The cytochrome bo complex is a heme-copper terminal quinol oxidase in the aerobic respiratory chain of Escherichia coli and contains low spin heme B, high spin heme O and Cu₅ as the redox metal centers in subunit I. Based on site-directed mutagenesis studies on the cyoE gene in the cytochrome bo operon, we have postulated that the cyoE gene encodes a protoheme IX farnesyltransferase (heme O synthase) (Saiki, K., Mogi, T., and Anraku, Y. (1992) Biochem. Biophys. Res. Commun. 189, 1491–1497).

The present study demonstrates that the CyoE protein is localized in the cytoplasmic membrane and that the CyoE-overproduced membranes efficiently catalyze a conversion of exogenous ferrous protoheme IX and farnesyl diphosphate to heme O in the presence of divalent cations such as Mg²⁺ or Ca²⁺. Thus, the cyoABCDE operon in E. coli encodes not only subunits of the cytochrome bo complex but also heme O synthase that is specifically required for functional expression of the bo-type quinol oxidase. Heme O seems to be an intermediate in heme A biosynthesis.

Despite of extensive genetic studies on the pathway and regulation of heme biosynthesis, only a little is known about heme A biosynthesis. In Bacillus subtilis (16), Staphylococcus aureus (17), and Saccharomyces cerevisiae (18), protoheme IX was shown to be a precursor of heme A but no intermediate was so far identified.

Recently, we have carried out systematic deletion (10) and alanine scanning mutagenesis studies (11) of the cyoE gene in the cytochrome bo operon (cyoABCDE) (19). We found that: 1) the cyoE gene is indispensable for heme O biosynthesis, 2) heme O is essential for the catalytic functions of the binuclear center (i.e. the dioxygen reduction chemistry), 3) overproduction of the CyoE protein elicits a conversion of endogenous protoheme IX to heme O in the cytoplasmic membranes, and 4) a binding motif for allylic polypropenyl diphosphate is conserved in a cytoplasmic loop of the CyoE protein family (11). Based on these observations, we have postulated that the cyoE gene encodes a novel enzyme, protoheme IX farnesyltransferase (heme O synthase).

This study established an assay system for heme O synthase and presented evidence that the CyoE protein synthesizes heme O from exogenous ferrous protoheme IX and farnesyl diphosphate (FPP) in the presence of Mg²⁺ or Ca²⁺. Thus, we concluded that the CyoE protein is heme O synthase in E. coli that catalyzes a transfer of a farnesyl moiety of FPP to position 2 of the vinyl group at pyrrole ring A of ferrous protoheme IX with concomitant addition of a hydroxyl group to position 1.

MATERIALS AND METHODS

Bacterial Strains, Growth Medium, and DNA Manipulations—E. coli strains and growth medium used in this study, and DNA manipulations were described previously (10–11). Strain ST4676/pMF01 (Δcyo-Cm'), where the whole cyoABCDE operon was expressed by a single copy expression vector, was grown in the presence of 15 µg/ml ampicillin and used as a wild-type control. For the growth of strain ST4676 (Δcyo-Cm') carrying pTTQ18 derivatives or pUR278-cyoE, 40 µg/ml ampicillin was supplemented to the culture medium.

Chemicals—Farnesyl diphosphate was synthesized by the method of Davison et al. (20) except that a trans,trans-farnesyl bromide (Aldrich) was used as a starting material instead of a chloride salt. Resulting products were analyzed by thin layer chromatography as described (20). Hemin was obtained from Sigma and dissolved at 4 mg/ml in 50% (v/v) ethanol containing 20 mM NaOH. Restriction endonucleases and other chemicals were as described previously (10–11).

Construction of pTTQ18-cyoE-2—In order to avoid a possible effect of the 5'-upstream region of the cyoE gene, we have constructed a new expression vector, pTTQ18-cyoE-2, where only the entire cyoE coding region and its Shine-Dalgarno sequence have been cloned downstream of the tac promoter. The gene-engineered SpI site in pCY063 (11) was blunt-ended by T4 DNA polymerase, and then the 1.0-kb (SpI/SalI fragment containing the cyoE gene was isolated and ligated with the 4.5-kb (EcoRI/SalI fragment of pTTQ18. The resultant plasmid, pTTQ18-cyoE-2, lacks the 3'-terminal half of the cyoD gene (0.3 kb) present in pTTQ18-cyoE (10). The expression level of the cyoE gene in ST4676/pTTQ18-cyoE-2 was examined by Western blotting analysis using the anti-LacZ-CyoE chimera antisemur and shown to be 2-fold higher than that of the previous construct (10).

Preparation of Membrane Vesicles—Cytoplasmic membrane vesicles were isolated from the IPTG-induced cells as described previously (10), and suspended in 10% (v/v) sucrose containing 3 mM EDTA (pH 8.0). Outer membranes and the cytoplasm were saved for analysis of local...
ization of the cyoE gene product. French press vesicles were prepared as described previously (21), and the content of the CyoE protein was estimated to be about 43% of the cytoplasmic membranes by 12.5% SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue R-250 (11). The content of the cytochrome bo complex was estimated by the CO-binding difference spectrum (10).

Preparation of Antiserum against the LacZ-CyoE Chimera Protein—For overproduction of the LacZ-CyoE chimera protein, we constructed an expression vector pUR278-cyoE as follows. The 1.2-kb BamHI-BglII fragment of pCYO61(11) containing the entire cyoE gene was subcloned into the BamHI site of pUR278 (22) to construct the in-frame lacZ-cyoE fusion gene. Strain JM109 harboring pUR278-cyoE was grown in a rich medium (10), and the expression of the chimera protein was induced for 2 h after addition of IPTG to a final concentration of 1 mM. The chimera protein with a molecular mass of about 120 kDa was separated from all the other cytoplasmic membrane proteins using 12.5% SDS-polyacrylamide gel electrophoresis, electroeluted from the gels, and used for immunization of rabbit (23). 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-week intervals. The titer of the antilacZ-CyoE chimera antiserum was examined by Western blotting analysis (11) using the CyoE-overproduced membranes and reached to the maximum level after eight boosts.

Assay of Heme O Synthase—A standard reaction mixture (200 μl) consists of 0.2 mM Tris-HCl (pH 7.4), membrane vesicles containing about 24 μg of the CyoE protein, 60 μM hemin, 120 μM FPP, and 1.5 mM MgSO4. The amount of the CyoE-overproduced membranes (350-600 μg of membrane protein; 24 μg of CyoE protein) in the mixture was normalized by the expression level of the CyoE protein, whereas the control membranes from strain ST4676/pTQT18 were used at 3 mg/ml. The amount of proteoheme IX derived from membrane-bound cytochromes in the reaction mixture was estimated to be about 1% of hemin; therefore, it can be negligible. Heme O synthase reaction was started by addition of a few crystals of sodium dithionite, then continued for 30 min at 37 °C, and terminated by addition of 30 μl of 37% (v/v) formaldehyde. The reaction was optimum at 50 °C and continued linearly up to 30 min at 37 °C (data not shown). Products were analyzed by pyridine hemochrome method or by reverse-phase HPLC as described previously (10-11).

RESULTS AND DISCUSSION

Expression and Localization of the CyoE Protein—The cyoE gene product has been predicted to have seven transmembrane regions and is expected to be in the cytoplasmic membrane (24). The 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. Upon addition of IPTG, a 26-kDa protein that cross-reacts with the anti-LacZ-CyoE chimera antiserum was specifically accumulated in cytoplasmic membranes from strain ST4676/pTQT18-cyoE-2 (∆cyo-Cm'/cyoE'), whereas no cross-reactive polypeptide was found either in the membranes from a negative control (ST4676/pTQT18) and from a wild-type control (ST4676/pMFO1; ∆cyo-Cm'/cyoABCDE') (Fig. 1) or in outer membranes and the cytoplasm of ST4676/pTQT18-cyoE-2 (data not shown). An apparent molecular mass of the CyoE protein was estimated to be 26 kDa in 12.5% SDS-polyacrylamide gel electrophoresis (Fig. 1) and 28 kDa in 18.75% urea-SDS-polyacrylamide gel electrophoresis (data not shown).

Thus, the deviation from a deduced molecular mass of 26 kDa of the CyoE protein was estimated to be negligible. Heme O synthase reaction was started by addition of a few crystals of sodium dithionite, then continued for 30 min at 37 °C (data not shown). Products were analyzed by pyridine hemochrome method or by reverse-phase HPLC as described previously (10-11).

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The expression level of the CyoE protein in the CyoE-overproduced strain amounted to 16% of the cytoplasmic membrane proteins (about 6 nmol/mg protein), whereas we were unable to detect the CyoE protein even immunochemically in the wild-type membranes. Since the content of the cytochrome bo complex in the wild-type cytoplasmic membranes was estimated to

2 H. Nakamura, K. Saiki, T. Mogi, and Y. Anraku, unpublished results.

3 H. Nakamura, K. Saiki, T. Mogi, and Y. Anraku, unpublished results.

be 0.41 nmol/mg protein (7% of the membrane proteins) from the CO-binding spectrum, the expression level of the structure genes for the oxidase complex (cyoABCD) and of the cyoE gene must be regulated in different manners although they are in the same operon. In fact, we have noticed that the expression level of the CyoE protein was much less than that of subunit I in the in vitro protein labeling system and that heme O synthase in vivo occurs even in the absence of a target protein (subunit I) or any other subunits of the cytochrome bo complex.2

The heme O level in the cytoplasmic membrane was found to be constant regardless of the absence of any subunit3 and may be determined simply by the concentration of free protoheme IX molecules in the cytoplasm. Alternatively, the amount of heme O synthase is controlled in tight association with expression of the cytochrome bo complex, or heme O synthase is unstable by some unknown reason. These results are consistent with our previous finding on the CyoE deletion mutant that the cyoE gene product is not a subunit of the cytochrome bo complex (11). Thus, we concluded that the CyoE protein is localized in the cytoplasmic membrane but not associated with the cytochrome bo complex.

In addition, we found that the overproduction of the CyoE protein resulted in the heme B to heme O conversion in vivo (10) and that the in vitro heme O synthase activity was associated only with the cytoplasmic membrane from the CyoE-overproduced strain and from the wild-type strain (Table I). Therefore, the CyoE protein was concluded to be heme O synthase in E. coli.

In Vitro Heme O Synthesis by the CyoE-overproduced Mem-
branes—Using the CyoE-overproduced membranes, we examined the assay conditions for heme O synthase activity. Products were analyzed by reverse-phase HPLC and heme species were identified based on the retention time of heme B (6.1 min) and heme O (8.3 min) from the purified E. coli cytochrome bo complex (10–11).

As shown in Tables II and III and Fig. 2, the CyoE-overproduced membranes can catalyze the in vitro heme O synthesis from exogenous heme (ferric protoporphyrin IX chloride salt) and FPP but only in the presence of sodium dithionite and divalent cations such as Mg\(^{2+}\) and Ca\(^{2+}\). Minor peaks were observed at 10.8 and 13.5 min (Fig. 2b) but no further characterization was carried out because of their low content (less than 5%). We also found that farnesol was unable to substitute for FPP (Fig. 2f) or to compete the reaction even at the concentration 500-fold higher than that of FPP (data not shown). It should be noted that the presence of reducing agents was essential, and dithionite was found to be most effective among reagents tested (i.e. dithiothreitol and ascorbate with phenazinemethosulfate).

Since protoporphyrin IX biosynthesis ends by ferrochelatase in the membrane (25, 26), ferrous protoporphyrin IX is likely a successive substrate of membrane-bound heme O synthase.

As suggested previously (11, 27), the diphasphate group of FPP is likely to interact with divalent cations, and then such a complex can be recognized by the allylic polypropenyl diphasphinate-binding motif present in the CyoE protein family. Alternatively, binding of Mg\(^{2+}\) with FPP may accelerate the cleavage of the diphasphoryl group, forming the farnesyl cation (28). The resulting polypropenyl cation undergoes a nucleophilic attack by the vinyl group of ferrous protoporphyrin IX, thus succeeding to a transfer of the farnesyl group to position 2 of the 2-vinyl group of ferrous protoporphyrin IX with concomitant addition of a hydroxyl group to position 1 (Fig. 3). Since the electron density at the 2-position of the vinyl group is assumed to be higher in ferrous protoporphyrin IX than in its ferric state, the former must be the real substrate for farnesylolation. Therefore, the reduction of hemin was indispensable for the in vitro reaction system.

As shown in chlorophyll biosynthesis (29), the methyl group at pyrrole ring D of heme O can be oxidized to a formyl group with molecular oxygen by a putative heme O monoxygenase (heme A synthase), so heme O is quite likely a direct precursor of heme A. Recently, the ctaAB genes in B. subtilis were suggested to be involved in heme A biosynthesis (30). The ctaA gene is present next to the cyoE homologue (ctaB gene) of the ctaBCDEFG operon for the caa\(_2\)-type cytochrome c oxidase (11, 12), so the ctaA gene product could be a candidate for heme O monoxygenase in heme A biosynthesis.

In conclusion, we demonstrated that the CyoE protein in vitro catalyzes a direct transfer of the farnesyl moiety from a FPP-Mg\(^{2+}\) complex to position 2 of the vinyl group at pyrrole

| Table II |
| --- |
| Components essential for heme O synthase assay |

The CyoE-overproduced French press vesicles containing 24 µg of the CyoE protein were incubated at 37°C in a 0.2 µm Tris-HCl buffer (pH 7.4) containing 60 µM hemin and 120 µM FPP with addition of salts at 1.5 mg. The heme O synthase activity with incubation of the membranes with MgSO\(_4\) refers to 100% (70 nmol of the CyoE protein/30 min).

| Assay condition | Heme O synthase activity (%) |
| --- | --- |
| Complete | 100 |
| - Hemin | 0 |
| - FPP | 3 |
| - MgSO\(_4\) | 13 |
| - Dithionite | 2 |
| + Farnesol/FPP | 2 |

| Table III |
| --- |
| Effect of metal ions on the heme O synthase reaction |

When the CyoE-overproduced French press vesicles were incubated at 37°C in 0.2 µm Tris-HCl buffer (pH 7.4) containing 60 µM hemin and 120 µM FPP with addition of salts at 1.5 mg. The heme O synthase activity with incubation of the membranes with MgSO\(_4\) refers to 100% (70 nmol of the CyoE protein/30 min).

| Salt added | Heme O synthase activity (%) |
| --- | --- |
| MgSO\(_4\) | 100 |
| MgCl\(_2\) | 102 |
| CaCl\(_2\) | 98 |
| ZnCl\(_2\) | 52 |
| CoCl\(_2\) | 45 |
| CdCl\(_2\) | 41 |
| NaCl | 20 |
| PdCl\(_2\) | 4 |
| CuSO\(_4\) | 3 |
| MnCl\(_2\) | NT* |

* Not tested since hemins were unable to extract.

![Fig. 2. Reverse-phase HPLC analysis of products from the heme O synthase reaction.](image)

![Fig. 3. Schematic model of heme O synthesis by the CyoE protein.](image)
In Vitro Heme O Synthesis by E. coli CyoE

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