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An *Escherichia coli cyoE* gene homologue in thermophilic *Bacillus* PS3 encodes a thermotolerant heme O synthase**

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Abstract The *cyoE* gene of the *Escherichia coli bo*-type quinol oxidase operon (*cyoABCDE*) has been previously shown to encode heme O synthase. To demonstrate a catalytic role of a *cyoE* homologue (the *caaE* gene) in the gene cluster for caa_3-type cytochrome *c* oxidase of thermophilic *Bacillus* PS3, we have carried out genetic complementation analysis using the chimeric operon *cyoABCD-caaE* and heme O synthase assay using the CaaE-overproduced *E. coli* membranes. We found that the *caaE* gene encodes a thermotolerant heme O synthase which provides an intermediate for heme A biosynthesis.

Key words: Heme O synthase; *bo*-Type quinol oxidase; Heme-copper terminal oxidase; Heme A biosynthesis; *E. coli cyoE* gene; Thermophilic *Bacillus* PS3

1. Introduction

Hemes O and A are derivatives of protoheme IX (heme B) in which the vinyl group at pyrrole ring A is substituted by a 17-carbon hydroxyethylfarnesyl group, in addition, heme A contains a formyl group in place of the methyl group at pyrrole ring D [1] (for a review see [2–4]). The farnesylated hemes and their variants are found exclusively in the heme-copper respiratory oxidases [1], and seem essential for the catalytic functions of the binuclear center [5–7], except for a newly found subfamily, cbb_3-type cytochrome *c* oxidase [8–10].

The *Escherichia coli bo*-type ubiquinol oxidase is a member of the heme-copper respiratory oxidases, thus it is closely related to mitochondrial and bacterial aa_3-type cytochrome *c* oxidases [11 for a review]. It contains a low-spin heme B, a high-spin heme O and the Cu_B center as the redox metal centers in subunit I and functions as a redox-coupled proton pump [11]. We have recently shown that defects of the *E. coli cyoE* gene of the *bo*-type quinol oxidase operon (*cyoABCDE* [12]) result in the conversion of a functional heme BO-type to a nonfunctional heme BB-type [5,6]. It was noticed that only a deletion of the *cyoE* gene among the five *cyo* genes caused a deficiency of heme O in cytoplasmic membranes (H. Nakamura, K. Saiki, T. Mogi and Y. Anraku, unpublished observations), whereas the overexpression of the *cyoE* gene in the *cyo* operon deletion strain resulted in the conversion of a half of protoheme IX molecules to heme O [5]. Furthermore, we have demonstrated that the CyoE-overproduced membranes can catalyze a transfer of the polyprenyl moiety of farnesyl diphosphate (FPP[1]) to the vinyl group of ferrous protoheme IX in the presence of divalent cations such as Mg^{2+} [13]. Based on these observations and consideration that all the CyoE homologues contain the putative polyprenyl diphosphate-binding motif in loop II/III, we concluded that the *cyoE* gene encodes heme O synthase, protoheme IX farnesyltransferase, and that heme O synthase is required for the supply of the prenylated heme specifically to the heme-copper binuclear center where dioxygen reduction and proton pumping take place [1,5,6,13].

The *cyoE* homologues (i.e., *ctaB, caaE,* ORF1) are present not only in the subunit II/III operon 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 *Thermus thermophilus*, *Bacillus subtilis*, alkaliphilic *B. firmus* OF4 (refs. cited in [1]), and thermophilic *Bacillus* PS3 [14]. It is also known as the yeast nuclear gene, *COX10*, which is essential for the functional expression of mitochondrial cytochrome *c* oxidase [15]. Defects in those of *P. denitrificans*, *Rb. sphaeroides* and yeast are known to cause a deficiency of cytochrome *a* absorption and of cytochrome *c* oxidase activity (refs. cited in [1]). Accordingly, the bacterial and eukaryotic *cyoE* homologues are likey to encode heme O synthase [5,6,13], and may be involved in heme A biosynthesis [6,13, 16].

In this study, we examined the functional role of the *cyoE* homologue (*caaE* gene) in the *caaF-caaE-caaABCD* gene cluster for caa_3-type cytochrome *c* oxidase in thermophilic *Bacillus* PS3 ([14] and M. Ishizuka, unpublished observations). From genetic complementation test in *E. coli* and the heme O synthase assay using the CaaE-overproduced cytoplasmic membranes, we found that the CaaE protein expressed in *E. coli* functions as a thermotolerant heme O synthase in vivo and in vitro and, suggested that the CaaE protein supplies heme O as an intermediate for heme A biosynthesis in thermophilic *Bacillus* PS3.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli strains ST4676 (*Δcyo*-Cm[r] *cyd*[+]) and ST2592 (*Δcyo*-Cm[r] *Δcyd*-Km[r]) were used for preparation of cytoplasmic membrane vesicles and for genetic complementation test, respectively [5,6,17]. Growth conditions were as described previously [5,6].

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Abbreviations: FPP, farnesyl diphosphate; IPTG, isopropyl-1-β-D-thiogalactopyranoside; HPLC, high performance liquid chromatography.

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*2.2. Construction of pCYO6-*caaE

To make plasmid pCYO65, the unique *Pst*I site was introduced in the *cyoE* gene of pCYO6 [6] by site-directed mutagenesis using an oligonucleotide, 5′-TTTCGTTACCTGCAGGTATTG-3′, corresponding to Gln^5 to Lys^{11} of the CyoE, as described previously [5,6]. To create the unique *Pst* I site at the 5′-terminal of the *caaE* gene, a 257 bp *Pst*I–*Bam*HI fragment was amplified by PCR using VENT DNA polymerase (New England BioLabs) and lambda clone λE2 DNA carrying the entire *caa* gene cluster as a template. Oligonucleotides used for PCR were 5′-AGAGAGTTACTGCAGGTTGTGAAAATCG GAATCGTC-3′ corresponding Arg^{27} to Val^{38} and 5′-GCGGATC-CATCCCGGTGACGGTCGG-3′ corresponding to Pro^{111} to Pro*103* of the CaaE. In the PCR product, codons for Ser^{30}-Ser^{31} were changed from 'TCG TCT' to 'CTG CAG'.

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 of pCYO62 [6] before the transcriptional terminator of the *cyo* operon. The resultant plasmid was named pCYO62-CO2–1. Finally, the 240 bp *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*. DNA sequences which have been subjected to the PCR amplification or site-directed mutagenesis were confirmed by sequencing analysis [5,6].

*2.3. Construction of pMFO21-*caaE *and pTTQ18-*caaE

For genetic complementation test, a chimeric operon, *cyoABCD-caaE*, was constructed by subcloning of the 1.5 kb *Eco*RI–*Sph*I fragment of pCYO6-*caaE* into the corresponding site of a single copy expression vector, pMFO21 [5,6], which carries the wild-type *cyoABCDE* operon. The resultant plasmid was named pMFO21-*caaE*. For overexpression of the *caaE* gene, the 1.2 kb *Eag*I–*Sph*I fragment of pCYO6-*caaE* was introduced into the corresponding site of pTTQ18-*cyoE* [13] to produce pTTQ18-*caaE* where the expression of the cloned gene is under control of the *tac* promoter.

2.4. Analysis of heme composition of the cytoplasmic membranes

Cytoplasmic membrane vesicles were isolated from ST4676 harboring pTTQ18 derivatives after induction with IPTG [5,13]. The hemes were extracted from the membranes by acid acetone and were separated by reverse phase HPLC [3,5]. The flow rate was 0.5 ml/min and the elution profile was monitored by the average absorbance at 396 to 402 nm [5].

2.5. Heme O synthase assay

The standard reaction mixture (200 μl) containing 30 to 120 μg of membrane proteins was incubated at 37°C for 30 min after addition of a few crystals of sodium dithionite [13]. After termination of the reaction, the hemes were immediately extracted from the reaction mixture and subjected to HPLC analysis [3,5]. The specific activity of these membranes were found to be nearly the same and the relative activities were expressed as% of the 37°C activity of the respective membranes.

3. Results

3.1. Genetic complementation analysis of the caaE *gene*

Using the *cyoABCD-caaE* chimeric operon on the single copy expression vector, we examined the catalytic activity of the *caaE* gene product in vivo. The control vector pHNF2 and the *cyoE* deletion plasmid pMF021-ΔE2 could not complement the defect of the aerobic growth of ST2592 on minimal/glycerol plates. Strain ST2592 lacks the operons for both *bo*- type and *bd*-type quinol oxidases, therefore, it can grow aerobically only via glycolysis not via oxidative phosphorylation. In contrast, plasmid pMFO21-*caaE* carrying the chimeric operon supported the aerobic growth of the terminal oxidase-deficient strain as did pMFO21 carrying the wild-type *cyo* operon (Fig. 1a). Spectroscopic analysis of the cytoplasmic membranes confirmed that properties of the low-spin and the high-spin hemes of *bo*- type quinol oxidase are the same in the latter two strains (data not shown). 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.

3.2. Overexpression of the caaE *gene in* E. coli

For efficient translation of a heterologous gene in *E. coli*, we took an advantage of the overexpression system established for the *E. coli cyoE* gene [5,13]. Thus, the *caaE* gene corresponding to Val32 to Trp309 (C-terminus) of thermophilic *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 [18].

When the *caaE* gene was expressed in ST4676 (*Δcyo cyd*$^+$)/pTTQ18-*caaE* by induction with IPTG, a 24.5 kDa polypeptide was specifically overproduced in the cytoplasmic membrane (Fig. 2, lane 3). The apparent molecular weight 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 [13]. It is probably due to aberrant electrophoretic mobility of hydrophobic mem-

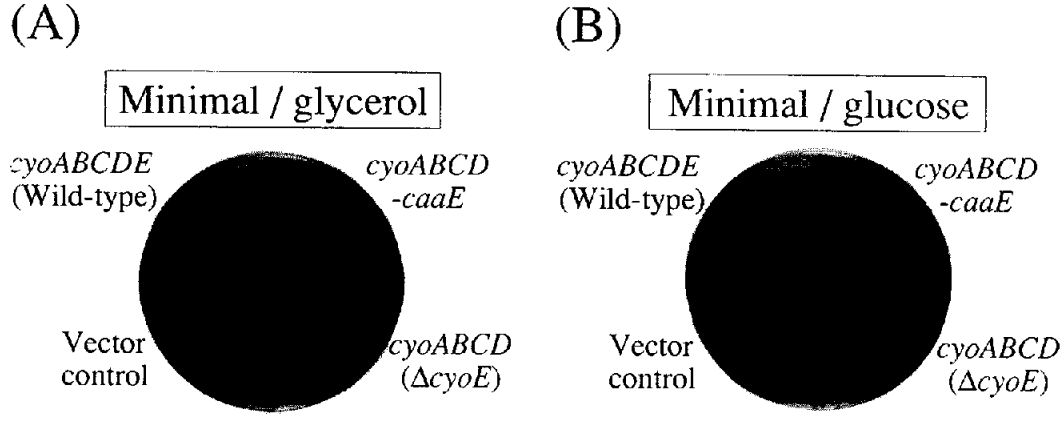

Fig. 1. Genetic complementation test of the *caaE* gene from thermophilic *Bacillus* PS3 in the terminal oxidase-deficient *E. coli* mutant using a single copy expression vector. ST2592 (*Δcyo Δcyd*) harboring pMFO21 (*cyoABCDE*$^+$ [5]), pMFO21-*caaE* (*cyoABCD*$^+$-*caaE*$^+$), pHNF2 (vector control [5,6]), and pMFO21-ΔE2 (*cyoABCD*$^+$ [5]) were grown aerobically on minimal medium plates containing 0.5% glycerol (A) or 0.5% glucose (B) for 5 days at 37°C.

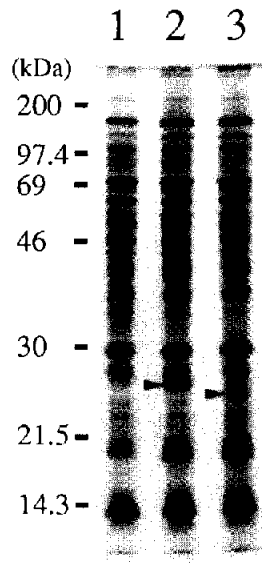

Fig. 2. Analysis of the CaaE protein expressed in *E. coli* by 12.5% SDS polyacrylamide gel electrophoresis. Ten μg of cytoplasmic membrane proteins of ST4676 harboring pTTQ18 (lane 1), pTTQ18-*cyoE* [5] (lane 2) and pTTQ18-*caaE* (lane 3) was loaded per lane and proteins were visualized by Coomassie brilliant blue R-250 [5,6]. *Arrow heads* in lanes 2 and 3 indicate the CyoE and CaaE proteins, respectively.

brane proteins. The expression level of the CaaE protein was estimated to be about 5% of membrane proteins by densitometric analysis and was less than that of the CyoE protein (ca. 10%; Fig. 2, lanes 2 and 3, respectively).

3.3. *Heme O synthesis accompanied by the expression of the* caaE *gene*

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 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 (data not shown). In contrast to the control membranes from ST4676/pTTQ18 (Fig. 3a), the conversion of protoheme IX to heme O occurred in those from ST4676/pTTQ18-*cyoE* (Fig. 3b) and from ST4676/pTTQ18-*caaE* (Fig. 3c). 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. 2).

3.4. *Heme O synthesis catalyzed by the CaaE-overproduced membranes*

In the presence of FPP, ferrous protoheme IX and Mg^{2+}, both the CyoE- and the CaaE-overproduced membranes catalyzed heme O synthesis (Fig. 4). 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, both membranes lost completely the heme O synthase activity.

4. Discussion

Based on site-directed mutagenesis [5,6] and biochemical studies [13], we have postulated that the *cyoE* gene of the *E. coli* cytochrome *bo* operon encodes heme O synthase which catalyzes transfer of a polyprenyl moiety of FPP to the vinyl group of pyrrole ring A of ferrous protoheme IX in the presence of Mg^{2+}. Accordingly, the *cyoE* gene homologues present in or adjacent to the structure genes for the heme-copper terminal oxidases (refs. cited in [1]) and yeast *COX10* [15] were suggeted to encode heme O synthase [5,6,13].

The genetic complementation analysis demonstrated that the *caaE* gene from thermophilic *Bacillus* PS3 can functionally substitute for the *cyoE* gene in *E. coli* (Fig. 1). Furthermore, the CaaE protein expressed in *E. coli* has been shown to catalyze the conversion of protoheme IX to heme O both in vivo and in vitro (Figs. 3 and 4). The observation that the CaaE protein was a thermotolerant heme O synthase (Fig. 4) provides a further support for our proposal. Thus, the *cyoE* gene homologues known as the *caaE*, *ctaB*, or *COX10* gene are concluded to be the structure gene for heme O synthase.

Recently, Svensson et al. [16] have shown that the deletion of the *ctaA*gene in the *ctaA-ctaB-ctaCDEF* gene cluster for the *B. subtilis* caa_3-type cytochrome *c* oxidase caused a defect in heme A biosynthesis whereas the expression of the *ctaA* and *ctaB* genes together in *E. coli* resulted in production of heme A. They have suggested that the *ctaA* and *ctaB* genes encode heme A and heme O synthases, respectively [16]. These results in *E. coli* and *B. subtilis* suggest that the expression of the genes coding for heme O and heme A synthases and for the heme-copper respiratory oxidase subunits must be coordinately regulated for functional expression of the terminal oxidase [1,6]. Heme A is a 8-formyl derivative of heme O [2], accordingly, heme A synthase seems to catalyze a mono-oxygenation of the methyl group at pyrrole ring D of heme O with molecular oxygen [1,13].

Finally, ongoing biochemical and genetic studies on heme O and heme A synthases will deepen our current understanding on their enzymatic mechanisms and regulations. Crystallogra-

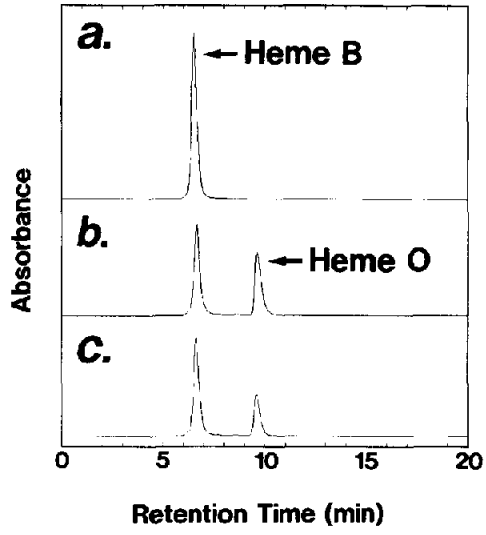

Fig. 3. Reverse phase HPLC analysis of the heme composition of cytoplasmic membranes isolated from ST4676 harboring pTTQ18 (a), pTTQ18-*cyoE* (b) and pTTQ18-*caaE* (c).

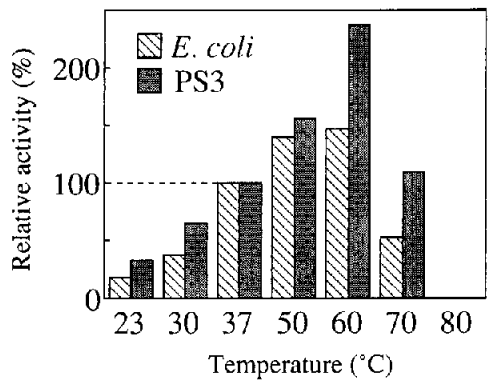

Fig. 4. Temperature-dependence of the in vitro heme O synthase activity of cytoplasmic membranes isolated from ST4676 harboring pTTQ18-*cyoE* (*E. coli*) and pTTQ18-*caaE* (PS3).

phic studies on the CyoE family proteins are anticipated to provide an insight into a mechanism for the site-specific prenyltransfer reaction of a protoporphyrin macrocycle, the product of which is indispensable for dioxygen-dependent energy transduction.

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