (about 30 ml was used). The salt was dried by lyophilization to yield 14.4 g of white solid. The white solid was washed in acetnitrile and evaporated 3 times to yield 13.8 g of Tris(tetra-*n*-butyl)ammonium hydrogen pyrophosphate. Used AG 50W-8X column was regenerated to hydrogen form with 300 ml of 1 M HCl, then, was changed to ammonium form by eluting the column with 54 ml of 1 M NH_4Cl solution. Column regeneration was checked by eluent of pH. In a frame-dried round bottom flask replaced by nitrogen gas, 13.8 g of Tris(tetra-*n*-butyl)ammonium hydrogen pyrophosphate was dissolved in 30 ml of dry acetonitrile and added 2.14 g of farnesyl bromide. The mixture was stirred for 2 hours at room temperature. Then, acetnitrile was removed by rotary evaporation at 45°C for up to 1 hour and the resultant (about 16 ml) was dissolved in 20 ml of 1:49 (v/v) isopropanol:25 mM ammonium bicarbonate (ion-exchange buffer). Half of crude product (about 18 ml) was passed through the AG 50W-8X column mentioned above (ammonium form) equilibrated with 200 ml of ion-exchange buffer. The white milky suspension (about 30 ml) that was eluted after about 40 ml of elution was collected and lyophilized to yield 7.3 g of pale white-yellow solid. This solid was further purified by a cellulose powder Whatman CF11 column and the FPP fractions were determined by thin layer chromatography as described (Davisson *et al.* 1985). Standard farnesyl diphosphate was a gift from Dr. K. Ogura (Tohoku Univ.). Finally, 0.9 g of farnesyl diphosphate was obtained.

Assay of heme O synthase

For a standard reaction, a mixture (200 µl) of 200 mM Tris-Cl pH7.4, membrane vesicles (350-600 µg for crude membranes and 20-60 µg for cytoplasmic membranes), 60 µM hemin, 120 µM FPP, and 1.5 mM $MgSO_4$ was prepared. Heme O synthase reaction was started by addition of a few crystals of sodium dithionite, then continued for 20-30 min. at 37°C, and terminated by addition of 30 µl of 37% (w/v) formaldehyde solution. The mixture could be stored at -80°C. Products were analyzed by pyridine hemochrome method or reverse-phase HPLC.

Others

SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (Laemmli 1970) using Rainbow™ colored protein molecular weight markers (weight range of 14.3-200 kDa) as

a standard (Amersham Co.). Silver stain was performed in room temperature and the procedures were as follows. Gel was soaked in 10% MeOH-5% acetate for 2 to 12 hours followed by fixing with 50% (v/v) MeOH-0.0185% (v/v) formaldehyde for 20 min. Gel was then incubated in 0.0325 mM DTT for 20 min. and was stained by 0.1% (w/v) $AgNO_3$ for 20 min. Then, gel was washed by DW for 3 times and was quickly rinsed by developing buffer (3% (w/v) Na_2CO_3, 0.0185% (v/v) formaldehyde) repeatedly until a brown color in buffer was disappeared. Visualization was performed in developing buffer and the reaction was stopped by soaking in 5% (v/v) acetate for around ten hours followed by soaking in 30% (v/v) EtOH for 2 hours. Estimation of molecular masses of membrane proteins were made by electrophoresis in 7.2 M urea containing 18.75% SDS-polyacrylamide gel (Kadenbach *et al.* 1983) followed by visualizing with Coomasie Brilliant Blue R250 or silver stain (Oakley *et al.* 1980). Molecular weight marker 69825 (peptide range of 2.5-17 kDa; Fluka AG, Switzerland) was used for estimations of low molecular weight proteins with correct values (Kratzin *et al.* 1989). Western immunoblotting and ELIZA were performed according to Towbin *et al.*. (Towbin *et al.* 1979) with slight modifications (Minagawa 1992). Protein concentration was determined by BCA kit (PIERCE, IL, USA) using bovine serum albumin as a standard.

Materials

Restriction endonucleases and modifying enzymes for DNA manipulations were purchased from Takara Shuzo (Kyoto, Japan) and New England BioLabs. Isopropyl thio-β-D galactopyranoside was from Nova Biochem.. Ligation and blunting of plasmid DNA fragments were performed by DNA Ligation Kit and DNA Blunting Kit, respectively (Takara Shuzo, Kyoto). λ DNA was purchased from Takara Shuzo (Kyoto) and its *Hin* DIII DNA fragments were used as a DNA size marker. Sep-Pak Cartridges were purchased from Waters (US). Other chemicals were commercial products of analytical grade.

(A) Deletion mutants

Mutant	Mutagenic oligonucleotide	Additional site
ΔE3	3'-ACAAATTGTTGATGTAG-CTACACGACCACTTTCCGG-5'	*Cla* I
ΔE4	3'-ACTAGCCGATGACACGA-TCGGACACCGTCTACG-5'	*Nhe* I
ΔE5	3'-GGCGAAATTCCTAATG-CATCATTTTCCGTAAAG-5'	*Sna* BI
ΔE6	3'-CGAGACGCGCCAATG-GACAAGCCGAAGAGATA-5'	*Bst* PI
ΔE7	3'-GAGCCACTACTACAGG-ATTCCATTGTTTTGGAGAGAT-5'	*Eco* 81I

(B) Missense mutants (Alanine scanning)

Mutant	Mutagenic oligonucleotide	Codon change
K11A	3'-GTTCATTGCCGTGGTCCGTAG-5'	AAA→GCA
L48A	3'-GACCAACCCCACAGTCGCCAACACCGCAGCCCA-5'	CTG→GCG
C54A	3'-CGCAGCCCACGACACAAATTG-5'	TGT→GCT
N57A	3'-ACACACAAACGGTTGATGTAG-5'	AAC→GCC
D61A	3'-CACAAATTGTTGATGTAGCGGTCCCTATAGCTGTCTTTC-5'	GAC→GCC
D63A	3'-TAGCTGTCCCGATAGCTGTCT-5'	GAT→GCT
D65A	3'-TCCCTATAGCGGTCTTTCTAC-5'	GAC→GCC
M68A	3'-CTGTCTTTCCGCCTTTCCTGC-5'	ATG→GCG
R70A	3'-TTCTACCTTCGCTGCTTCTTA-5'	AGG→GCG
T71A	3'-TACCTTTCCCGCTTCTTAGCC-5'	ACG→GCG
R74A	3'-TGCTTCTTACGCCACGACCAC-5'	CGG→GCG
G79A	3'-GACCACTTTCGGGACTAGAGA-5'	GGC→GCC
L101A	3'-CGAAATACGACCGCACCAAACCGC-5'	CTG→GCG
C110A	3'-GGCGACCGGCGGACCGACCCC-5'	TGC→GCC
Y120A	3'-AAACACCAACGACAGCCGCAA-5'	TAT→GCT
Y124A	3'-CAGCCGCAACGATCGGACATG-5'	TAT→GCT
K129A	3'-GACATGTACCGTGCGGTGAGA-5'	AAA→GCA
R130A	3'-ATGTACTTTCGCGTGAGACAG-5'	CGC→GCG
H131A	3'-TACTTTGCGCGGAGACAGATG-5'	CAC→GCC
G139A	3'-TGCAACTAACGAAGCGAGAGG-5'	GGT→GCT
G143A	3'-AGCGAGAGGCGGCGACGCGGC-5'	GGC→GCC
P146A	3'-CCGCGACGCCGGGGCCACTAG-5'	CCG→GCC
P147A	3'-CGACGCGGCCGCCACTAGCCG-5'	CCG→GCG
G150A	3'-GGCCACTAGCGGATGACACGC-5'	GGC→GCC
Y151A	3'-GCCACTAGCCGCGGACACGCCATT-5'	TAC→GCC
W172A	3'-AAGTCGGACCGCGTCTACGGA-5'	TGG→GCG
P175A	3'-ACCGTCTACCGAGTGAGGATA-5'	CCT→GCT
H176A	3'-GTCTACGGACGGAGGATACGG-5'	CAC→GCC
D187A	3'-GCGAAATTCCGAATGGTCCGC-5'	GAT→GCT
Y188A	3'-AAATTCCTACGGGTCCGCCGT-5'	TAC→GCC
K200A	3'-GGTCACCATCGTCCGTAAAGC-5'	AAA→GCA
K206A	3'-AGCCACCGCCGCTTAGTGTAG-5'	AAG→GCG
H208A	3'-AGCCACCGCTTCTTACGGTAGTGCGACATATAG-5'	CAC→GCC
K232A	3'-CGACCCATACGTATAGACCAC-5'	AAA→GCA
D256A	3'-TTTCAACGACGACTGTCTTAG-5'	GAT→GCT
D257A	3'-CAACGACTACGGTCTTAGACC-5'	GAC→GCC
F265A	3'-CGCGCGTTCGACCGGCCGAAGAGATAG-5'	TTC→GCC
S268A	3'-AAGCCGAAGCGATAGTAGCGG-5'	TCT→GCT
D282A	3'-TACAGGCAACGAAAATACCAT-5'	GAT→GCT
D287A	3'-TACCATGGCCGGAGCGTATGC-5'	GAC→GCC

Table II-1 Mutagenic oligonucleotides used for site-directed mutagenesis in CyoE
Sequences of mutagenic primers complementary to the *cyoE* sense strand. The mutagenized codon are underlined and the changed nucleotides are shown by boldtype.

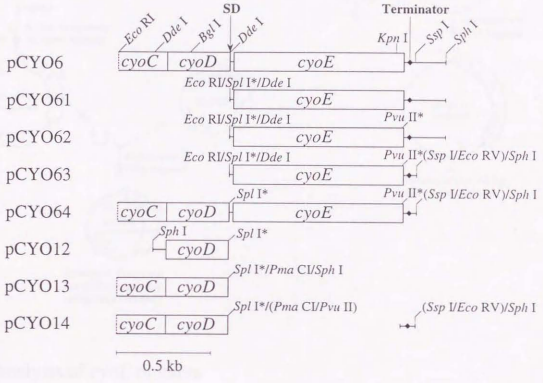


Fig. II-1 Physical map of the vector plasmids used
The inserted DNA regions of a subcloned *cyo* gene and the unique restriction sites are indicated. The restriction sites artificially introduced are marked by asterisks.

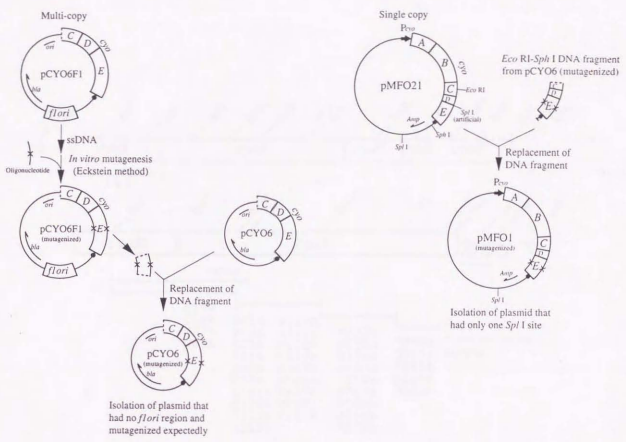


Fig. II-2 Strategies for analysis of CyoE mutants

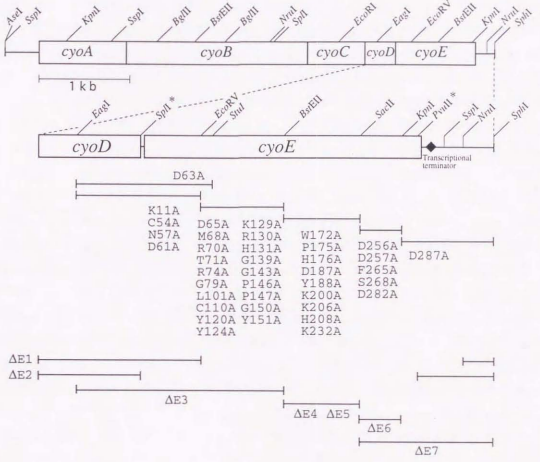


Fig. II-3 Strategies for DNA sequencing of Ala replacement and deletion mutants in CyoE

The DNA fragments introduced with the single mutation indicated were execized at the minimal length using the indicated restriction enzyme sites, and the resulting DNA fragments were introduced pCYO6 (in Material and methods). The expected mutation in mutagenized DNA region was confirmed by DNA sequencing.

Chapter III. Introduction of alanine-scanning and deletion mutagenesis into the *cyoE* gene

Introduction

Bacterial and mitochondrial terminal oxidases have significant homologies mainly with subunit I and partially with subunit II and III but not with other minor subunits. Active bacterial terminal oxidases were first purified with two subunit preparations (Yamanaka *et al.* 1979, Ludwig and Schatz 1980) whereas mammalian (Capaldi 1990) and yeast (Taanman and Capaldi 1992) mitochondrial aa_3-type oxidases contained up to 13 subunits (Capaldi 1990). It is now known that subunit I, II and III are encoded by mitochondrial genome and other ten subunits are encoded by the respective genes in the chromosome. Recent molecular biological studies have indicated that the gene clusters for heme-copper terminal oxidases in eubacteria are organized with 4 to 6 ORFs although up to 4 subunits were biochemically identified in many purified oxidase complexes. The genes other than those for subunits I to III and IVB may encode the CyoE family homologues and for the CtaA family homologues, however, the function of these genes was largely unknown. The *Escherichia coli cyo* operon consists of five ORFs (Chepuri *et al.*, 1990, Nakamura 1990). The *cyoA, B* and *C* encode subunit II, I and III, respectively, of the cytochrome *bo* complex (Nakamura *et al.* 1990, Minghetti *et al.* 1992) and the *cyoD* gene surely encodes subunit IVB (Nakamura 1990). The CyoD homologues in thermophilic bacterium PS3 (Sone *et al.* 1990) and *Acetobacter aceti* (Fukaya *et al.* 1993) were identified as subunit IVB by peptide sequencing. All 5 ORFs in the *cyo* operon are required for functional expression of the oxidase complex (Nakamura *et al.*, 1990) although the active oxidases have been purified as two subunit (Kita *et al.* 1984) and four subunit enzymes (Matsushita *et al.* 1984, Uno *et al.* 1994). In our laboratory, a five subunit preparation of the *bo* complex could be obtained using dodecylsucrose SM-1200 (Nakamura 1990). The mobility of the fifth subunit in SDS-PAGE analysis appeared to respond to that of CyoE expressed in mini cell system (Nakamura 1990), suggesting that subunit V may be encoded by the *cyoE* gene. Recent molecular biological studies have revealed that the *cyoE* gene homologues exist not only in the operons and the gene clusters of eubacteria but also in the nuclear genome in *Saccharomyces cerevisiae* (**Fig. III-1**). But any molecular biological approaches in the CyoE protein family were never reported yet.

In *Escherichia coli*, the fast and progressive molecular biological and biochemical approaches are applicable than in any other cells. In this chapter, I focused my efforts to identify the essential residues and regions of the CyoE protein. I have constructed 6 CyoE deletion mutants and individually substituted alanines for 40 out of 296 amino acid residues of the CyoE protein, including 22 out of 23 invariant residues. Furthermore, spectroscopic and biochemical characterizations of the CyoE deficient mutants were carried out to analyze the functional roles of the CyoE protein.

Results

Construction of CyoE deletion mutants

The CyoE protein is composed of 296 amino acid residues and has putative seven membrane spanning regions (Chepuri *et al.*, 1990) (**Fig. III-2**). The topology of CyoE was determined by gene fusion experiments (Chepuri and Gennis, 1991). According to this model, all the long loops

including the loop IV-V (71 residues) were located in the cytoplasm. The CyoE protein family has 20-30% amino acid sequence identity. The amino acid alignment shows that 24 amino acid residues are completely conserved (**Fig. III-1**). Among them, 17 residues are located in the cytoplasmic loops and 6 residues are in the membrane spanning regions in CyoE. Loops II-III, IV-V and VI-VII contain 5, 12 and 0 conserved residues (**Fig. III-2**). This suggests that the cytoplasmic loops and the membrane spanning regions are both important for the function of CyoE. To confirm and minimize the essential regions of CyoE, intramolecular deletion mutants of CyoE were constructed. ΔE3 (Δ62-74) was deleted 13 residues in loop II-III (22 residues) and contains all the 5 conserved residues. ΔE4 (Δ154-169) was deleted 16 residues in the middle of loop IV-V (71 residues) and contains no homologous residues. ΔE5 (Δ189-197) was deleted 9 residues in loop IV-V and contains 1 conserved residue. ΔE6 (Δ253-263) was deleted 11 residues in loop VI-VII (21 residues). ΔE7 (Δ281-end) was deleted half of the membrane spanning region VII to the end (**Fig. III-3**). These deletions were introduced by site-directed mutagenesis. At the same time, the complete CyoE deletion mutant, ΔE2 (Δ1-end), was constructed since ΔE1 (Δ64-end) was also deleted the transcriptional terminator of the *cyo* operon, too (Nakamura 1990) (**Fig. III-3**). As described later, all the deletion mutants were defective in the cytochrome *bo* oxidase activity completely so that I could not minimize the essential region of CyoE. In Δ E1 mutant, the expression level of subunit I (CyoB) in the cytoplasmic membranes was estimated to be 50% of the wild-type cells by Western blotting analysis with anti-subunit I rabbit serum. On the other hand, other CyoE deletion mutants retained comparable levels (72-97% of wild-type) of subunit I (Saiki *et al.* 1992).

Alanine scanning in CyoE

In the CyoE protein family, 23 amino acid residues in total (including Ala191) have been shown to be strictly conserved and mainly localized in the putative cytoplasmic loops (**Fig. III-2**): Asn-57, Asp-65, Met-68, Arg-70, Thr-71, and Arg-74 in loop II/III (domain 1, NNYXDRDIDXXMERTKXRP); Lys-129, Arg-130, Gly-138, Gly-143, Pro-146, Pro-147, and Gly-150 in loop IV/V (domain 2a, KRXXXXNTVVGSXSGAVPPXIGW); and Trp-172, Pro-175, His-176, Tyr-188, and Ala-191 in loop IV/V (domain 2b, WQXPHFXALAXXXXXDYXAAGIPML), suggesting their functional and/or structural significance. Other invariant residues are scattered in transmembrane regions (Leu-48, Gly-79, Tyr-120, and Tyr-124) or in the periplasmic loop III/IV (Leu-101). To identify the domain structures or the essential residues in the catalytic center of the CyoE protein, I introduced alanine scanning mutagenesis into the CyoE protein and examined the effects of 40 amino acid substitutions in a 296-residue-long polypeptide chain. Alanine was selected as a substituting amino acid since it is less likely to perturb packing or long-range interactions in proteins and therefore is suitable for the examination of the role of amino acid side chains. Substitutions of all the invariant residues except Ala-191 and 16 charged residues plus Phe-265 and Ser-268 (**Fig. III-2**) have been introduced via oligonucleotide-directed site-specific mutagenesis, and DNA sequences of the mutant multicopy plasmids were confirmed throughly not to contain any unexpected mutations. The mutant *cyo* operons were expressed by the single copy vector pMFO1, a derivative of the F-sex factor, to avoid a

lethal effect resulting from overproduction of membrane proteins or the gene dosage effects of the cytochrome *bo* complex.

Complementation test for aerobic growth of the constructed mutants

The catalytic activities of the mutant oxidases were expected to correlate with the rates of aerobic growth. Thus, I examined whether they are able to support the aerobic growth of the *cyo cyd* double deletion mutant using the single-copy expression vector. The *cyo* gene containing CyoE deletion mutants were replaced with that of pMFO21, a single-copy mini-F plasmid, and a resultant plasmid was introduced into ST2592 (W3110 Δ*cyo* Δ*cyd*) for analyzing *in vivo* aerobic growth capacity as described in "Experimental Procedures". At all the growth temperatures tested (23, 30, 37 and 42°C), none of the *cyoE* mutants exhibited a temperature-sensitive phenotype. Based on the growth phenotypes, the *cyoE* mutants were divided into two groups: the wild-type mutants that can grow aerobically both on minimal glycerol and minimal glucose plates via oxidative phosphorylation (17 out of 40 mutants constructed) and the defective mutants that can grow only on minimal glucose plates via glycolysis (**Table III-1**). The latter was all the deletion mutants (ΔE1-ΔE7) and 23 alanine replacement mutants (the defective mutants) and the former was 17 alanine replacement mutants (the wild-type mutants). Out of these essential residues, 18 residues were located in the cytoplasmic loops; those were Asn-57, Asp-61, Asp-63, Asp-65, Arg-70 and Arg-74 in loop II-III, Lys-129, Gly-143, Pro-146, Tyr-151, Trp-172, Pro-175, His-176, Asp-187 and Tyr-188 in loop IV-V, and Asp-256, Asp-257 and Ser-268 in loop VI-VII. The other 5 residues were Lys-11, Tyr-120, Tyr-124, Lys-206 and Asp-282 and were located in the membrane spanning regions (**Fig. III-4**). On the other hand, non-essential residues were identified as 8, 8 and 1 residues in the cytoplasmic loops, membrane spanning regions and periplasmic loops, respectively. Furthermore, out of 17 conserved residues analyzed in the cytoplasmic loops, 11 residues were identified to be essential. These results suggested that the conserved essential residues and the cytoplasmic loops II-III, IV-V and VI-VII were important for the function of CyoE (Saiki *et al.* 1993b). From these observations, 3 putative domains, domain 1 in loop II-III and domain 2a and 2b in loop IV-V were supposed in CyoE function (Saiki *et al.* 1993b) (**Fig. III-4, III-14**).

Expression level of subunit I and II in the *cyoE* mutant cytochrome membranes

A correlation of a loss of the catalytic activity with the expression level of the mutant oxidases was examined by immunochemical quantitative analysis of the mutant oxidases. The cytoplasmic membranes were prepared from ST4676 (Δ*cyo*) harboring pMFO21 derivatives and subjected to Western blot analysis using rabbit polyclonal anti-subunit I antiserum. The amounts of subunit I polypeptides in the mutant membranes were the same as those in the wild-type membranes (Saiki *et al.* 1992 and 1993b) (summarized in **Table III-1**). This indicates that loss or instability of subunit I polypeptides can be ruled out as a major cause of the defective mutations. In the *COX10* deletion mutants in *Saccharomyces cerevisiae*, specific degradation of subunit I was observed (Nobrega *et al.* 1990), but any observable degradation of subunit I was not detected in the *cyoE* mutants.

Effect of wild-type mutations on metal centers of the mutant oxidases in cytoplasmic membranes

Spectroscopic properties and copper contents of the mutant oxidases were studied in cytoplasmic membrane vesicles prepared from ST4676 (W3110 Δ*cyo*) harboring pMFO21 derivatives (Δ*cyo cyd*$^+$.*cyo*$^-$). The effects of amino acid substitutions on the low-spin and the high-spin hemes were studied by UV-visible spectroscopy. The low-spin heme was qualified as cytochrome $b_{563.5}$ by the amplitude of a 563.5 nm peak in the second-order finite spectra of dithionite-reduced *minus* air-oxidized difference spectra at 77 K. The high-spin heme was estimated as cytochrome *o* from the CO-reduced *minus* reduced difference spectra at room temperature. Cytochrome *o* has typical features, with a peak of at 416 nm and a trough at 430 nm in the CO-binding spectra. The content of the Cu_B center was estimated as the amount of copper in the cytoplasmic membranes since the cytochrome *bo* complex is a major copper-binding protein in the *Escherichia coli* cytoplasmic membranes. Besides the mutant cytochrome *bo* complex, the aerobically growing cells express the cytochrome *bd* complex and cytochrome b_{556} of succinate dehydrogenase. Thus, vigorously aerating cultures were harvested at the early late log phase (OD_{650}=0.7-0.8) to minimize the expression of the cytochrome *bd*. The wild-type mutant oxidases showed the 563.5 nm peak for the low-spin heme and the typical CO-binding spectra as the wild-type oxidase, and the amounts of cytochrome *o* and copper in the mutant membranes were similar to those (0.39 and 0.33 nmol/mg protein, respectively) in the wild-type membranes. Therefore, the metal centers present in the wild-type mutant oxidases are all normal, in agreement with the results of the genetic complementation test (Saiki *et al.* 1993b) (**Fig. III-5**, **Table III-1**).

Effects of defective mutations on the metal centers of the mutant oxidases in cytoplasmic membranes

The mutations and deletions that caused a defect in the *in vivo* activity of the mutant oxidases (the defective mutant group) reduced the amounts of all the metal centers. Interestingly, all the defective mutants showed the same results. The 563.5 nm peak of the low-spin heme in the 77 K redox difference spectra and the copper content of the defective mutant membranes were reduced to two-thirds of the wild-type level. The CO-binding activity of the high-spin heme in the defective mutant oxidases were affected more severely and was reduced to one-third of the wild-type level, accompanied by 2-4 nm red shifts of the Soret peak in the reduced CO-bound form (Saiki *et al.* 1993b) (**Fig. III-6**, **Table III-1**). These features were exactly the same as those of the *cyoE* deletion ΔE2 (Saiki *et al.* 1992). Very interestingly, any subunit deletion of the *bo* complex never caused these red shifts of CO-binding spectra (Nakamura 1990) so that this red shift was specific for the *cyoE* defective mutants. These results suggested that 1) the replacement of any one of the essential residues caused complete loss of the function of CyoE, 2) the severe effect on high-spin heme meant that severe damage took place in the high-spin heme binding site, 3) but the degradation of subunit I was not affected at all. It should be stressed that the observed red shift of CO-binding spectra was specific in the *cyoE* mutants. Therefore, defective oxidases should be purified for further biochemical and enzymatic analyses.

Ubiquinol oxidase activity and subunit composition of partially purified defective mutant oxidases

Biochemical and biophysical analyses with the use of partially purified mutant oxidases were carried out. Out of the defective mutants, Asp65, Tyr120 and Trp172 were selected as representative of three different topological domains, cytoplasmic loop II-III (domain 1), membrane-spanning helix IV, and cytoplasmic loop IV-V (domain 2b), respectively (**Fig. III-4**). Additionally, the *cyoE* gene deletion mutant, ΔE2 was selected as a control mutant oxidase. Cytochrome *bo* complex is expressed advantageously in the early phase of growth prior to the switching to the expression of cytochrome *bd* complex. To reduced the expression level of the cytochrome *bd* complex, the cells were grown aerobically in a jar fermenter with vigorous agitation and high aeration, and harvested at the early exponential phase of growth. To avoid any artificial effect on the expressed oxidases, expression of the *cyo* operon was on the single-copy vector plasmid and the cells were harvested at the early phase of growth. Cytoplasmic membranes were prepared from 60 liter cultures of ST4676 (Δ*cyo*) harboring pMFO1, pMFO1-D65A, -Y120A, -W172A and -ΔE2 and purified as described. All the partially purified mutant oxidases were found to be defective in the ubiquinol-1 oxidase activity, consistent with the genetic complementation test, indicating that these *cyoE* mutations completely eliminated the catalytic function of the cytoplasmic *bo* complex (**Table III-2**). Next, these partially purified oxidases were subjected to SDS-15% polyacrylamide gel electrophoresis followed by silver staining to analyze subunit compositions of the oxidase complexes. In the wild-type oxidase, it composed of five subunits (apparent molecular masses of 46, 32, 21, 13 and 26.5 kDa). The 26.5 kDa polypeptides were thought to be a candidate of CyoE protein (Mogi and Anraku 1990, Nakamura 1990, Minghetti *et al.* 1992). All the other mutant oxidase complexes showed the same subunit composition with the wild-type oxidase complex (Saiki *et al.* 1993b) (**Fig. III-7**).

Analysis of the metal centers of the partially purified defective mutant oxidases

The second-order finite difference spectra of the 77K redox difference spectra showed two negative peaks at 563.5 and 555 nm and are qualitatively identified to those of the wild-type oxidase (**Fig. III-8**). The spectra also indicate that there is no other cytochromes contaminating in the purified mutant oxidase preparations (*i.e.* the cytochrome *bd* complex and cytochrome b_{556}). The CO-binding difference spectra confirmed that the 3 nm blue shift of the high-spin heme Soret peak appeared under the reduced CO-bound conditions that was observed in the cytoplasmic membranes. The amplitudes of the absorbance change upon binding of carbon monoxide in the CO-binding difference spectra were reduced to one-third of the wild-type level. These results were essentially the same with those in the defective cytoplasmic membranes and indicated that the CO-bound high spin heme portion was affected by defective *cyoE* mutations. Copper and heme contents were determined by atomic absorption spectroscopy and the pyridine hemochromogen method, respectively. The mutant oxidases contained 0.71-1.02 mol of copper atoms/2 mol of heme prosthetic groups, and the heme content of the mutant oxidases was 82-91% of that of the wild-type oxidase when normalized with subunit I content (**Table III-2**). This indicates that the defective *cyoE* mutations lowered the

affinity of the Cu_B-binding site for copper and that the supplement of copper ions in the culture completely restored the partial loss of the Cu_B center. The contents of Cu_B are likely unaffected by *cyoE* defective mutations. Three metal centers in the defective oxidases were further analyzed by EPR spectra of the mutant oxidases using air-oxidized, resting enzyme at 15 K (**Fig. III-9**). All the mutant oxidases contained both the high spin and low spin hemes, which are assignable by the axial g=6.0 and 3.0 signals, respectively (Hata *et al.* 1985, Tsubaki *et al.* 1993). The enhancement of the high spin g=6.0 signal in the mutant oxidases relative to that in the wild-type oxidase may be attributed to a partial loss of the spin coupling between the Fe_O (S=5/2) and Cu_B (S=1/2) (Tsubaki *et al.* 1993). All these results showed apparently that the high-spin heme sites were severally damaged in the defective mutants so that the oxidases lost their enzymatic activity (Saiki *et al.* 1992 and 1993b).

Heme composition of the partially purified defective mutant oxidases

The cytochrome *bo* complex has one molecule of heme B and one molecule of heme O in subunit I. These heme compositions in the defective mutant oxidases were analyzed. Heme B shows the α and β peaks of the pyridine-hemochrome spectra at 556.5 and 525 nm, respectively, while heme O shows those at 553 and 521 nm, respectively (Puustinen and Wikström 1991). The α and β peaks of the partially purified defective mutant oxidases were shifted compared with the wild-type oxidase from 555 to 556.5 and from 523 to 525 nm, respectively. These results suggested that the heme species bound in the mutant oxidases were two molecules of heme B instead of one heme B and one heme O. It should be stressed that total amount of hemes of the mutant oxidases, determined by the pyridine-hemochrome spectra using an extinction coefficient of protoheme IX, were comparable with that of the wild-type oxidase (**Table III-2**). Further evidence was proved by reverse phase HPLC. As heme O is prenylated, it can be separated from heme B by a C_{18} column reverse phase HPLC (Puustinen and Wikström 1991). Hemes eluted by acetone/HCl and diethyl ether were separated on a C_{18} column and were detected by their Soret absorption. Reverse phase HPLC of heme elution profiles definitively showed that hemes in the mutant oxidases eluted with one peak and their retention times were coincided with those of heme B of the wild-type *bo* complex or protoheme IX of bovine hemoglobin (**Fig. III-10**). To eliminate the possibility that heme O was lost or changed to heme B through the purification process, heme composition of mutant cytoplasmic membranes were analyzed. The reverse phase HPLC profile of hemes eluted from the defective cytoplasmic membranes showed clearly that heme O was changed to heme B in the defective mutant oxidases membranes (**Fig. III-11**). These results indicated that the defective *cyoE* mutants replaced heme O with heme B in the high-spin heme binding site in subunit I of the *bo* complex and resulted in the loss of oxidase activity (Saiki *et al.* 1992 and 1993b).

Heme compositions of *cyo* gene deletion mutants

H. Nakamura constructed the *cyoA*, *B*, *C*, *D*, and *E* deletion mutants and showed that all the five ORFs of the *cyo* gene were essential for the active enzyme complex (Nakamura 1990). Then, heme compositions of these cytoplasmic membranes were analyzed using reverse phase HPLC heme

analysis system. Apparently, only the control and the *cyoE* deletion mutant membranes lost heme O completely whereas other deletion mutant membranes contained heme O besides heme B (**Fig. III-12**). The fact that the *cyoB* deletion mutant membranes still contained heme O suggested that heme O is localized in the cytoplasmic membranes. These results indicate that CyoE is required for heme O biosynthesis and that other CyoABCD polypeptides are not.

Conclusion

I have constructed 6 CyoE deletion mutants and have individually substituted 40 amino acid residues of the CyoE protein including 22 invariant residues with alanines. I found that the 6 deletion and 23 alanine substitution mutant oxidases were nonfunctional and showed a specific loss of the CO binding activity at the site of the high-spin heme. Characterizations of the partially purified D65A, Y120A, and W172A mutant oxidases, which have the mutations of different topological domains, and the *cyoE* deletion mutant oxidase revealed that their defects are attributable to substitution of protoheme IX for heme O present in the high-spin heme-binding site (**Fig. III-13**). Based on these observations, I propose that the conserved amino acid residues present in the cytoplasmic loops II/III and IV/V are part of the catalytic center of the CyoE protein.

Discussion

Importance of heme O in the cytochrome *bo* complex

The cytochrome *bo* complex had long been considered as a heme BB-type quinol oxidase (Kita *et al.* 1984, Matsushita *et al.* 1984, Chepuri *et al.* 1990). Recently, Bolgiano *et al.* (1991) and Puustinen and Wikström (1991) noticed the atypical pyridine ferrohemochrome spectrum of hemes in membranes and in the purified oxidase that had been isolated from the cytochrome *bo* complex-overproducing strains. Puustinen and Wikström (1991) demonstrated that the cytochrome *bo* complex contains a novel heme species, heme O. It was shown to be a 2-hydroxyethylfarnesyl derivative of heme B by fast-atom bombardment mass spectroscopy (*Mr* of 839), ^{1}H-NMR (nuclear magnetic resonance), infrared and resonance Raman spectroscopies (Puustinen and Wikström 1991, Wu *et al.* 1992). Heme O contains a methyl group in place of the formyl group at position 8 in heme A (Caughey *et al.* 1975). This makes heme O more hydrophobic and causes its hemochrome to shift more to the blue range than that of heme A or heme B. Although Puustinen and Wikström (1991) originally thought that the cytochrome *bo* complex had two heme O molecules (as a heme OO-type oxidase; cytochrome *oo3*), it later turned out that this was an artifact resulting from the use of the oxidase-overproducing strain (Puustinen *et al.* 1992). The cytochrome *bo* complex isolated from the wild-type strains or from a merodiploid strain of the cytochrome *bo* operon reproducibly contains one mole each of heme B and heme O (heme BO-type) (Saiki *et al.* 1992, Tsubaki *et al.* 1993, Puustinen *et al.* 1992). In this chapter, I found that the defective *cyoE* mutants showed a conversion of heme BO-type to heme BB-type (**Fig. III-13**). These inactive heme BB-type oxidases showed the specific defect in the high-spin heme. Therefore, my study have shown that heme B and heme O are

bound to the low-spin and the high-spin heme-binding sites, respectively (Saiki *et al.* 1992 and 1993b, Hill *et al.* 1992). Furthermore, heme O in the high spin heme site is essential for the functional cytochrome *bo* oxidase complex (Saiki *et al.* 1992 and 1993b).

The *cyoE* gene product (with a deduced molecular mass of 32 kDa) is predicted to have seven transmembrane helices (Chepuri *et al.* 1990, Chepuri and Gennis 1990) and has been suggested to be subunit V of the oxidase complex (with an apparent molecular mass of 28 kDa on SDS/urea-polyacrylamide gels) (Nakamura 1990, Minghetti *et al.* 1992), although subunit V is not required for the oxidase functions *in vitro* (Kita *et al.* 1984, Matsushita *et al.* 1984). Size-exclusion HPLC analysis has shown that subunit V is tightly associated with the wild-type oxidase complex (Mogi, T., Nakamura, H., and Anraku, Y, unpublished results). In this work, I found that this 28-kDa polypeptide is present even in the *cyoE* complete deletion mutant oxidase (ΔE2) in SDS-polyacrylamide gel electrophoresis analysis. This indicates that the CyoE protein is not an assembly factor of the cytochrome *bo* complex and that subunit V is unlikely to be the *cyoE* gene product.

Functional role of CyoE

Previously, it was reported that deletion of the *cyoE* homologue in the *aa*3-type cytochrome *c* oxidase genes of *Paracoccus denitrificans* (Steirücke *et al.* 1991) and *Rhodobacter sphaeroides* (Cao *et al.* 1992) or of the yeast nuclear gene *COX10* (Nobrega *et al.* 1990) eliminates typical cytochrome *a* absorption at around 605 nm and destabilizes subunit I to which two heme A molecules are bound. Deletion/complementation studies in yeast (Nobrega *et al.* 1990, Tzagoloff *et al.* 1990), *P. denitrificans* (Steirüke *et al.* 1991) and *R. sphaeroides* (Cao *et al.* 1992) have suggested that the *ctaB/COX10* and also *ctaG/COX11* gene products function as assembly factors (i.e. they either confer the proper tertiary structure on the subunit polypeptide or allow the ordered association of subunit proteins to form the *hetero*-oligomeric complex). Alternatively, since the farnesylated heme A and heme O are specific for the heme-copper respiratory oxidases among known cytochromes, the CtaB/COX10p protein may participate in a pathway of heme A biosynthesis or in the insertion of the heme and/or copper prosthetic groups into subunit I (Nobrega *et al.* 1990, Tzagoloff *et al.* 1990, Steinrücke *et al.* 1991).

To elucidate the functional role of the *cyoE/ctaB/COX10* gene, I have carried out systematic deletion and alanine scanning mutagenesis of the *E. coli cyoE* gene (Saiki *et al.* 1992, 1993b). I found that the *cyoE* mutations that caused a defect of *bo*-type quinol oxidase activity resulted in conversion of an active heme BO-type oxidase to a non-functional heme BB-type oxidase (**Fig. III-13**) (Saiki *et al.* 1992 and 1993b, Hill *et al.* 1992). Since the conversion of the heme type was associated with marked perturbations of the binuclear center (i.e. decrease in the CO-binding activity and 3-4 nm red-shifts of the Soret peak), these results supported that the CyoE protein is necessary for heme O biosynthesis in *E. coli*.

Based on the results of alanine scanning mutagenesis in CyoE, I found that three essential domains (domain 1, 2a and 2b) are present in the CyoE cytoplasmic loops (**Fig. III-14**). Recently, the *E. coli ubiA* gene which encodes a 4-hydroxybenzoate octaprenyltransferase involved in ubiquinone-8 biosynthesis has been sequenced and was found to be a homologue of the *cyoE* gene

(21% identical in the protein level) (Nichols and Green 1992, Siebert *et al.* 1992). The proposed tertiary structures of CyoE and UbiA (or COQ2) have high resemblance, suggesting that they were derived from the same origin. Interestingly, observed homologous regions between CyoE protein family and UbiA/COQ2 are concentrated in domain 1, 2a and 2b of the cytoplasmic loops in CyoE (**Fig. III-14**). The most highest homologous region falls in the loop II-III of CyoE, which includes an aspartic acid rich sequence (DRDID in CyoE and DRKFD in UbiA). This motif was proposed for the interaction of hexaprenyl-pyrophosphate synthase and farnesyl-pyrophosphate synthase with Mg^{2+} ion bound to the pyrophosphate moiety (Ashby and Edwards 1990). A stretch of the hydrophobic residues that followed this region was ascribed to the binding site for the farnesyl moiety (Ashby and Edwards 1990). Farnesyl diphosphate synthase is a polyprenyl transferase and catalyzed the sequential 1'-4 condensation of the 5-carbon isoprenoid compounds isopentenyl diphosphate (IPP) and dimethylallyl diphosphate to form the 10-carbon geranyl diphosphate (GPP), which is then condensed with another IPP molecule to form the 15-carbon FPP. It contains two DDXXD motifs, which are DDXXDXXXXRRG in Domain I and DDXXD in domain II in FPP synthases (Song and Poulter 1994). The first aspartate residue in the DDXXD motif of both domains I and II of FPP synthase were shown to be essential for the catalysis by farnesyl-pyrophosphate synthase whereas the third aspartate is dispensable for the functions (Marrero *et al.* 1992, Joly and Edwards 1993, Song and Poulter 1994). The conserved arginine residue followed by DDXXD motifs in domain I of FPP synthase are also essential for the functions of FPP synthase (Joly and Edwards 1993). In domain 1 of the CyoE protein, all the aspartate and arginine residues (DXDIDXXMXRTXXR) appear to be essential for its catalytic functions (in chapter III of this thesis). The aspartate and arginine residues in domain 1 of CyoE are well conserved in the CyoE protein family and polyprenyltransferase of ubiquinone biosynthesis (UbiA/COQ2). Interestingly, the DDXXD motif is found in oxidosqualene cyclase that catalyzes the cyclization of oxidosqurene to lanosterol. Using the suicide substrate of oxidosquarene, tritium-labeled 29-MOS, the first and second aspartate residues of the DDXXD motif of oxidosquarene were labeled (Abe and Prestwich 1994). This result indicates that these aspartate residues are concerned directly with the catalytic reaction by stabilization of tertiary or allylic carbocations (Abe and Prestwich 1994). Based on the sequence similarity, Nichols and Green suggested that the CyoE protein may be the interaction site with ubiquinone-8 or ubiquinol-8 in the cytochrome *bo* complex (Nichols and Green 1992). However, this possibility is unlikely since the four-subunit oxidase complex composed of CyoA, B, C and D polypeptides is fully active in the ubiquinol-1 oxidation (Matsushita *et al.* 1984). All these results strongly suggest that the CyoE protein has an ability to conduct polyprenyl transfer. Therefore, in the following chapters, I attempted to verify this hypothesis that the CyoE protein is heme O synthase.

Table. III-1

Mutant	Aerobic growth	cyt o (nmol/mg protein)	Cu (nmol/mg protein)	Low spin heme	Subunit I
Wild type	+	0.39	0.33	+++	+++
control	-	0.00	0.01	-	-
Wild-type mutants					
L48A[a]	+	0.46	0.38	+++	+++
C54A	+	0.46	0.38	+++	+++
M68A	+	0.36	0.28	+++	+++
T71A	+	0.35	0.26	+++	+++
G79A	+	0.49	0.34	++	++
L101A	+	0.38	0.38	+++	+++
C110A	+	0.26	0.29	++	+++
R130A	+	0.37	0.34	++	+++
H131A	+	0.49	0.45	+++	+++
G139A	+	0.30	0.37	++	+++
P147A	+	0.42	0.47	++	++++
G150A	+	0.40	0.42	+++	++++
K200A	+	0.32	0.37	+++	+++
H208A	+	0.21	0.34	++	+++
K232A	+	0.40	0.35	+++	+++
F265A	+	0.44	0.45	+++	+++
D287A	+	0.38	0.50	+++	+++
Defective mutants (deletions)					
ΔE1	-	0.11	0.19	+	++
ΔE2	-	0.14	0.18	++	+++
ΔE2'	-	0.08	0.14	-	+
ΔE3	-	0.15	0.21	++	+++
ΔE4	-	0.13	0.18	++	+++
ΔE5	-	0.13	0.13	++	++
ΔE6	-	0.17	0.21	++	+++
ΔE7	-	0.19	0.26	++	+++
Defective mutants (Ala substitutions)					
K11A	-	0.15	0.16	++	+++
N57A	-	0.15	0.27	++	+++
D61A	-	0.14	0.17	++	++
D63A	-	0.10	0.14	++	+++
D65A	-	0.12	0.12	++	+++
R70A	-	0.15	0.15	++	+++
R74A	-	0.16	0.19	++	+++
Y120A	-	0.12	0.20	++	+++
Y124A	-	0.13	0.20	++	+++
K129A	-	0.16	0.19	++	+++
G143A	-	0.13	0.25	++	++
P146A	-	0.15	0.21	++	+++
Y151A	-	0.21	0.23	++	+++
W172A	-	0.13	0.22	++	+++
P175A	-	0.20	0.24	++	++
H176A	-	0.14	0.26	++	+++
D187A	-	0.21	0.21	++	+++
Y188A	-	0.19	0.21	++	+++
K206A	-	0.16	0.21	++	+++
D256A	-	0.19	0.28	++	+++
D257A	-	0.21	0.29	++	+++
S268A	-	0.19	0.25	++	++
D282A	-	0.13	0.26	++	++

[a]Mutants of the conserved amino acid residues are shown in boldface type.

Table III-1 Characterizations of the Mutant Oxidases in the Cytoplasmic Membranes

The CyoE mtants were classified into 2 groups based on the effect on the catalytic activity of the mutant oxidases determined by the genetic *in vivo* complementation test of the aerobic growth. The amounts of cytochrome *o* (high-spin heme) and low spin heme (cytochrome $b_{563.5}$) were determined by CO-binding difference spectra and 77 K redox difference spectra, respectively. Copper content and the expression level of subunit I were determined as described in "Experimental Procedures". Strain ST4676 carrying mini-F plasmid pMFO1, and pHNF2,was used as the wild-type control (Wild-type) and negative controls, respectively.

	cyt o	Cu	heme	Subunit I	Quinol oxidase
	nmol/mg protein[a]				%
Wild type	2.3	2.8	6.5	100	100
DE2	0.5	2.8	5.5	74	<0.1
D65A	0.7	2.3	5.9	88	<0.1
Y120A	0.7	1.9	5.3	90	<0.1
W172A	0.7	2.4	5.7	75	<0.1

[a]The amounts of the metal centers in the mutant oxidases were normalized with the amounts of subunit I polypeptide in each preparation.

Table III-2 Characterizations of the purified mutant oxidases

The amounts of cytochrome *o* and copper in the mutant oxidases were determined as described in "Experimental Procedures". Heme contents were measured by the pyridine hemochromogen method using an extinction coefficient of protoheme IX for heme O and are expressed as the sum of heme B and heme O. The relative amounts of subunit I are expressed as a percent of that in the purified cytochrome *bo* complex. Ubiquinol oxidase activity was measured using ubiquinol-1 as substrate and is expressed as a percent of the wild-type control (54.1 mmol of ubiquinol-1 oxidized per min/mg protein).

Fig. III-1 Sequence alignment of CyoE with homologous sequences

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B. sub.  :                                                MANSRILNDTAIDGQIEET
B. fir.  :                                              MNKSNTAIDPTNVIEAGPDSSVADVQQ
T. PS3   :                                                MAELKAVHQDAADAGHRSHVSVK
S. cer.  :    ...STSSGSEATTDASTQLPFNVKLVDPMVRKSKRSHAISEGLNMKTLKK
E. UbiA  :                                                MEWSLTQNKL
S. COQ2  :    ...SSSSSPSSKESAPVFTSKELEVARKERLDGLGPFVSRLPKKWIPYAE

                      -
E. coli  :    MMFKQYLQVTKPGIIFGNLISVIGG----FLL-A-SKGSI---D-Y----   3
P. den.  :                MSLVVFTAFVG----LWI-A-PQPVN---P-F----
B. sub.  :    TAWKDFLSLIKIGIVNSNLITTFTG----MSV-ALHISGL----SFLGNI
B. fir.  :    KSWKDYLVLAKQGIVTSNLITTFAG---IYLAIV-YTGTVFTMH-L----
T. PS3   :    TVWRELSSVVKIGIVNSNLITTFAGMWLAFYF-T--GEHF---LENL---
S. cer.  :    KVIMPYLQLTKPRLTILVMLSAICS----YAL-S-PYPAS---V-N----
E. UbiA  :    LAFHRLMRTDKPIGALLLLWPTLWA----LWV-A-TPGVP---Q-L----
S. COQ2  :    LMRLEKPVGTWLLYLPCSWSILMGA----MMQ-G-ATLSA---T-A----

                        +       +  -  -  -  +  -+   -       +
E. coli  :    PLFIYTLVGVSLVVASGCVFNNYIDRDIDRKMERTKNRVLVKGLISPAVS   8
P. den.  :    VAF-CAVLFIALGGGASGALNMWYDADIDAVMRRTAGRPVPSGRVTSQEP
B. sub.  :    NTVLLTLIGSSLIIAGSCAINNWYDRDIDHLMERTKVRPTVTGKIQPSQA
B. fir.  :    DTMIFALLGAALVMAGGCTLNNYIDRDIDHLMERTKERPTVTGRFSAKHV
T. PS3   :    HLVFFTLFGAALVIAGSCAINNYIDRDIDQYMERTKARPTVTGTMDPRRV
S. cer.  :    ELLCLT-VGTTLCSGSANAINMGREPEFDRQMVRTQARPVVRGDVTPTQA
E. UbiA  :    WILAVFVAGVWLMRAAGCVVNDYADRKFDGHVKRTANRPLPSGAVTEKEA
S. COQ2  :    GMLGIFGVGALVMRGAGCTINDFLDRKLDQRVIRSVERPIASGRVSPRRA

                          +                +       -    -   -++
E. coli  :    LVYATLLGIAGFMLLWFGANPLACWLGVMGFVVYVGVYSLYMKRHSVYGT 13
P. den.  :    LAVGIALSGLSVMMLGAGGNWFAAGFLAFTIFFYAVVYTIWLKRSTPQNI
B. sub.  :    LWSGILLVALGLIML-LMTTVMAAVIGFIGVFTYVVLYTMWTKRRYTINT
B. fir.  :    LLVGLAQAALGIIFLAL-TTPTAAVIGLIGLFIYVVLYTMWTKRTTTLNT
T. PS3   :    LWLGIGLVAIGEMSLLMTTVTAAV-VGLIGMVTYVFLYTLWTKRHYTITT
S. cer.  :    FEFAALIGTLGVSILYFGVNPTVAILGASNIALYGWAYT-SMKRKHIINT
E. UbiA  :    RALFVVLVLISFLLV-LTLNTMTILLSIAALAL-AWVYP-FMKRYTHLPQ
S. COQ2  :    LVFLGAQTLVGMGVLSL--LPAQCWWLGLASLPIVFTYPLF-KRFTYYPQ

                +   -  -+  +-                        -   --
E. coli  :    LIGSLSGAAPPVIGYCAVTGEFDSGAAILLAIFSLWQMPHSYAIAIFRFK 18
P. den.  :    VIGGAAGAFPPMIGWALPTGGIGIESLLMFALIFFWTPPHFWALALFMKD
B. sub.  :    VVGSVSGAVPPLIGWTAVEGNIGVVAWVLFMILFIWQIPHFLALAIKKTE
B. fir.  :    IVGSFSGAVPPLIGWAAIDGGLHLYAWLLFFIMFLWQPPHFLALAMKRVE
T. PS3   :    VVGSISGAVPPFIGWTAVDPEFHIVPLILFLIMFLWQPPHFLALAMKPCE
S. cer.  :    WLGALVGMVPPLMGWAAASPLSHPGSWCLAGLLFAWQFPHFNTLSHNIRN
E. UbiA  :    VVLGAAFGWSIPMAFAAVSESVPLSCWLMFLANILWAVAYDTQYAMVDRD
S. COQ2  :    AALSACFNWGALLGFPAMGVMSWPTMIPLYLSSYLWCMTYDTIYAHQDKK

                --             +     - +                    +
E. coli  :    DYQAANIPVLPVVKGISVAKNHITLYIIAFAVATLMLSLGGYA-GYKYLV 23
P. den.  :    DYSKAGVPMLTVTHGRKVTRCHIFAYTLVLAPFALWLGFTSVGG-PLYLA
B. sub.  :    DYRAANIPMLPDVYGFEVTKRQIIVWVACL--MPLPFFLGS-L-GLPIVI
B. fir.  :    EYRAAGIPMLPVVAGFEMTKRQMVVYVAALLPVSLM--LYPF--GLVYTI
T. PS3   :    EYRAAGIPMLPVVHGFAMTKRQIIVWVACL----LPLPFYLFSLGVPFLV
S. cer.  :    EYKNAGYVMTAWKNPLLNARVSLRYSILMFPL-CFGLSYFNIT-DWYYAQ
E. UbiA  :    DDVKIGIKSTAILFGQYDKLIIGILQIGVLALMAIIGELNGLGWGYYWSI
S. COQ2  :    FDIKAGIKSTALAWGPRTKSIMKAMSASQIALLAVAGLNSGLLWGPGFIG
```

Fig. III-1

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                                        --          +  -           -
E. coli  :      VAAAVSVWWLGMALRGYKVADDRIWARKLFGFSIIAITALSVMMSVDFMV 28
P. den.  :      VSVVLNALFIAGGWQILRRSEDQAQADGYRVEKRYFRLSLYYTFLHFLAL
B. sub.  :      LGLLLNIGWLILGLMGFRSKNIMKWATQMFVYSLNYMTIYFVAMVVLTLF
B. fir.  :      VAAVLGVGWLALGIAGFKMKDDIKWARLMFVYSLNYLTILFVLMVIVHF
T. PS3   :      VATLLNVGWLFLGLWGLKMKDDLKWAKWMFVYSLNYLTILFVAMIIATLW
S. cer.  :      IDSGLINAWLTFWAFKFYWQQRINYSAKTLKDNVKFNKGLSVANIYARKT
E. UbiA  :      LVAGALFVYQQKLIANRREACFKAFMNNNYVGLVLFLGLAMSYWHF
S. COQ2  :      GLGVFAYRLFSMIKKVDLDNPKKNCW--KYFNANINTGLYFTYALAVDYI

                 +
E. coli  :      PDSHTLLAAVW                                        29
P. den.  :      LVQHWVGGW
S. cer.  :      FMASVLHLPAILILAIIHKKGRWDWIYPGEAKRPQERF
S. COQ2  :      LRLFGFL
```

E. coli ; *Escherichia coli* ***cyoE*** gene product
P. den. ; *Paracoccus denitrificans* **ORF1** gene product...21%
B. sub. ; *Bacillus subtilis* ***ctaB*** gene product.....34%
B. fir. ; *Bacillus firmus* OF4 ***ctaB*** gene product.....37%
T. PS3 ; thermophilic *Bacillus* PS3 ***caaE*** gene product..33%
S. cer ; *Saccharomyces cerevisiae* ***COX10*** gene product..22%
E. UbiA ; *Esvherichia coli* ***ubiA*** gene product.....17%
S. COQ2 ; *Saccharomyces cerevisiae* ***COQ2*** gene product...19%

Fig. III-1 Sequence alignment of CyoE with homologous sequences
The amino acid sequences of a 4-hydroxybenzoate polyprenyltransferase in ubiquinone biosynthesis a also aligned. Each amino acid is aligned with CyoE sequence one by one and the results are then aligned. Conserved residues are showed in bold type. Residues absolutely conserved in CyoE protein family of the heme-copper oxidase superfamily are showed by underline in CyoE protein sequence. T results of complementation test in CyoE Ala mutants are indicated above the CyoE sequence. +, complemented; and -, not complemented the aerobic growth of ST2592, respectively.

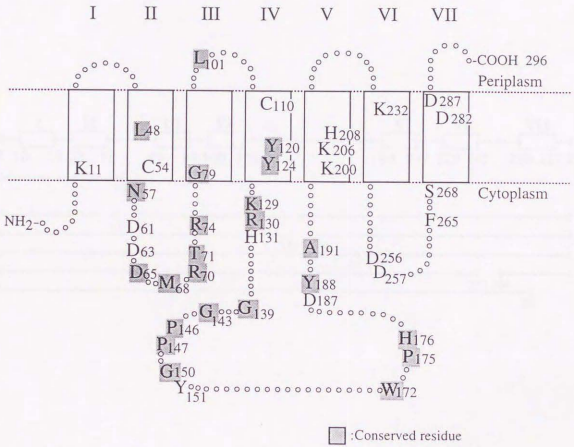


Fig. III-2 Topological model of CyoE
Target 40 residues except 191 alanine for alanine replacement experiment are indicated
Conserved 23 residues in Fig. III-1 are shown by shadow.

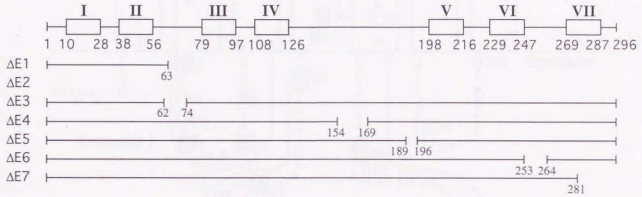


Fig. III-3 Locations of CyoE deletion mutants
Putative trans-membrane regions (I-VII) of CyoE in Fig. III-2 are indicated by box. Amino acid numbers are indicated below.

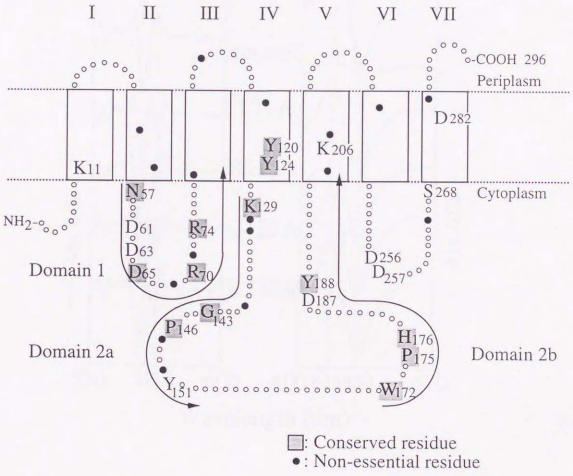


Fig. III-4 Location of essential residues by Ala scanning test
Essential residues that were determined by the results of the genetic aerobic complementation test are indicated. Domain 1, 2a and 2b are also shown.

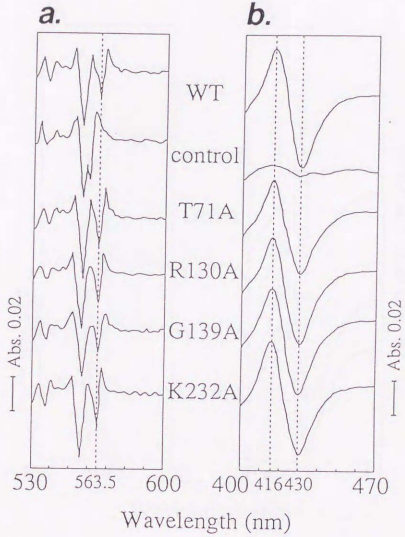


Fig. III-5 Second-order finite spectra of dithionite-reduced *minus* air-oxidaized difference spectra (left) and CO-reduced *minus* reduced difference spectra (right) of cytoplasmic membranes prepared from wild-type *cyoE* mutants.
Left panel, spectra were recorded with a Shimadzu UV-3000 double-wavelength spectrophotometer at 77K, and the protein concentration was 3 mg of protein/ml of 120 mM Tris-Cl (pH7.4). Measurements were done with a spaectral bandwidth of 1 nm, a light path of 2 mm, and a scan speed of 50 nm/min. *Right panel*, conditions used were the same as those described above, except that the measurements were carried out at room temperature at a protein concentrations of 0.5 mg of protein/ml with a light path of 10 mm. Strain ST4676 carrying mini-F plasmid pMFO1 or pHNF2 were used as the wild-type control (*WT*) and a negative control (*control*), respectively.

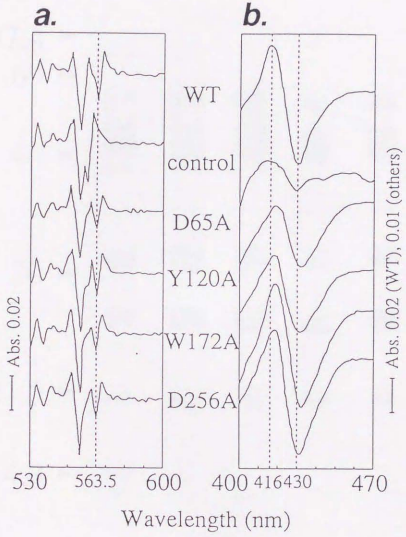


Fig. III-6 Second-order finite spectra of dithionite-reduced *minus* air-oxidaized difference spectra (left) and CO-reduced *minus* reduced difference spectra (right) of cytoplasmic membranes prepared from defective *cyoE* mutants.
Conditions are the same as with the legend in Fig. III-5.

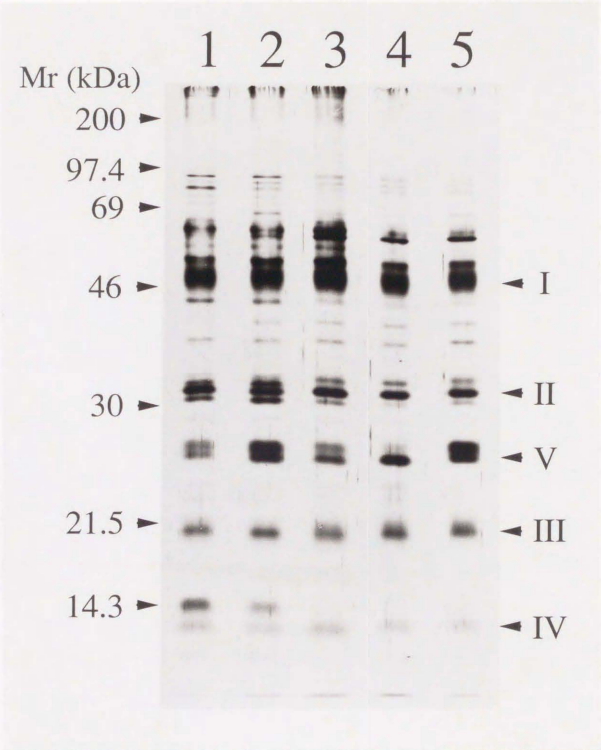


Fig. III-7 SDS-polyacrylamide gel electrophoresis analysis of partially purified *bo* complex of *cyo* deficient mutants
The partially purified *bo* complex (0.5 μg of protein) of wild-type; lane 1, ΔE2; lane2, D65A; lane3, Y120A; lane4, and W172A; lane5 were subjected to SDS-PAGE analysis followed by silver stainning. Molecular weight size and subunits of the *bo* complex were indicated in left and right sides, respectively, of the gel.

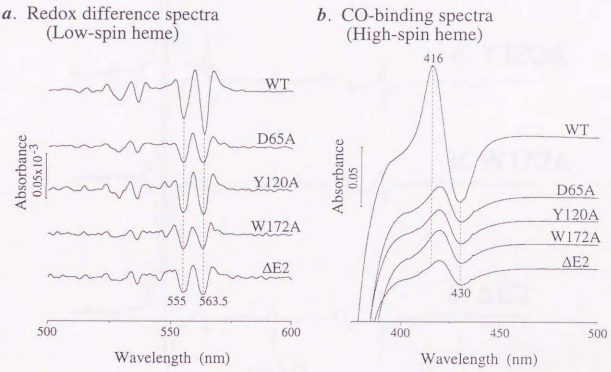


Fig. III-8 Second-order finite spectra of dithionite-reduced *minus* air-oxidized difference spectra (a) and CO-reduced *minus* reduced difference spectra (b) of partially purified wild-type and *cyoE* mutant oxidases

The protein concentrations used for measurements of 77K redox difference spectra and CO binding spectra were 1.0 and 0.2 mg, respectively, of protein/ml of 120 mM Tris-Cl (pH7.4) containing 0.1% sucrose monolaurate. Other detailes are described in the legend to Fig. III-5. *WT*; wild-type.

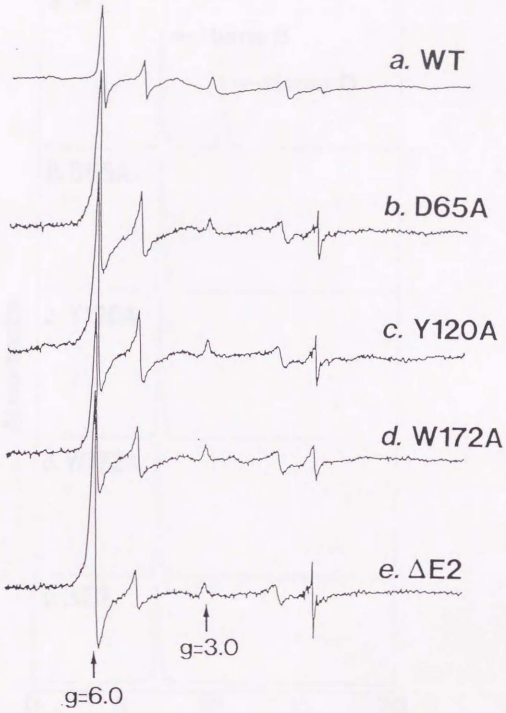


Fig. III-9 EPR spectra of partially purified *cyoE* mutant oxidases in air-oxidized, resting form

Spectra were taken at 15K using an oxidase solution (100 μM heme) in 50 mM Tris-Cl (pH7.4) containing 0.1% sucrose monolaurate and arbitrarily normalized with respect to the amplitude of the resonance at g=3 of the low-spin heme. Spectrometer conditions were as follows: modulation amplitude, 10 G; modulation frequency, 100kHz; microwave power, 5 milliwatts; and microwave frequency, 9224.6 MhHz. Accuracy of the g-values was approximately ±0.001. *WT*, wild-type.

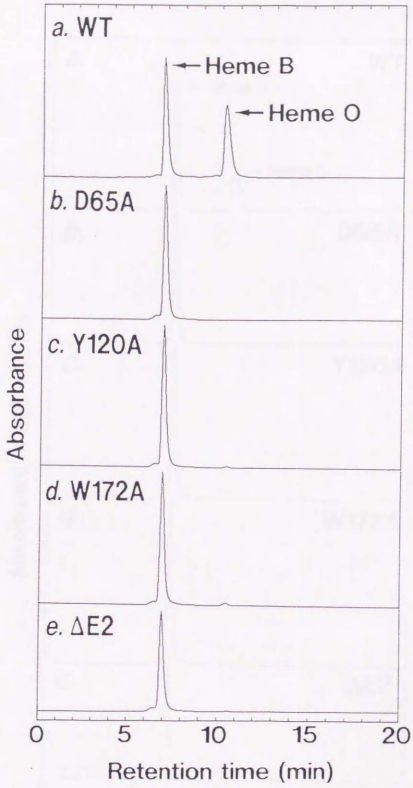


Fig. III-10 Elution profile of hemes extracted from purified wild-type (a) and *cyoE* mutant (b-e) oxidases by reverse-phase HPLC
The hemes extracted from the patially purified oxidases were separated by reverse-phase HPLC using a Shimadzu Model LC-9A system and an Altex Ultrasphere ODS column (4.6 mm, inner diameter, × 25 cm). The solvent was 95% ethanol/acetic acid/water (70:17:7), and the flow rate was 0.5 ml/min. The elution profile was monitored at the average absorbance of 396-402 nm using a Shimadzu SPD-M6A photodiode array detector. *WT*, wild-type.

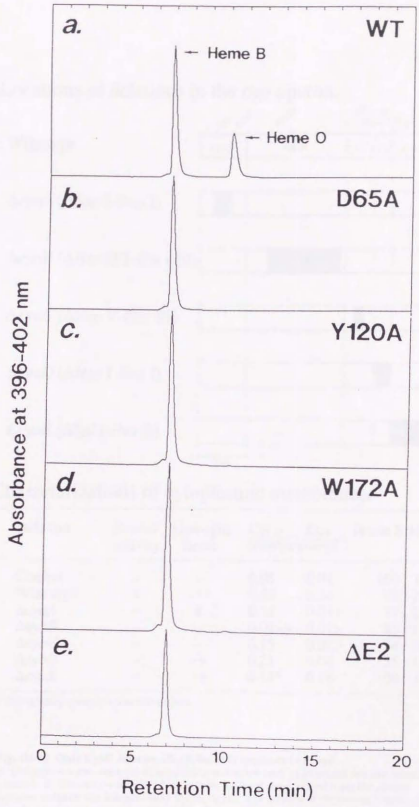
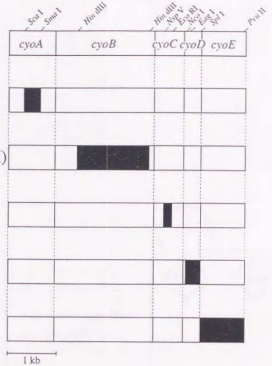


Fig. III-11 Elution profile of hemes extracted from cytoplasmic membranes of wild-type (a) and *cyoE* mutant (b-e) oxidases by reverse-phase HPLC
As in the legend of Fig. III-10. *WT*, wild-type.

A. Locations of deletions in the *cyo* operon.



B. Characterizations of cytoplasmic membranes.

Deletion	*In vivo* activity	Low-spin heme	Cyt *o* (nmol/mg protein)	CuB (nmol/mg protein)	Heme B:Heme O
Control	–	–	0.05	0.01	100 : 0
Wild type	+	+++	0.35	0.34	72 : 28
Δ*cyoA*	–	+	0.14	0.01>	77 : 23
Δ*cyoB*	–	–	0.01>>	0.01>	85 : 15
Δ*cyoC*	–	–	0.15	0.01>	74 : 26
Δ*cyoD*	–	++	0.25	0.04	85 : 15
Δ*cyoE*	–	++	0.14[a]	0.18	100 : 0

[a] CO-binding spectrum was red-shifted.

Fig. III-12 Only CyoE deletion affects heme O synthesis in *E. coli*
A. Deletion mutants were constructed using restriction sites as indicated and expressed by mini-F plasmid. B. The *in vivo* activity of the mutant oxidases were judged from the genetic complementation test using ST4683 (Δ*cyo*, Δ*cyd*). The content of the low-spin heme of the cytochrome *bo* complex was estimated by the absorbance at 563.5 nm in the redox difference spectrum at 77K. The amounts of the CO-binding high-spin heme (cytochrome *o*) were determined by CO difference spectra. The amounts of CuB were determined by atomic absorption spectroscopy. Hemes in cytoplasmic membranes were extracted by acid acetone and separated by reverse-phase HPLC. Hemes B and O were monitored by average absorbance at 396-402 nm. All the results of Δ*cyoA-D* deletion mutants except heme species analysis were cited from thesis of Nakamura (Nakamura 1991).

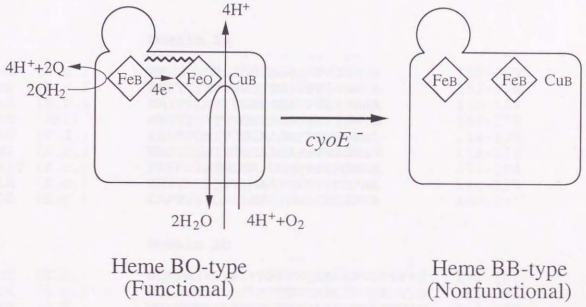


Fig. III-13 The scheme of the *cyoE* deficient mutants
The *cyoE* deficient mutants results in replacement of heme O to heme B and lose the catalytic activity of the *bo* complex. So, heme O is essential for the dioxygen reduction chemistry at the binuclear center.

```
                    Domain 1
                    -   - - -  + -+  -    +
CyoE    (E.c.)      NNYIDRDIDRKMERTKNRVLVKG          57 -79
CtaB    (B.s.)      NNWYDRDIDHLMERTKVRPTVTG          81 -103
CtaB    (B.f.)      NNYIDRDIDHLMERTKERPTVTG          89 -111
CaaE    (PS3)       NNYIDRDIDQYMERTKARPTVTG          85 -107
CtaB    (P.d.)      NMWYDADIDAVMRRTAGRPVPSG          42 -64
CtaB    (R.s.)      NMWSHEDIDRVMKRTRNRPVPSG          42 -64
COX10   (S.c.)      NMGREPEFDRQMVRTQARPVVRG          199-222
UbiA    (E.c.)      NDYADRKFDGHVKRTANRPLPSG          67 -89
COQ2    (S.c.)      NDFLDRKLDQRVIRSVERPIASG          142-164
                      Polyprenyl-PPi
                      binding motif

                    Domain 2a
                    -++       +   -  -+  +-
CyoE    (E.c.)      KRHSVYGTLIGSLSGAAPPVIGYCA        129-153
CtaB    (B.s.)      KRRYTINTVVGSVSGAVPPLIGWTA        152-176
CtaB    (B.f.)      KRTTTLNTIVGSFSGAVPPLIGWAA        160-184
CaaE    (PS3)       KRHYTITTVVGSISGAVPPFIGWTA        155-179
CtaB    (P.d.)      KRSTPQNIVIGGAAGAFPPMIGWAL        114-138
CtaB    (R.s.)      KRTTPQNIVIGGAAGAFPPMIGWAV        114-138
COX10   (S.c.)      KRKHIINTWLGALVGMVPPLMGWAA        270-294
UbiA    (E.c.)      KRYTHLPQVVLGAAFGWSIPMAFAA        114-138
COQ2    (S.c.)      KRFTYYPQAALSACFNWGALLGFPA        189-213

                    Domain 2b
                    -  --          --          +
CyoE    (E.c.)      WQMPHSYAIAIFRFKDYQAANIPVLPVVKG   172-201
CtaB    (B.s.)      WQIPHFLALAIKKTEDYRAANIPMLPDVYG   195-224
CtaB    (B.f.)      WQPPHFLALAMKRVEEYRAAGIPMLPVVAG   203-232
CaaE    (PS3)       WQPPHFLALAMKPCEEYRAAGIPMLPVVHG   198-227
CtaB    (P.d.)      WTPPHFWALALFMKDDYSKAGVPMLTVTHG   157-186
CtaB    (R.s.)      WTPPHFWSLALFMKSDYSDAGVPMLTVTHG   157-186
COX10   (S.c.)      WQFPHFNTLSHNIRNEYKNAGYVMTAWKNP   313-342
UbiA    (E.c.)      WAVAYDTQYAMVDRDDDVKIGIKSTAILFG   157-186
COQ2    (S.c.)      WCMTYDTIYAHQDKKFDIKAGIKSTALAWG   232-261
```

Fig. III-14 Sequence alignments of domain 1, 2a and 2b present in CyoE homologues and 4-hydroxybenzoate polyprenyltransferases

Amino acid sequences of the conserved domains 1, 2a and 2b of the *E. coli* CyoE protein are aligned with those of the CyoE homologues. The posotions of the amino acid residues are indicated. Results of the genetic complementation test in *E. coli* are shown above the CyoE sequence, and the putative allylic polyprenyl pyrophosphate-binding motif in domain 1 is *underlined*. B.s., *Bacillus subtilis*; B.f., alkalophilic *Bacillus firmus*; PS3, thermophilic *Bacillus* PS3; P.d., *Paracoccus denitrificans*; R.s., *Rhodobacter sphaeroides*; S.c., *Saccharomyces cerevisiae*.

Chapter IV. Establishment of overproduction system of CyoE *in vivo*

Introduction

Recent molecular biological studies have indicated that the *E. coli cyoE* homologues (Chepuri *et al.* 1990) are present in the genes for terminal oxidases of eubacteria (Ratio *et al.* 1987, Ishizuka *et al.* 1990, Saraste *et al.* 1991, Quirk *et al* . 1993) and in eukaryotes (Nobrega *et al.* 1990, Glerum and Tzagoloff 1994) although the identification of most gene products has not succeeded yet. These results suggest that the CyoE proteins are likely present in very low level. In this chapter, I described the CyoE overproduction system using H. Nakamura's plasmid series (Nakamura 1990, **Fig. IV-1**) and preparation of the antiserum against the chimera LacZ/CyoE protein to show the localization of the CyoE protein. Furthermore, I studied the distribution of heme O molecules in the cell.

Results

CyoE overproduction system

In 1990, H. Nakamura constructed pTTQ18 derivative, pHN31 (pTTQ18-*cyoE*) to over-express the *cyoE* gene product in a minicell system and identified a CyoE protein as a 28 kDa protein in 18.75% urea-SDS polyacrylamide gels (Nakamura 1990). I introduced pHN31 into strain ST4676 (W3110 Δ*cyo*) and the cells were grown with IPTG. The expression level of the CyoE protein was, however, too low to detect it as a protein band on SDS-PAGE gel and was estimated to be less than one percent of cytoplasmic membrane proteins. The CyoE protein was also difficult to detect by Western immunoblotting using polyclonal anti-LacZ/CyoE fusion protein.

I thought that pHN31 contained the 3'-half of the *cyoD* gene between the *tac* promoter of pTTQ18 and the *cyoE* gene and it might minimize the efficiency of CyoE production. Thus, I introduced the entire DNA region of the *cyoE* gene into pTTQ18. The resultant plasmid, pTTQ18-*cyoE*-2, did not contain the 3'-half of the *cyoD* gene, but the expression level of the CyoE protein in ST4676/pTTQ18-*cyoE*-2 was still low, suggesting that a loss of the plasmid may take place during culture of the cells. After several trials and errors, I found that a higher expression level of the CyoE protein could be obtained when the strains were cultured in a rich medium containing 40 μg/ml ampicillin or L-broth medium containing 0.5% glucose. Under the latter conditions, the LacZ fused proline carrier protein was successfully over-produced (Hanada *et al.* 1987). Finally, I established the growth conditions under which the expression level of the CyoE protein increased up to more than 10% of cytoplasmic membrane proteins and it could be detected by Western immunoblotting (**Fig. IV-2**) (Saiki *et al.* 1993a).

Production, identification and localization of the CyoE protein

Localization of the CyoE protein was examined in the CyoE-overproducing strain, since the expression level of the chromosomal *cyoE* gene was too low to detect immunologically in wild-type strains. Cells were divided into 3 fractions of the cytoplasm, cytoplasmic membranes and outer membranes according to the Yamato's method with slight modifications (Minagawa 1992) and the localization of CyoE protein was analyzed. Upon addition of IPTG, a 26 kDa protein that cross-

reacts with the anti-LacZ-CyoE chimera antiserum was specifically accumulated in the cytoplasmic membranes from strain ST4676/pTTQ18-*cyoE*-2, whereas no cross-reactive polypeptide was found either in the membranes from a negative control (ST4676/pTTQ18) and from a wild-type control (ST4676/pMFO1; Δ*cyo*/*cyoABCDE*) or in the outer membranes and in the cytoplasm of ST4676/pTTQ18-*cyoE*-2. An apparent molecular mass of the CyoE protein was estimated to be 26 kDa in 12.5% SDS-PAGE (**Fig. IV-2**) and 28 kDa in 18.75% urea-SDS-PAGE. The deviation from a deduced molecular mass of 32 kDa from DNA sequence (Chepuri *et al.* 1990) is likely due to anomalous mobility of membrane proteins in SDS-PAGE. So I concluded that CyoE protein is localized in the cytoplasmic membranes (Saiki *et al.* 1993a). The expression level of the CyoE protein in the CyoE-overproducing strain amounted to about 16% of the cytoplasmic membrane proteins (about 6 nmol/mg protein), whereas it was unable to detect even immunochemically in the wild-type membranes. Since the content of the cytochrome *bo* complex in the wild-type cytoplasmic membranes was estimated to be 0.41 nmol/mg protein (about 7% of the membrane proteins) from the CO-binding spectrum, the expression level of the *cyoE* gene must be regulated in a different manner although the *cyoABCD* genes are in the same operon.

Heme contents in the CyoE overproduced cytoplasmic membranes

Although localization of hemes were not known exactly, heme O seemed to be present in membranes due to its hydrophobic structure. Heme analysis was performed using cytoplasmic membranes. Hemes were eluted from the cytoplasmic membranes of the CyoE overproducing strain ST4676/pTTQ18-*cyoE*-2 and control strain ST4676/pTTQ18. Eluted hemes were separated by reverse phase HPLC and detected by their Soret absorption. Contents of heme B and heme O were determined using retention times of protoheme IX from bovine hemoglobin and heme O of the cytochrome *bo* complex as standards. I found that the CyoE overproduced cytoplasmic membranes contained two species of hemes that were coincided with heme B and heme O but control membranes contained only heme B (**Fig. IV-3**). Additionally, the α and β peaks of pyridine ferrohemochrome spectra of the CyoE overproduced membranes was shifted 2-3.5 nm to blue since those of heme O were 3-4 nm blue shifted compared with those of protoheme IX. Furthermore, isolated non-heme B detected in the CyoE overproduced membranes showed the same pyridine ferrohemochrome spectra of heme O reported previously (Puustinen and Wikström 1991). Therefore, I concluded that the new heme species observed in the CyoE overproduced cytoplasmic membranes is heme O molecules. It should be stressed that, although the heme content (1.8 nmol/mg protein) was not affected by overproduction of the CyoE protein, heme species in control strain was only protoheme IX so that heme O synthase activity was nearly absent in this strain.

Conclusion

The identification and localization of the CyoE protein were determined in the CyoE overproducing *E. coli* strains. The CyoE protein was identified as a 26 kDa protein in 12.5% SDS-polyacrylamide gels, and was localized mainly in the cytoplasmic membranes. In parallel with

overproduction of the CyoE protein, heme O molecules accumulated in the cytoplasmic membranes. Heme O was not observed in the cytoplasmic membranes of the *cyo* deletion strains. The CyoE protein increases the production of heme O but the other CyoABCD products are not needed for heme O synthesis.

Discussion

The *cyoE* gene product was predicted to be a hydrophobic protein with seven transmembrane helices and a large hydrophilic loop connecting helices IV and V (Chepuri *et al.* 1990). The predicted topology of the CyoE protein was verified by gene-fusion experiments (Chepuri and Gennis 1990). As shown in **Fig. III-4**, the majority of conserved residues in the CyoE/CtaB/COX10p proteins (18 out of 23 residues) appear in the cytoplasmic loops II/III and IV/V, indicating their topological importance.

Immunochemical studies using the CyoE-overproducing strain demonstrated that the *cyoE* gene product is a cytoplasmic membrane protein (Saiki *et al.* 1993a) whose molecular mass was identical to 28 kDa of the ^{35}S-labeled *cyoE* gene product in *E. coli* minicells (Nakamura 1990). However, the CyoE protein could not be detected by Western blotting analysis either in the cytoplasmic membranes from a wild-type strain or in the outer membrane and the cytoplasmic fractions of the CyoE-overproducing strain (**Fig. IV-2**). The deviation of the apparent *Mr* (28 kDa) from the deduced *Mr* of the *cyoE* gene product (32 kDa; Chepuri *et al.* 1990) is probably due to anomalous mobility of intrinsic membrane proteins in SDS-polyacrylamide gels or to post-translational modification. The yeast COX10p and COX11p are also expected to locate in the mitochondrial inner membrane because of their target sequences at the *N*-termini (Nobrega *et al.* 1990, Tzagoloff *et al.* 1990). In fact, COX11p was identified as a 28 kDa polypeptide in the membrane (Tzagoloff *et al.* 1990). These observations further support a CyoE function in the bacterial cytoplasmic membrane.

I found that heme O in the *E. coli* cytoplasmic membrane is maintained at a level similar to that in the cytochrome *bo* complex of wild-type bacteria even when oxidase subunits are absent. As the content of the cytochrome *bo* complex in wild-type cytoplasmic membranes was estimated from the CO-binding spectrum to be about 0.4 nmol/mg protein (*c.* 7% of membrane proteins), the CyoE protein should be readily detectable if it was indeed identical to subunit V of the oxidase complex. However, my immunoblotting analysis of the *cyoE* gene product (Saiki *et al.* 1993b) and minicell protein labeling experiments on the *cyoABCDE* gene products (Nakamura 1990) indicated much lower expression levels of the CyoE protein than of the oxidase subunits. Quirk *et al.* have reported that mRNA corresponding to the *cyoE* homologue in alkalophilic *Bacillus firmus* OF4 (the *ctaB* gene) was in relatively low abundance compared with the *ctaCDEF* mRNA coding for the *caa*3-type cytochrome *c* oxidase (Quirk *et al.* 1993).

These results suggest that a catalytic amount of the CyoE/CtaB protein is required for synthesis/assembly of the functional oxidase complex and that the expression of the genes coding for the oxidase subunits and of the *cyoE* gene must be regulated in different manners, even though they

are present in the same operon. Terminator-like structures, which are found between the *ctaB* gene and the structure genes for the *Bacillus caa*3-type oxidase (Saraste *et al.* 1991, Ishizuka and Nakajima 1992, Quirk *et al.* 1993) and behind the subunit I (*cyoB*) gene in the *E. coli cyo* operon (Chepuri *et al.* 1990), may be responsible for the differential expression of the oxidase subunits and the *cyoE* gene family. The production of the *cyoE* gene family is probably controlled transcriptionally (Quirk *et al.* 1993) or translationally in tight association with the assembly of the oxidase complex. Thus, the steady-state amount of the CyoE protein may be below the level detectable by conventional methods.

I found that the heme content (1.8 nmol/mg protein) was not affected by overproduction of the CyoE protein, suggesting that protoheme IX is a precursor and may be converted to heme O by a CyoE protein.

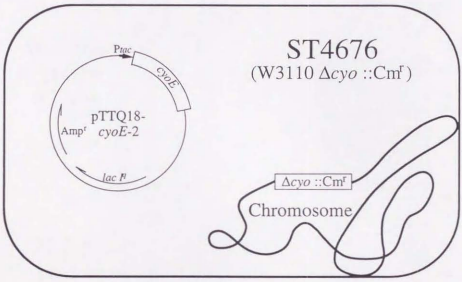


Fig. IV-1 Strategy for overproduction of the CyoE protein
The *cyoE* gene was subcloned into expression vector plasmid pTTQ18 and the resultant plasmid pTTQ18-*cyoE*-2 was introduced into *E. coli* strain ST4676 (Δ*cyo*). The *cyoE* gene was induced by the addition of IPTG.

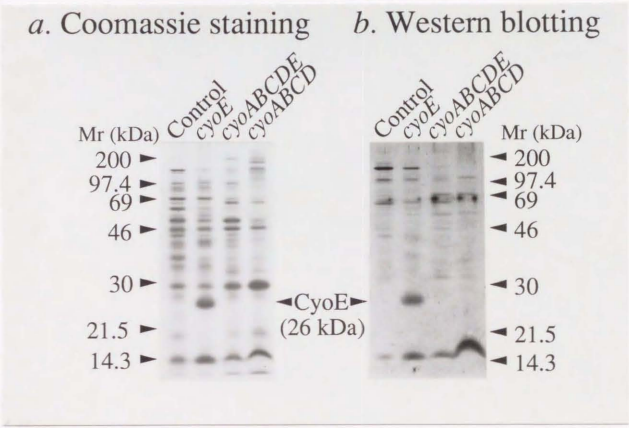


Fig. IV-2 Analysis of the expression level of the CyoE protein in the cytoplasmic membrane vesicles

a, protein staining; *b*, Western blotting analysis using the anti-LacZ-CyoE chimera antiserum. The cytoplasmic membranes were isolated from ST4676/pTTQ18 (Δ*cyoABCDE*/vector; *lanes 1* and *5*), ST4676/pTTQ18-*cyoE*-2 (Δ*cyoABCDE*/*cyoE*+; *lanes 2* and *6*), ST4676/pMFO1 (Δ*cyoABCDE*/*cyoABCDE*+; *lanes 3* and7), and ST4676/pMFO1-ΔE2 (Δ*cyoABCDE*/*cyoABCD*+; *lanes 4* and *8*), and 10 μg of membrane proteins were loaded per lane on 12.5% SDS-polyacrylamide gels. The CyoE protein was visualized by Coomassie Brilliant Blue R250 (a) or by using alkaline phosphatase-conjugated goat anti-rabbit IgG (b).

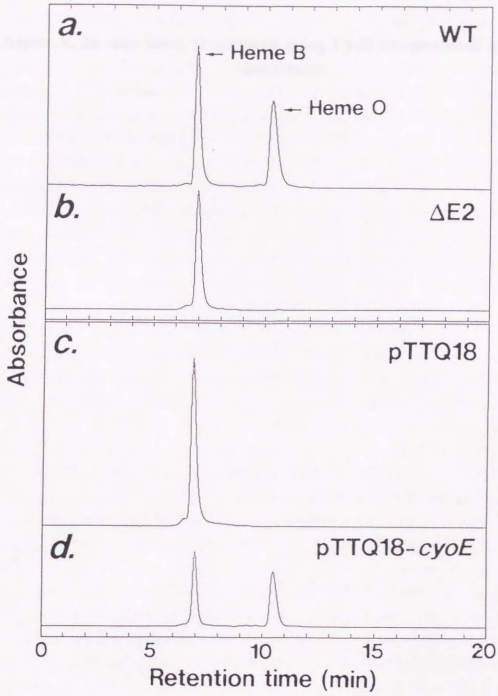


Fig. IV-3 Reverse-phase HPLC analysis of heme composition
Hemes extracted from the purified wild-type (a) and ΔE2 (b) and from cytoplasmic membrane visicles of strain ST4676 harboring pTTQ18 (c) and ST4676 harboring pTTQ18-*cyoE*-2 (d).Elute positions of hemes B and O are indicated. Heme B was identified with protoheme IX from bovine serum hemoglobin.

Chapter V. *In vitro* heme O synthesis using CyoE overproduced cytoplasmic membranes

Introduction

The biosyntheses of hemes O and A are largely unknown, however, tetrapyrroles are generally supposed to be synthesized using the protoheme IX biosynthetic pathway. Especially, heme A is strongly suggested to be synthesized or derived from heme B by some biophysical studies. To the contrast, owing to the very recent discovery and relatively minor distribution in nature of heme O, no studies have been reported yet about the biosynthesis of heme O. Based on structural similarities of heme O and heme A, heme O is supposed to be an intermediary product in the biosynthetic pathway of heme A (Lynen's model in **Fig. I-3**). Actually, formylated heme B is structurally unstable and can't be present in nature (Sono *et al.* 1991), suggesting that the 17-carbon moiety of heme A is first transferred to heme B.

I indicated in chapter III that the putative allylic polyprenyl diphosphate-binding motif was found in the CyoE protein family. Alanine scanning test in this region (domain 1 in CyoE) indicated that this region is essential for the functions of the CyoE protein. In chapter IV, I describe that an overproduction of the CyoE protein results in accumulation of heme O. These results strongly support that the CyoE protein is a novel enzyme, heme O synthase. I also found that FPP is transferred to the 2-vinyl group of protoheme IX by the heme O synthase (**Fig. V-1**).

This chapter reports that a construction of the *in vitro* heme O synthesis system to identify heme O synthase. Properties of the enzyme including substrate specificity are also presented.

Results

Construction of *in vitro* heme O synthetic reaction system

Using CyoE-overproduced cytoplasmic membranes, the assay conditions for heme O synthase activity were examined. Products were analyzed by reverse-phase HPLC and heme species were identified based on the retention time of heme B from bovine hemoglobin and heme O from the purified *E. coli* cytochrome *bo* complex (**Fig. V-2**). As shown in **Table V-1** and **Fig. V-3 b**, the CyoE-overproduced membranes can catalyze the *in vitro* heme O synthesis from exogenous hemin and FPP in the presence of sodium dithionite and divalent cations such as Mg^{2+} and Ca^{2+} (Saiki *et al.* 1993a). This reaction was dependent on the amount of CyoE protein (**Fig. V-3 a**) and the presence of reducing agent such as dithionite (**c**), divalent metal cation (**d**), or FPP (**e**). The hydroxyl-compound, farnesol was unable to substitute for FPP (**f**). The heme O synthetic activity was absent in the outer membranes and cytoplasm of CyoE-overproduced strain (**Table V-2**).

The conditions for the *in vitro* heme O synthetic reaction were as follows:

[Reducing reagent]

It should be noted that reducing reagent was essential in the *in vitro* heme O synthetic reaction, and dithionite was found to be the most effective among reagents tested (*i.e.* dithiothreitol, 2-mercaptoethanol, and sodium ascorbate). Heme species in the presence of dithionite were rather stable in short incubation time (**Fig. V-4**). At the end of incubation, formaldehyde was added to consume residual dithionite and to convert unstable ferro-hemes to stable ferri-hemes. This step was

critical since ferro-hemes were unstable and degraded in the heme extraction step. The heme O synthetic activity was enhanced about 10 times by addition of an electron transmitter, phenazinemethosulfate (PMS) (**Table V-3**).

[Divalent metal cation]

Since Mg^{2+} was required for the heme O synthetic reaction, effects of other 8 divalent metal cations were tested, such as $CaCl_2$, $ZnCl_2$, $CoCl_2$, $CdCl_2$, $FeCl_2$, $PdCl_2$, $CuCl_2$, and $MnCl_2$. As shown in **Table V-4**, Ca^{2+} was equally effective as Mg^{2+}, whereas Zn^{2+}, Co^{2+} and Cd^{2+} were not. Optimum concentrations of Mg^{2+} and Ca^{2+} were 1.5 mM (**Fig. V-5**). Both sulfate and chloride salts of magnesium gave the same result (**Table V-4**), indicating that the divalent metal cations are required for the heme O synthetic reaction.

[pH and buffer]

Optimum pH for the heme O synthetic reaction was pH7.4 (**Fig. V-6**). No remarkable difference was observed when Tris-HCl or sodium phosphate buffers were used at around pH7.4 (**Fig. V-6**). Optimum concentration of Tris-HCl pH7.4 buffer was 200 mM (**Fig. V-7**).

[Others]

The heme O synthetic activity linearly increased for 30 min at 37 °C although the total heme species were decreased (**Fig. V-8**). In the presence of detergent, the activity was increased when a detergent concentration was very low (around 0.1%) (**Fig. V-9**). Sucrose monolaurate SM-1200 and Tween 80 activated the activity whereas octyl glucoside and Triton X-100 were inhibitory for the activity when the detergent concentration was high (up to 1%) (**Fig. V-9**).

Substrate specificity and kinetic parameters

GPP and GGPP were also transferred to protoheme IX by CyoE over-produced membranes but the efficiency of both reactions was low, compared with that of FPP (**Fig. V-10**). The *in vitro* heme O synthetic reaction proceeded reliably down to 5 μM of hemes as a minimum amount. But the initial velocity of heme O synthetic reaction was saturated at 5 μM FPP so that a Michaelis constant was unable to determine (**Table V-5**).

Orientation of a catalytic active site for heme O synthase

The CyoE protein has long cytoplasmic loops (the loop II-III, IV-V, and VI-VII). Essential residues and conserved residues for the CyoE protein are located in these regions as shown by the alanine scanning (chapter IV). The putative polyprenyldiphosphate binding region was predicted to locate in the loop II-III. So the active site of CyoE protein was expected to expose in the cytoplasm. To test this, right-side-out and inside-out vesicles were prepared from CyoE-overproduced strain and the activities of these membranes were analyzed. As shown in **Table V-6**, no difference was observed in heme O synthetic activity.

Purification of CyoE

The CyoE protein was easily aggregated and lost heme O synthetic activity completely after solubilization with Tween 20, Tween 80, Triton X-100, sodium Sarkosyl, CHAPS, octyl glucoside

or sucrose monolaurate (SM-1200). The CyoE protein became completely inactive once aggregated. No renaturing procedures from an aggregated form of the CyoE protein could be obtained. In the presence of 30-60% glycerol, only SM-1200 could solubilized it. Those conditions were 50 mM Tris-Cl pH6.8-7.4, 1% SM-1200, 30-60 % glycerol, 24 μM FPP, 0.3 M NaCl, 1 mM PMSF. Glycerol and FPP were needed absolutely to keep the CyoE protein active throughout a purification study. But ion-exchange column chromatographies (DEAE Sephacell and CM Sepharose) resulted in no separation of proteins by a high concentration of glycerol. So I tried purification of a His-tagged CyoE protein. Expressed His-tagged CyoE located in both outer and inner membranes fractions and only the His-tagged CyoE in the cytoplasmic membranes showed heme O synthetic activity. Then, the enzyme was solubilized in the same conditions mentioned above and purified using Ni-NTA resin (QIAGEN Inc., CA, USA). After binding to the resin, buffer was changed to 50 mM Tris-Cl pH6.8, 0.1% SM-1200, 30-60% glycerol, 24 μM FPP, 1.5 mM $MgCl_2$, 0.3 M NaCl, 0.1 mM PMSF, and eluted the column with the same buffer containing 100 mM imidazole. As a result, His-tagged CyoE was co-eluted with many proteins. After several trials and errors, further purification of the enzyme was unsuccessful, even under conditions that were best for solubilization.

Conclusion

In vitro heme O synthetic reaction system was successfully constructed using CyoE-overproduced cytoplasmic membranes. Using this system, I found for the first time that heme O can be synthesized from dithionite-reduced ferro-protoheme IX and FPP in the presence of divalent metal cations such as Mg^{2+} or Ca^{2+}. The observed heme O synthetic activity completely depends on the presence of CyoE protein. Therefore, I concluded that the CyoE protein is a novel enzyme, heme O synthase.

Discussion

In vitro heme O biosynthetic reaction

I found that the heme O synthetic activity was localized solely in the cytoplasmic membranes and depended on the amount of CyoE protein (**Fig. IV-2**, **Table V-2**). This result indicates that the CyoE protein is heme O synthase. Heme O was synthesized from protoheme IX and FPP in the presence of divalent cations such as Mg^{2+} or Ca^{2+}.

The oxidized form of protoheme IX (ferric iron protoporphyrin; ferriheme) is very stable although it is known to form a dimer in aqueous solution. The aggregated hemes are much less active than the monomeric form (Brown and Shillcock 1976, Brown *et al.* 1980). On the other hand, ferrous iron protoporphyrin IX (ferroheme) is very unstable and, in the presence of molecular oxygen, it degrades and changes to the corresponding biliverdins by elimination of one of four methene bridges of tetrapyrrole macromolecule, though it is the final product in the protoheme IX biosynthetic pathway (Porra and Jones 1963). I found that the heme O synthase activity was increased about 10 times by the presence of PMS in the reaction mixture (**Table V-3**). Since

ascorbate/PMS reduces ferri-protoheme IX to ferro-protoheme IX, the latter is a substrate for heme O synthase (**Fig. V-11**).

Addition of formaldehyde was essential to stop the reaction not only to destroy excess dithionite but also to inhibit the conversion of ferro-hemes to ferri-hemes. By this way, I could separate and determine the amount of ferro-hemes produced.

Farnesol was unable to substitute for FPP (**Fig. V-3 f**) and was not an inhibitor even with 500-fold excess of FPP. FPP is synthesized from the isomer of IPP (isopentenylpyrophosphate), dimetylallylpyrophosphate, by FPP synthase in cells. I found that GPP and GGPP are substrates for the heme O synthase. This means that the diphosphate group of FPP is essential for heme O synthetic reaction. Divalent cations are also needed to the reaction. Mg^{2+} is most abundant divalent metal cation in cells so that heme O is synthesized from ferro-protoheme IX and FPP/Mg^{2+} in *E. coli* cells.

Mechanism of heme O synthesis

Based on the present observations and results, I propose that FPP/Mg^{2+} can be recognized by an allylic polyprenyl diphosphate-binding motif present in the CyoE protein family (**Fig. IV-1**). Alternatively, FPP/Mg^{2+} facilitates cleavage of the diphosphoryl group, forming a farnesyl cation. The resulting farnesyl cation undergoes a nucleophilic attack by the vinyl group of ferrous protoheme IX, thus succeeding to a transfer of the farnesyl group to position 2 of the 2-vinyl group of ferrous protoheme IX with concomitant addition of a hydroxyl group to position 1 (**Fig. V-12**). Since the electron density at the 2-position of the vinyl group is assumed to be higher in ferrous protoheme IX than in its ferric state, the former must be the real substrate for farnesylation. As a result, the CyoE protein catalyzes a direct transfer of the farnesyl moiety from the FPP/Mg^{2+} complex to position 2 of the vinyl group at pyrrole ring D of ferrous protoheme IX, possibly by a one-step reaction (**Fig. V-12**).

Assembly model of the *bo* complex

Here, I should emphasize that the bacterial operons for heme-copper terminal oxidases have a unique structural feature and encode not only subunits of the oxidase complex but also the key enzyme for biosynthesis of its own prosthetic group. In *E. coli* cytochromes, heme O is specifically found in the cytochrome *bo* complex that is encoded by the *cyo* operon (*cyoABCDE* gene). Subunit I-III have been assigned to be the products of the *cyoB* , *cyoA* and *cyoC* genes, respectively (Nakamura *et al.* 1990, Minghetti *et al.* 1992). Subunit IV was identified to be the product of the *cyoD* gene (Nakamura, 1990). Therefore, molecular assembly of the cytochrome *bo* complex and biosynthesis of heme O may be coordinately regulated to facilitate the aerobic respiration under high oxygen tension (**Fig. V-13**).

Orientation of the active site of CyoE

A topological model of CyoE has been proposed using gene fusion techniques (Chepuri and Gennis 1990). CyoE has seven membrane spanning regions and composed of 296 amino acids. Interestingly, about 42% (123 residues) of amino acids are exposed in the cytoplasm. Most of them

(119 residues) are present in loop II-III, IV-V and VI-VII. The loop II-III contains the putative allylic polyprenyl binding motif and is expected to form a FPP/Mg^{2+} binding region. About 80% of the conserved amino acid residues are located in these cytoplasmic loops. So they may be a site(s) of the heme O synthetic reaction center. However, I found that the heme O synthetic activity of right-side-out and inside-out vesicles were the same. Heme is a very hydrophobic compound and may be able to migrate across the phospholipid bilayer and reach easily to a heme-binding site in proteins (Rose *et al.* 1985). Although FPP and Mg^{2+} are membrane non-permeable due to its pyrophosphate moiety and positive charge, respectively, FPP/Mg^{2+} was reported to be able to penetrate into cells (Taketsuji *et al.* 1983). If this would be the case, all substrates for heme O synthesis are membrane-permeable, and accessible to the catalytic site even if the right-side-out membranes are used.

Assay conditions	Heme O synthase activity
	(%)
Complete	100
-Hemin	0
-FPP	3
-$MgSO_4$	13
-Ditionite	2
+Farnesol/-FPP	2

Table V-1 Components essential for heme O synthase assay
CyoE-overproduced cytoplasmic membranes were incubated at 37°C in the standard reaction mixture in the absence of each component. Farnesol was added at a final concentration of 60 mM in place of FPP. The heme O synthase activity in the complete reaction mixture refers to 100%.

Vector [a]	Fraction	Enzyme activity (nmol/mg protein/30 min)
pTTQ18-*cyoE*-2	IM	4.9
	OM	0.01>[b]
	Cytoplasm	0.01>[b]
pTTQ18	IM	0.01>
pMFO1(*cyoABCDE*$^+$)	IM	0.19

[a] Host strain, ST4676 (Δ*cyo*, *cyd*$^+$)
[b] Hemes degraded partially.

Table V-2 Localization and overproduction of heme O synthase in *E. coli*
The reaction mixture (200 μl) containing 0.12 mg of cytoplasmic membrane proteins or 0.3 mg of ouyer membrane proteins or cytoplasmic proteins was incubated at 37°C for 30 min. The heme O synthase activity was estimated from the relative peak area of heme O to that of protoheme IX on reverse-phase HPLC.

Ferroprotoheme IX (reduced hemin) is needed for heme O synthase activity

Reductant	Relative activity(%)
Dithionite	100
Ascorbate*	2
Ascorbate/PMS*	23
PMS*	1
None	1

*Sodium ascorbate : 5 mM, PMS : 0.05 mM

Table V-3 Effect of reducing agents on heme O synthase reaction
The reactions were carried out in the standard heme O synthase reaction assay.

Salt added	Heme O synthase activity (%)
$MgSO_4$	100
$MgCl_2$	102
$CaCl_2$	98
$ZnCl_2$	52
$CoCl_2$	45
$CdCl_2$	41
$FeSO_4$	20
$PdCl_2$	4
$CuSO_4$	3
$MnCl_2$	NT

NT, not tested since hemes were unable to extract.

Table V-4 Effect of metal ions on the heme O synthase reaction
The reactions were perfomed in the standard heme O synthase reaction assay in the presence of each salts at 1.5 mM. The heme O synthase activity with incubation of the membranes with $MgSO_4$ refers to 100%.

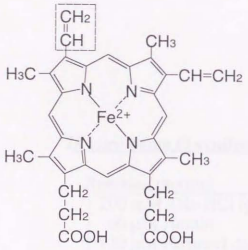
Conditions	Substrate concentration (μM) 5	10	15	20	30	40	50	60	300	Km/V (μM)/(μM/min)
FPP										
pH7.4	0.242	0.250	0.339	0.250	0.318				0.290	ND
pH6.4	1.046	0.978		1.133	1.056	1.125	1.112		0.845	ND
GPP										
pH7.4		0.137		0.160	0.186	0.201	0.188			7.5/0.12
pH6.4	-	0.084		0.195	0.273	0.241	0.316			ND
GGPP										
pH7.4	-	0.037		0.032	0.035	0.033	0.035			ND
Hemin										
pH7.4				0.253	0.254	0.318	0.265	0.291		ND
pH6.4				0.857	0.883	0.839	0.847	0.847		ND

Table V-5 Analysis of kinetic parameters for heme O synthase

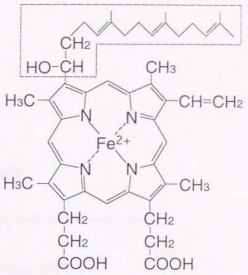
In vitro heme O synthetic reaction was performed in 0.2 M Tris-Cl pH7.4 or 0.2 M sodium phosphate pH6.4, 1.5 mM $MgSO_4$, 0.1% sucrose monolaurate, 60 μM hemin-Cl or 300 μM FPP, 90 μg/ml (pH7.4) or 360 μg/ml (pH6.4) of CyoE membranes, and indicated concentrations of substrates at 23°C. Heme O synthetic reaction was started by addition of a few crystals of sodium dithionite, and terminated by addition of f.c. 7.4% formaldehyde followed by N2 liq.-freezing. Amount of the product was caluculated by ratio of Abs. at 390-410 nm. Each values showed were average of two (pH7.4) or three (pH6.4) data. Molar extinction coefficients of prenyrated hemes were assumed to be the same with that of protoheme IX. All the values of GGPP at pH6.4 were not detected so that ommited. -; Not detected, ND; Not determined.

Toluene treatment	Relative activity (%)			
	Ferricyanide reductase		Heme O synthase	
	ISOV	RSOV	ISOV	RSOV
–	100 [a]	26	100 [a]	97
+	108	96	83	63

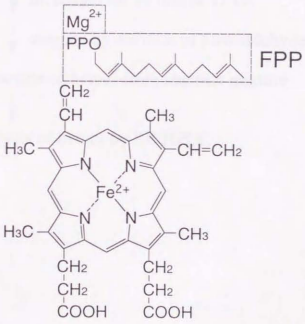
Table V-6 Heme O syntase activity of CyoE overproduced ISOV and RSOV
Toluene treatment was done in that membranes were incubated with 1% toluene at 37°C for 20 min before measurements. a, the activity refers to 100%. The heme O synthase activity was measureed in the standard assay conditions.



Heme B



Heme O



Heme B

Fig. V-1 Hypothetical model of heme O synthetic reaction
Heme O synthetic reaction was assumed based on structural correlation of heme B and heme O and Lynen's heme A synthesis model.

***In vitro* heme O synthase assay**

Reaction mixture

200 mM Tris-HCl (pH7.4)
60 μM Hemin
120 μM Farnesyl diphosphate (FPP)
CyoE overproduced cytoplasmic membranes

↓ started by addition of $Na_2S_2O_4$

↓ incubated for 30 min at 37°C

↓ stopped by addition of formaldehyde

Extraction of hemes from reaction mixture

↓

Analysis of hemes by RP-HPLC

Fig. V-2 Strategy for *in vitro* heme O synthase analysis

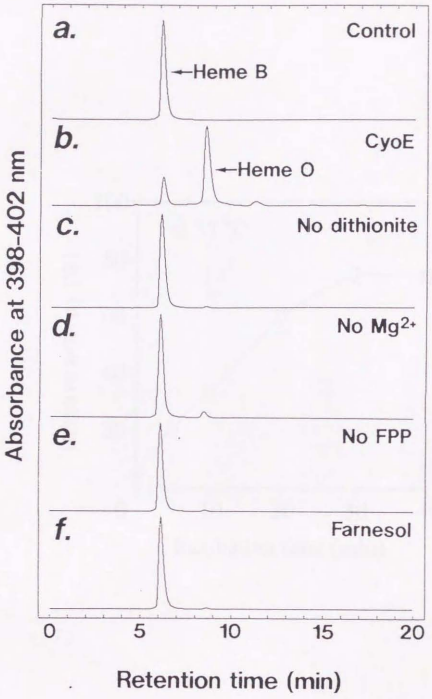


Fig. V-3 Reverse-phase HPLC analysis of products from the heme O synthase reaction
Hemes were extracted by acid acetone from the standard reaction mixture containing the control membranes from ST4676/pTTQ18 (a) or the CyoE-overproduced membranes from ST4676/pTTQ18-*cyoE*-2 (b-f). The reactions were also carried out in the absence of dithionite (c), MgSO4 (d), or FPP (e) or in the presence of farnesol in place of FPP (f). hemes B and O were separated by reverse-phase HPLC and elution profiles were monitored by average absorbance at 396-402 nm.

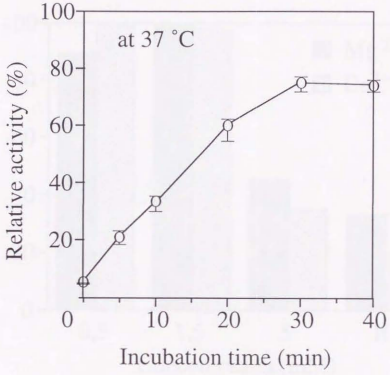


Fig. V-4 Time course of heme O synthase activity
In vitro heme O synthase reation was performed in the standard reaction conditions.

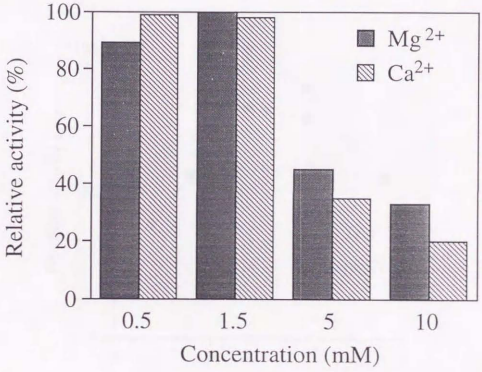


Fig. V-5 Effect of metal concentrations on heme O synthase reaction
The reactions were performed in the standard heme O synthase reaction assay in the presence of indicated concentrations of $MgSO_4$ or $CaCl_2$. The heme O synthase activity with 1.5 mM of $MgSO_4$ refers to 100%.

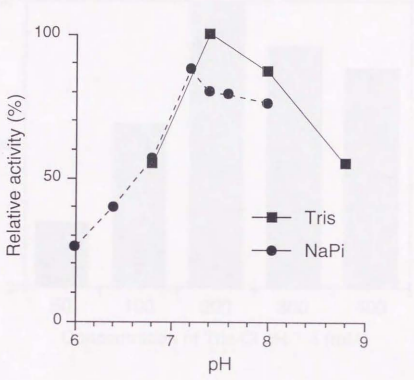


Fig. V-6 Effect of pH on *in vitro* heme O synthase activity
The reactions were carried out in the standard heme O synthase assay in buffers with pH indicated. The heme O synthase activity in 200 mM Tris-Cl pH7.4 refers to 100%.

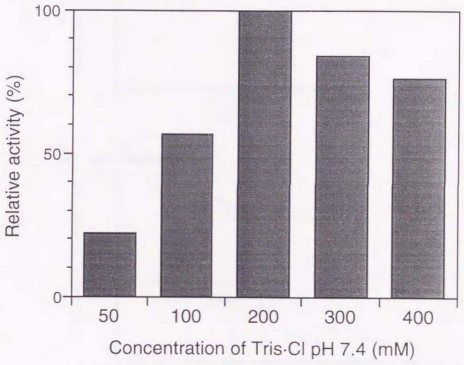


Fig. V-7 Effect of concentrations of Tris-Cl buffer on *in vitro* heme O synthase activity
The reactions were perofrmed in the standard heme O synthase assay in indicated concentrations of Tris-Cl pH7.4 buffer. The heme O synthase activity in 200 mM Tris-Cl pH7.4 refers to 100%.

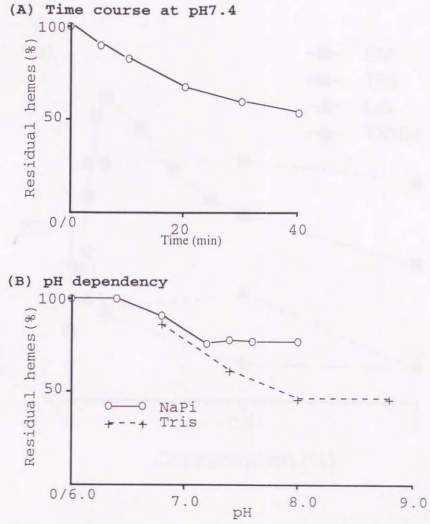


Fig.V-8 Effects of pH on hemes stability in the heme O synthase reaction

In vitro heme O synthetic analysis was performed in the standard heme O synthase reaction assay conditions except that reaction was performed at 37°C (for 20 min in (B)). (**A**) Residual hemes under non-reduced conditions refer to 100%. Ratios of heme O produced at 5, 10, 20, 30, 40 min were 5.4, 19.5, 30.7, 55.6, 72.6, 72.6%, respectively, of total residual hemes. Under no FPP control, hemes remained after the reaction were 75%. (**B**) The amount of oxidized protoheme IX refers to 100%.

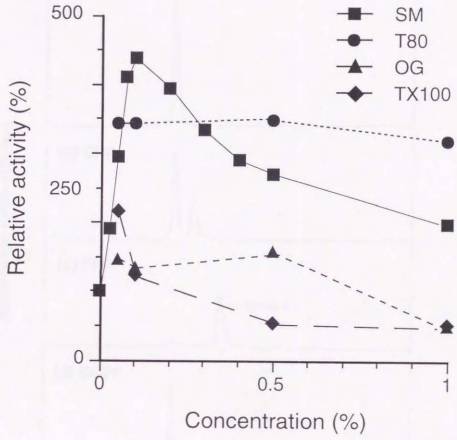


Fig. V-9 Effect of detergent on heme O synthase activity
The reactions were performed in the standard heme O synthase reaction assay conditions in the presence of indicated concentrations of detergents. The heme O synthase activity in the absence of detergent refers to 100%.

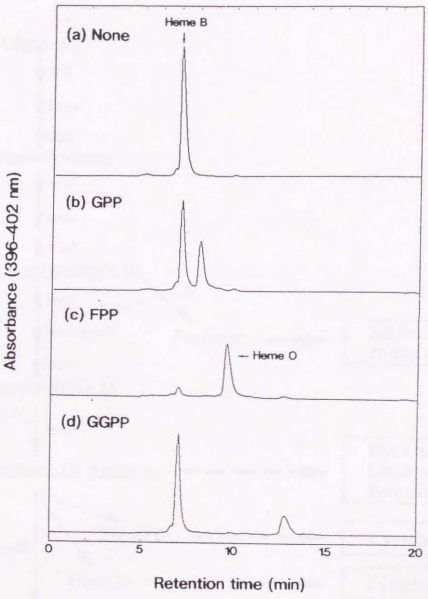


Fig. V-10 Reverse-phase HPLC analysis of various heme products by the heme O synthase reaction

The reactions were performed under the standard heme O synthase reaction assay (c) using various polyprenyl diphosophate (b and d). In place of FPP, 120 μM of GPP (b), and GGPP (d) were used. Hemes were separated by reverse-phase HPLC and elution profiles were monitored by average absorbance at 396-402 nm.

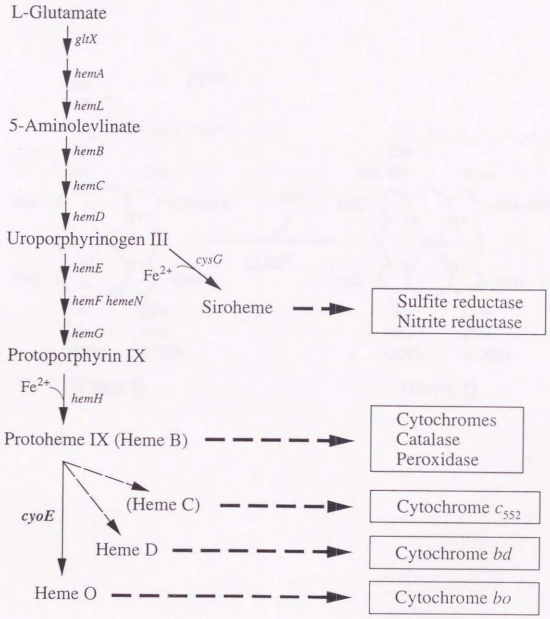


Fig. V-11 Biosynthesis and distribution of hemes in *Escherichia coli*

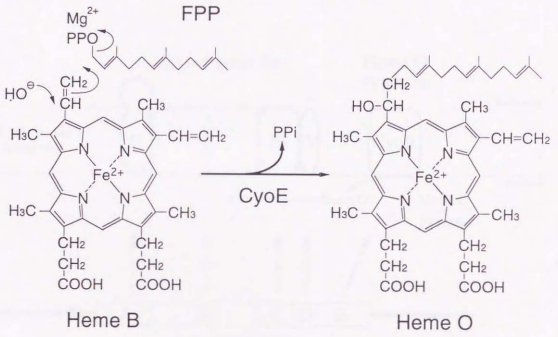


Fig. V-12 Schematic model of heme O synthase by the CyoE protein
PPO and *PPi* indicate diphosphoryl group and diphosphate, respectively

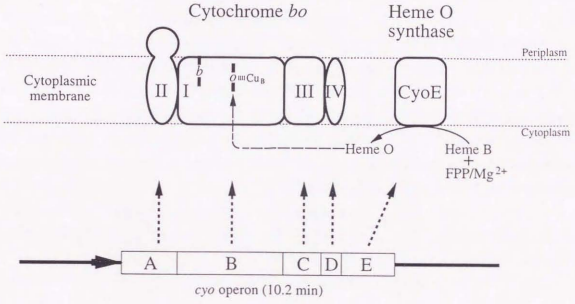


Fig. V-13 Heme O biosynthesis is coupled to functional expression of the cytochrome *bo* complex

Chapter VI. *CaaE* of thermophilic *Bacillus* PS3, a CyoE protein homologue, is heme O synthase

Introduction

Despite past investigations on the biosynthetic pathway and regulation of heme A, little has been known about heme A synthesis. Protoheme IX was considered as a precursor of heme A (Sinclear *et al.* 1967, Weinstein *et al.* 1986). A possible mechanism for the formation of the side-chain at the 2-vinyl group that uses FPP has been proposed (Grassl *et al.* 1963, Leeper, 1985). Genetic studies with *Bacillus subtilis* (Hansson and von Wachenfeldt, 1993), *Staphylococcus aureus* (Sinclear *et al.* 1967) and *Saccharomyces cerevisiae* (Gollub *et al.* 1977) have provided evidence that heme B was a precursor of heme A. If heme A is, in fact, derived from protoheme IX, one may expect to find a class of mutants with defects in heme A biosynthesis that would result in a loss of cytochrome *c* oxidase activity or in the accumulation of a heme A precursor. Until recently (Svensson *et al.* 1993), no one had succeeded in detecting such intermediates or enzymatic defects related to heme A biosynthesis. In bacteria, the isolation of mutants defective in heme A biosynthesis may be hampered by the presence of an alternative terminal oxidase(s).

Recently, Matsushita *et al.*(1992) showed that a change in the culture conditions for *Acetobactor aceti*, from static to shaking or vice versa, elicited a change of cell type which is associated with a change of the terminal oxidase from a heme BA-type to a heme BO-type. The heme CAA-type cytochrome *c* oxidase in thermophilic *Bacillus* PS3 also changes to the heme CAO-type oxidase under slightly air-limited growth conditions (Sone and Fujiwara 1991). Since heme O is structurally related to heme A in terms of the presence of the 2-hydroxyethylfarnesyl group, heme O may well be a precursor of heme A. If this is the case, conversion of the 8-methyl group of heme O to a formyl group may be suppressed under low-oxygen tension.

The *cyoE* homologues (i.e., *ctaB, caaE,* ORF1) are present not only in the subunit II/III operons for *aa*3-type cytochrome *c* oxidase in *Paracoccus denitrificans* and *Rhodobacter sphaeroides* but also adjacent to the *caa*3-type cytochrome *c* oxidase operon in *Bacillus* species including thermophilic *Bacillus* PS3 (Ishizuka *et al.* 1990). It is also known as the yeast nuclear gene, *COX10*, which is essential for the functional expression of mitochondrial cytochrome *c* oxidase (Nobrega *et al.* 1990). Accordingly, the bacterial and eukaryotic *cyoE* homologues are likely to encode heme O synthase, and may be involved in heme A biosynthesis. Therefore, the use of the *Bacillus* PS3 *caaE* gene, a homologue of the *cyoE* gene in *E. coli* and the *ctaB* gene in *Bacillus* species, was thought to be advantageous for biochemical experiment to elucidate its function. Furthermore, the use of my CyoE over-expression system of *E. coli* will promise sufficient production of the CaaE protein. In this chapter I describe the functional role of the *caaE* gene of *Bacillus* PS3 in heme O synthesis.

Results

Genetic complementation test of the *cyoABCD-caaE* chimera operon

Using the *cyoABCD-caaE* chimeric operon on the single copy expression vector (pMFO21-*caaE*), I examined the catalytic activity of the *caaE* gene product *in vivo* (**Fig. VI-1**). Strain ST2592 lacks the operons for both *bo*- type and *bd*-type quinol oxidases, which are the terminal oxidases of

aerobic respiratory chain in *E. coli*, and it can grow aerobically only via glycolysis. The control vector pHNF2 and the *cyoE* gene deletion plasmid (*cyoABCD*) could not complement the defect of the aerobic growth of ST2592 on minimal/glycerol plates (Saiki *et al.* 1992). In contrast, plasmid pMFO21-*caaE* carrying the wild-type *cyoABCD* gene and the *caaE* gene in the place of the *E. coli cyoE* gene (*cyoABCD-caaE*) supported extensively the aerobic growth of the terminal oxidase-deficient strain as did pMFO21 carrying the intact wild-type *cyo* operon (*cyoABCDE*) as in **Fig. VI-1**. Spectroscopic analysis of the cytoplasmic membranes prepared from ST4676 (Δ*cyo*)/pMFO21-*caaE* confirmed that properties of the low-spin and the high-spin hemes of the *bo*-type quinol oxidase are the same to those of wild-type strain (ST4676/pMFO21) as in **Fig. VI-2**. These results indicate that the role of the *cyoE* gene in the functional expression of *bo*-type quinol oxidase can be substituted by the *caaE* gene from thermophilic *Bacillus* PS3 (Saiki *et al.,* 1994).

Over-expression of the *caaE* gene in *E. coli*

For efficient translation of a heterogeneous gene in *E. coli*, I took an advantage of the over-expression system established for the *E. coli cyoE* gene (Saiki *et al.* 1992 and 1993a). Thus, the *caaE* gene corresponding to Val-32 to Trp-309 of *Bacillus* PS3 was placed behind the 5'-terminal sequence corresponding to Gln-8 of the CyoE. The junction site was chosen as a putative end of the N-terminal protruding region. When the *caaE* gene was expressed in ST4676 (Δ*cyo*)/pTTQ18-*caaE* by induction with IPTG, a 24.5 kDa polypeptide was specifically overproduced in the cytoplasmic membranes (**Fig. VI-3, lane 3**). The apparent molecular mass of the CaaE protein in 12.5% SDS polyacrylamide gel electrophoresis was smaller than that deduced from the DNA sequence (32.3 kDa), as reported for the *E coli cyoE* gene product (Saiki *et al.* 1993a). It is probably due to aberrant electrophoretic mobility of hydrophobic membrane proteins. The expression level of the CaaE protein was estimated to be about 5% of membrane proteins by densitometric analysis.

In vivo* heme O synthesis accompanied by CaaE-overproduction in *E. coli

To examine *in vivo* activity of the *caaE* gene product, cytoplasmic membrane vesicles were prepared from the IPTG-induced ST4676/pTTQ18-*caaE* cells. Hemes were extracted from the membranes and subjected to reverse-phase HPLC analysis. Assignment of the eluted peaks was done by running separately hemes extracted from the purified *E. coli bo*-type quinol oxidase and from bovine hemoglobin. In contrast to the control membranes from ST4676/pTTQ18 (**Fig. IV-4 a**), the conversion of protoheme IX to heme O occurred with those from ST4676/pTTQ18-*caaE* (**Fig. VI-4 c**) like those of CyoE overproduced cytoplasmic membranes (**Fig. VI-4 b**). A difference in relative amounts of heme O between the latter two membranes (40% and 30% of total hemes, respectively) could be partly due to that in the expression level of the cloned genes (**Fig. VI-3**).

***In vitro* heme O synthetic activity of CaaE-overproduced cytoplasmic membranes**

In the presence of FPP, ferrous protoheme IX and Mg^{2+}, both the CyoE- and CaaE-overproduced cytoplasmic membranes catalyzed heme O synthesis *in vitro* (**Fig. VI-5**). Interestingly, the optimum temperature of the reaction with the CaaE membranes was found to be

about 60°C and is higher than about 50°C with the CyoE membranes. It should be noted that the CaaE membranes retained the activity even at 70°C at a level comparable to that at 37°C whereas the CyoE membranes lost a half of the 37°C activity at 70°C. At higher temperatures (at 80°C), both membranes lost completely the heme O synthase activity.

Conclusion

The genetic complementation test demonstrated that CyoE and CaaE proteins are essentially and functionally the same enzyme. CaaE overproduced *E. coli* cells showed a CaaE protein dependent production of heme O and CaaE overproduced cytoplasmic membranes could catalyze the *in vitro* heme O synthetic reaction like CyoE. These results definitively indicate that CyoE and CaaE proteins are heme O synthase and support that the single enzyme of heme O synthase is responsible for heme O synthesis *in vivo*.

Discussion

Heme O biosynthesis in eubacteria and eukaryote

The genetic complementation analysis demonstrated that the *caaE* gene from thermophilic *Bacillus* PS3 can functionally substitute for the *cyoE* gene in *E. coli*. Furthermore, the CaaE protein expressed in *E. coli* was shown to catalyze the conversion of protoheme IX to heme O both *in vivo* and *in vitro*. The observation that the CaaE protein is a thermotolerant heme O synthase provides a further support for my proposal. Thus, the *cyoE* gene homologues known as the *caaE*, *ctaB*, or ORF1 genes are concluded to be the structure gene for heme O synthase (**Fig. VI-6**). Recently, Svensson *et al.* have shown that the *ctaB* gene of *Bacillus subtilis* expressed in *E. coli* resulted in no apparent production of heme O in the cytoplasmic membrane (Svensson *et al.* 1994). Heme O was not detected in wild-type *Bacillus subtilis* strains while the deletion of the *ctaA* gene caused a production of heme O in the cytoplasmic membrane (Svensson *et al.* 1994). Interestingly, co-expression of the *ctaA* gene with the *ctaB* gene of *Bacillus subtilis* in *E. coli* resulted in accumulation of heme A in the cytoplasmic membrane (Svensson *et al.* 1994). These results indicate that heme O synthesis by CtaB protein of *Bacillus subtilis* needs the presence of the CtaA protein for unknown reasons when expressed in *E. coli*. Physiologically, *Bacillus subtilis* doesn't need heme O for the function of terminal oxidases so that the function of the CtaB protein is controlled by the CtaA protein for effective conversion of heme O to heme A. In contrast, thermophilic *Bacillus* PS3 is known to produce heme O when cultured under air-limited conditions (Sone *et al.* 1990), suggesting that the cells cannot synthesize heme A under limited oxygen tension (**Fig. VI-6**).

Heme A biosynthesis in eubacteria

Up to now, studies of heme A synthase have only been reported in the *Bacillus subtilis* CtaA protein. Svensson *et al.* reported that the *ctaA* gene is needed for the heme A biosynthesis in the catalytic step of formylation of heme O (Svensson *et al.* 1994). CtaA homologues were found in the

terminal oxidase genes of *Bacillus subtilis* (Mueller and Taber 1989, Saraste *et al.* 1991) and *Bacillus firmus* OF4 (Quirk *et al.* 1993). In general, plural terminal oxidases are found in bacterial aerobic respiratory chain (Anraku 1988). Recent gene clonings for these terminal oxidases showed that the *ctaA* and *ctaB* genes are co-existed in the same gene locus in both *Bacillus* species. Svensson *et al.* have shown that the deletion of the *ctaA* gene in the *ctaA-ctaB-ctaCDEF* gene cluster for the *Bacillus subtilis caa*3-type cytochrome *c* oxidase causes a defect in heme A biosynthesis whereas the expression of the *ctaA* gene or the *ctaA* and *ctaB* genes together in *E. coli* resulted in production of heme A (Svensson *et al.* 1994). These results suggest that the formylation at position 8 of heme O take place in the presence of the CtaA protein *in vivo* (Svensson *et al.* 1994). Successively, *Bacillus subtilis* CtaA protein was purified as a heme-containing membrane protein, however, the reaction mechanism of heme A synthesis has not been established (Svensson and Hederstedt 1994). Heme A is a 8-formyl derivatives of heme O (Caughey *et al.* 1975), accordingly, heme A synthase seems to catalyze mono-oxygenation of the methyl group at pyrrole ring D of heme O. Therefore, heme O is a direct precursor of heme A (**Fig. VI-7**) (Saiki *et al.* 1993b and 1994, Mogi *et al.*, 1994). Interestingly, origin of the formyl-group oxygen of chlorophyll *b* was indicated to be directly derived from atmospheric oxygen using $^{18}O_2$ isotope labeling experiments (Schneegurt and Beale 1992, Porra *et al.* 1993).

Heme A biosynthesis in eukaryotes

The *ctaA* gene homologues have not been found in eukaryotes and α-subgroup of the purple bacteria such as the gene cluster of the *aa*3-type cytochrome *c* oxidases in *Paracoccus denitrificans* (Raitio *et al.* 1987) and *Rhodobacter sphaeroides* (Hosler *et al.* 1992, Cao *et al.* 1992). In *Saccharomyces cerevisiae*, the deletion of the *COX10* gene cause the degradation of subunit I (Nobrega *et al.* 1990). If the gene for heme A synthase (heme O mono-oxygenase) was deleted, such mutant strain would be expected to show the same phenotype to the *COX10* gene deletion strain. In fact, mutations in the *COX11* gene showed the very similar phenotype (Tzagoloff *et al.* 1990). The *COX11* gene was cloned and sequenced, and found that the COX11 protein is homologous to ORF3 (or the *ctaG* gene) in the *ctaCBGE* gene cluster for the *aa*3-type cytochrome *c* oxidase of *Paracoccus denitrificans* (Tzagoloff *et al.* 1990). The CtaG proteins of *Paracoccus denitrificans* and *Rhodobacter sphaeroides* and the COX11 protein of *Saccharomyces cerevisiae* are homologous but function of those proteins have not been characterized yet. At present, CtaG/COX11 proteins are the strong candidate for heme A synthase, a heme O mono-oxygenase (Saiki *et al.* 1993b, Mogi *et al.* 1994). If this is the case, the biosynthesis of heme A in eubacteria and eukaryotes are catalyzed by the different enzymes.

Functional role and universal appearance of prenylated hemes in terminal oxidases

Hemes O and A are found only in terminal oxidases and are obligatorily required for the catalytic function of the binuclear center, whereas the low-spin heme binding site of the *E. coli* cytochrome *bo* complex is known to be promiscuous with respect to heme type and can accept heme O when it is over-expressed by a multicopy expression vector (Puustinen *et al.*, 1992). The presence

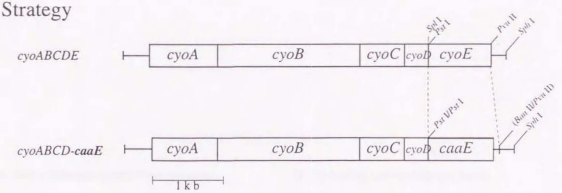
of heme O at the binuclear center of the heme-copper terminal oxidase clearly excludes the possible involvement of a formyl group in the proton pumping mechanism via formation of a Shiff base (Ondrias and Babcock, 1980) or hydrogen bonding between the formyl group and amino acid residues of the oxidase (Babcock and Callahn, 1983). Caughey *et al* suggested that the polyisoprenoid chain of heme A serves as a lipophilic anchor to cytochrome *c* oxidase or participates in conformationally controlled electron transfer over long distances via overlaps of π-electrons of double bonds and porphyrin (Caughey *et al.,* 1975). Anchoring the farnesyl moiety to the protein moiety may stabilize geometric constraints of the high-spin heme iron relative to the Cu_B center or the low-spin heme during the dioxygen reduction chemistry. It is possible that 2-hydroxyethylfarnesyl chain of the high-spin heme extends to the low-spin heme, thereby providing a continuous covalent bond system that ensures an efficient electron transfer between two heme molecules via the supper exchange mechanism (Beratan *et al.,* 1991). In the photosynthetic reaction center, Allen *et al.* have suggested a possible role of the phytyl chain of bacterial chlorophyll in intramolecular electron transfer (Allen *et al.,* 1987). Woodruff *et al.* postulated that the farnesyl moiety of the high-spin heme (or residues nearby at the binuclear center) functions as the ligand shuttle and controls the accessibility and coordination chemistry of exogenous ligands at the binuclear center (Woodruff *et al.,* 1991). Alternatively, it may provide a specific and conformationally controlled route for dioxygen entry to the binuclear center or for protons to be pumped out to the periplasm.

Until recently, prenylated hemes at the high-spin heme binding site were believed to be essential in heme-copper terminal oxidase superfamily, however, peculiar terminal oxidases that bind two heme B molecules in subunit I were identified in *Bradyrhizobium japonicum* (Preisig *et al.,* 1993), *Rhodobacter capsulatus* (Gray *et al.,* 1994, Thöny-Meyer *et al.,* 1994), *Rhodobacter sphaeroides* (García-Horsman *et al.,* 1994), and *Paracoccus denitrificans* (de Gier *et al.,* 1994). These oxidases are homologous with only subunit I of other heme-copper terminal oxidase superfamily. Very interestingly, proton pump capacity was identified in the *cbb*3-type cytochrome *c* oxidase of *Paracoccus denitrificans* (de Gier *et al.,* 1994, Ratio and Wikström 1994). These results indicate that the farnesyl moiety of hemes O and A are not necessary for the proton pump activity in terminal oxidases of this superfamily (Calhoun *et al.,* 1994, García-Horsman *et al.,* 1994). Very recently, the crystal structures of the *aa*3-type cytochrome *c* oxidases of bovine mitochondria and the soil bacterium *Paracoccus denitrificans* were determined at 2.8 Å resolution (Tsukihara *et al.* 1995. Iwata *et al.* 1995). The hydroxyethylfarnesyl group of heme a_3 is twisted to form a U-shaped arm and located between helices VIII and IX, and no interaction with the redox centers or the porphyrin was observed (Tsukihara *et al.* 1995, Iwata *et al.* 1995), suggesting the no catalytic role in electron transfer of the 17-carbon moiety of the high-spin hemes.

Recently, the heme-copper terminal oxidases are suggested to have been evolved directly from denitrification enzymes (Saraste *et al.,* 1994, Castresana *et al.,* 1994). They also proposed that aerobic metabolism in organisms with cytochrome oxidases has a monophyletic and ancient origin, prior to the appearance of eubacterial oxygenic photosynthetic organisms (Castresana *et al.,* 1994). The ancestral oxidase presumably functioned as a primitive terminal oxidase using another molecules as electron acceptors (for example, nitric or nitrous oxide) (Castresana *et al* .,1994). Before the

appearance of photosynthesis systems, a low tension of oxygen had been produced as a result of water photolysis in a biosphere. Therefore, some other ancestral oxidases had to function just to get rid of oxygen that would be poisonous for cells when exposed to a localized high tension of oxygen (Castresana *et al* .,1994). Incorporation of oxygen molecules into heme O might work as an oxygen sensor or regulator of the ancestral oxidase. Since merely formylated protoheme IX is very unstable (Sono *et al.*, 1991), prenyl residue was needed to be transferred into protoheme IX prior to addition of formyl group (Saiki *et al.*, 1993a, Mogi *et al.*, 1995).

(A) Strategy



(B) Complementation test of aerobic growth

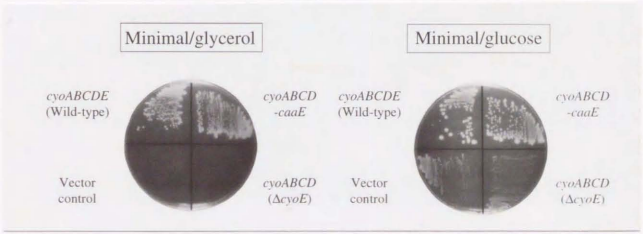
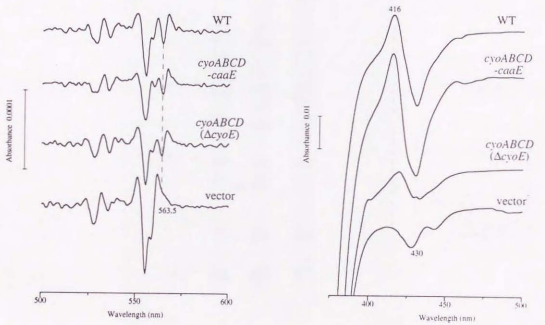


Fig. VI-1 Genetic complementation test of the *caaE* gene from thermophilic *Bacillus* PS3 in the terminal oxidase-deficient *E. coli* mutant using a single copy expression vector
(A) Strategy for construction of the *cyoABCD-caaE* chimeric operon. (B) ST2592 (Δ*cyo* Δ*cyd*) harboring pMFO21 (*cyoABCDE*), pMFO21-*caaE* (*cyoABCD-caaE*), pHNF2 (vector control), abd pMFO21-ΔE2 (*cyoABCD*) were grown aerobically on minimal medium plates containing 0.5% glycerol or 0.5% glucose for 5 days at 37°C.



Fif. VI-2 Second-order finite spectra of dithionite-reduced *minus* air-oxidized difference spectra (A) and CO-reduced *minus* reduced difference spectra (B) of cytoplasmic membranes
The protein concentrations were 3 (A) and 0.5 mg of protein/ml, respectively. Strain ST4676 carrying mini-F plasmid pMFO21 or pHNF2 was used as the wild-type control (*WT*) and a negative control (*control*), respectively.

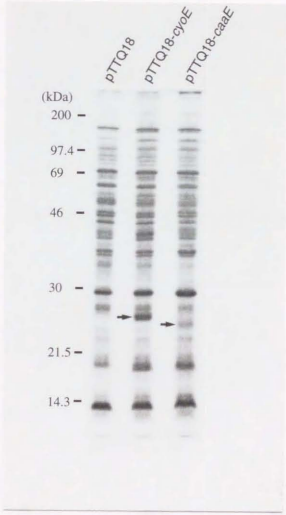


Fig. VI-3 Analysis of the CaaE protein expressed in *E. coli* by 12.5% SDS polyacrylamide gel electrophoresis

Ten μg of cytoplasmic membrane proteins of ST4676 harboring pTTQ18 (vector control), pTTQ18-*cyoE*-2, and pTTQ18-*caaE* was loaded per lane. Proteins were visualized by Coomassie brilliant blue R-250. *Arrow* in lanes indicated the CyoE and CaaE proteins, respectively.

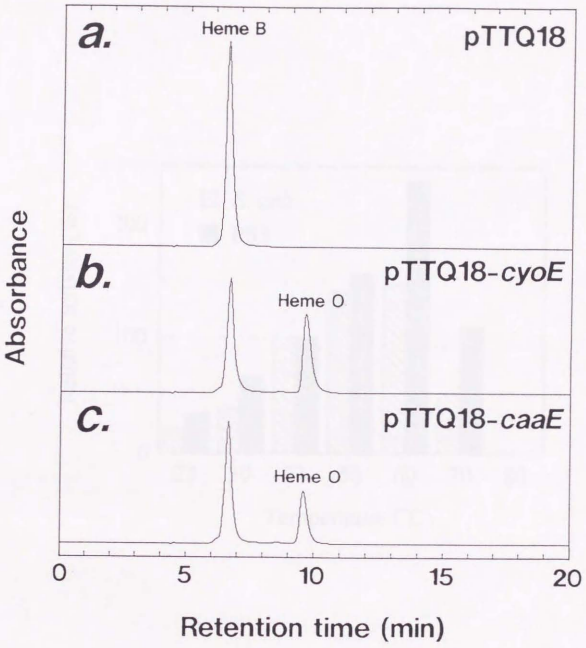


Fig. VI-4 Reverse phase HPLC analysis of the heme composition of cytoplasmic membranes
Hemes extracted from cytoplasmic membranes were analyzed by reverse phase HPLC. (a) ST4676 harboring pTTQ18 (vector control), (b) pTTQ18-*cyoE*-2, and (c) pTTQ18-*caaE*, respectively.

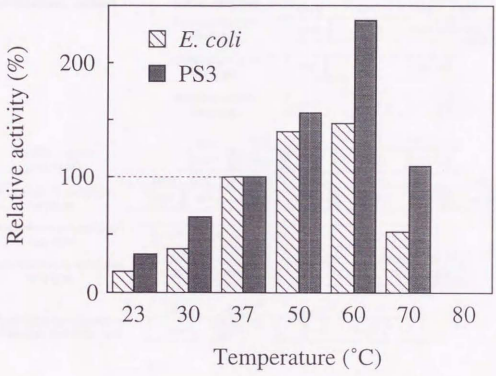


Fig. VI-5 Temperature-dependence of the *in vitro* heme O synthase activity of cytoplasmic membranes isolated from ST4676 harboring pTTQ18-*cyoE*-2 (*E. coli*) and pTTQ18-*caaE* (PS3)
The activity at 37°C refer to 100%. At 80°C, both activity were not determined.

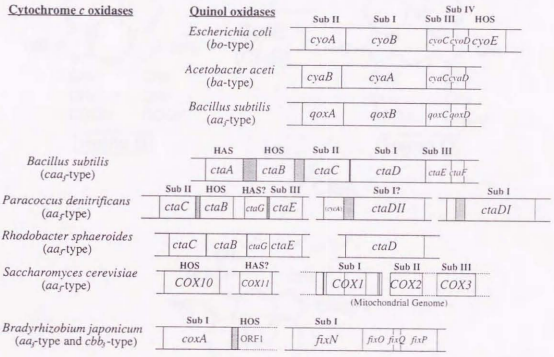


Fig. VI-6 Organization of genes coding or required for the heme-copper terminal oxidases
Genetic loci are shown for quinol oxidases in *E. coli*, *A. acti*, and *B. subtilis*; and cytochrome *c* oxidases in *B. subtilis*, *P. denitrificans*, *R. sphaeroides*, *Saccharomyces cerevisiae*, and *Bradyrhizobium japonicum*. Sub I, Sub II, and Sub III and Sub IV shown above the gene loci indicate subunits of the terminal oxidases. HOS and HAS indicate heme O synthase and heme A synthase, respectively.

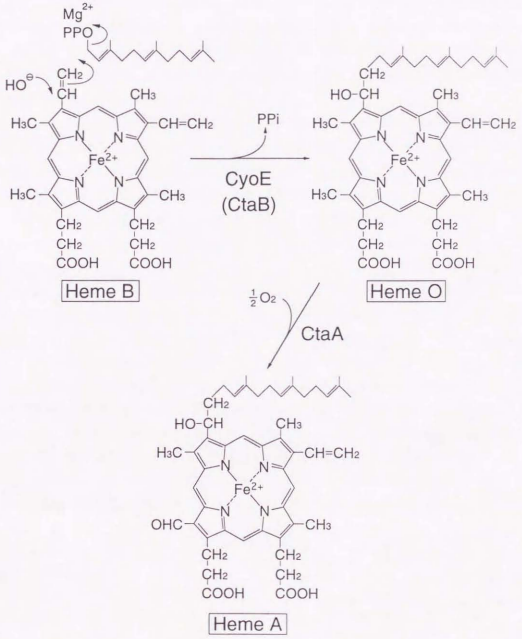


Fig. VI-7 Schematic model of heme O and heme A biosynthesis
Heme O synthesis is catalyzed by the CyoE/CtaB/COX10 protein ferrous protoheme IX farnesyltransferase, and heme A synthesis by the CtaA protein heme O monooxygenase. PPO and PPi indicate diphosphate group and diphosphate, respectively.

Chapter VII. Conclusion

The conclusions obtained throughout my thesis work are as follows:

1. I have constructed 6 CyoE deletion mutants and have individually substituted 40 amino acid residues of the CyoE protein including 22 invariant residues with alanines. I found that the 6 deletion and 23 alanine substitution mutant oxidases are nonfunctional and show a specific loss of the CO binding activity at the site of the high-spin heme. Characterizations of the partially purified D65A, Y120A, and W172A mutant oxidases, which have the mutations of different topological domains, and the *cyoE* deletion mutant oxidase revealed that their defects are attributable to substitution of protoheme IX for heme O present in the high-spin heme-binding site. Based on these observations, the conserved amino acid residues present in the cytoplasmic loops II/III and IV/V are suggested to be part of the catalytic center of the CyoE protein.

2. The identification and localization of the CyoE protein were determined in the CyoE overproducing *E. coli* strains. The CyoE protein was identified as a 26 kDa protein in 12.5% SDS-polyacrylamide gels, and was found to localize mainly in the cytoplasmic membranes. In parallel with overproduction of the CyoE protein, the heme O molecules accumulated in the cytoplasmic membranes. Heme O was not observed in the cytoplasmic membranes of the *cyo* deletion strains. The CyoE protein increases the production of heme O but the other CyoABCD products are not needed for heme O synthesis.

3. *In vitro* heme O synthetic reaction system was successfully constructed using CyoE-overproduced cytoplasmic membranes. Using this system, I found for the first time that heme O can be synthesized from dithionite-reduced ferro-protoheme IX and FPP in the presence of divalent metal cations such as Mg^{2+} or Ca^{2+}. The observed heme O synthetic activity completely depends on the presence of CyoE protein. These results indicate that CyoE protein is a novel enzyme, heme O synthase.

4. The genetic complementation test demonstrated that CyoE and CaaE proteins are essentially and functionally the same enzyme. CaaE overproduced *E. coli* cells show a CaaE protein dependent production of heme O and CaaE overproduced cytoplasmic membranes can catalyze the *in vitro* heme O synthetic reaction like CyoE. These results definitively indicate that CyoE and CaaE proteins are heme O synthase and support that the single enzyme of heme O synthase is responsible for heme O synthesis *in vivo*.

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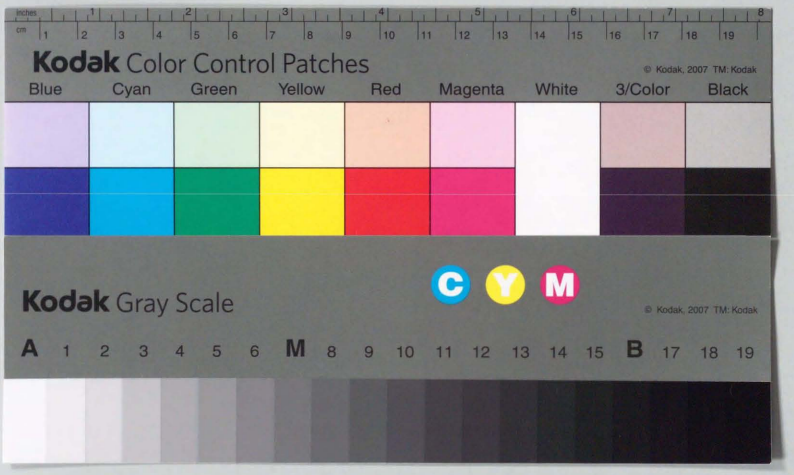
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