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Probing Structure of Heme A Synthase from *Bacillus subtilis* by Site-Directed Mutagenesis

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Biosynthesis of heme A from heme B is catalysed by two enzymes, heme O and heme A synthases, in the membrane. Heme A synthase in *Bacillus subtilis* (CtaA) has eight transmembrane helices and oxidizes a methyl group on pyrrole ring D of heme O to an aldehyde. In this study, to explore structure of heme binding site(s) in heme A synthase, we overproduced the *B. subtilis* His_6-CtaA in *Escherichia coli* and characterized spectroscopic properties of the purified CtaA. On the contrary to a previous report (Svensson, B., Andersson, K.K., and Hederstedt, L. (1996) Low-spin heme A in the heme A biosynthetic protein CtaA from *Bacillus subtilis*. *Eur. J. Biochem*. 238, 287–295), we found that two molecules of heme B were bound to CtaA. Further, we demonstrated that substitutions of His60 and His126 did not affect heme binding while His216 and His278 in the carboxy-halves are essential in heme binding. And we found that Ala substitutions of Cys191 and Cys197 in loop 5/6 reduced heme content to a half of the wild-type level. On the basis of our findings, we proposed a helical-wheel-projection model of CtaA.

Key words: *Bacillus subtilis*, heme A synthase, terminal oxidase, dioxygen reduction, mitochondria.

Abbreviations: HPLC, high-performance liquid chromatography; SML, sucrose monolaurate.

Hemes O and A are 'key compounds' of the aerobic respiration in mitochondria and bacteria and used for the dioxygen reduction site of the heme-copper terminal oxidases (*i.e*. cytochrome *c* oxidase and quinol oxidase) while heme D (chrolin) is preferred for the dioxygen reduction by bacterial *bd*-type quinol oxidase (*1*). Heme O and heme A carry a 17-hydroxyethylfarnesyl group at position 2 of pyrrole ring A and heme A is an 8-formyl derivative of heme O (*2*, *3*) (Fig. 1). In *Escherichia coli*, we discovered that the last ORF (*cyoE*) in the cytochrome *bo* operon encodes heme O synthase (*4–6*). CyoE synthesizes and supplies heme O just enough for the heme *o*-Cu_B binuclear centre of cytochrome *bo*-type quinol oxidase (CyoABCD). Δ*cyoE* mutations eliminated completely heme O in the cytoplasmic membranes, and resulted in a non-functional heme *bb*-type oxidase (*5–7*). Alanine scan mutagenesis revealed the presence of a catalytic site in cytoplasmic loops (*6*). We demonstrated that in the presence of Mg^{2+} or Ca^{2+} CyoE-overproduced membranes catalysed the transfer of a farnesyl group from farnesyl diphosphate to the 2-vinyl group on pyrrole ring A of ferrous heme B (*4*) (Fig. 1). We found also that the *cyoE* homologue (*ctaB*) in the *ctaA-ctaB-ctaCDEF* gene cluster of the thermophilic *Bacillus* PS3 encodes a thermotolerant heme O synthase (*8*). Thus, we concluded that the *cyoE*/*ctaB* gene, which is often associated with the bacterial oxidase operon, encodes a novel enzyme, heme O synthase. Mutational and expression studies on the *ctaB* gene in *Bacillus subtilis* (*9*, *10*) and *Bacillus cereus* (*11*, *12*) and the *COX10* gene in *Paracoccus denitrificans* (*13*), *Rhodobacter sphaeroides* (*14*), yeast (*15*, *16*) and human (*17*, *18*) reached to the same conclusion.

In *B. subtilis*, Svensson *et al*. (*9*, *10*) showed that the *ctaA* gene is involved in heme A biosynthesis where CtaA (heme A synthase) oxidizes the 8-methyl group of heme O to a formyl group (Fig. 1). CtaA homologue (Cox15p) in yeast (*19*, *20*) and human (*21*) plays a similar role in heme A synthesis. Svensson and Hederstedt (*22*) expressed the *B. subtilis* CtaA and CtaB in *E. coli* (BL21/pET-3a:*ctaB*/pET-9d:*ctaA*) at some extent and determined structures of hemes produced in the cells. They postulated that CtaA catalyses the oxidation of the 8-methyl group to a formyl group through a C8 alcohol intermediate *via* successive P450-like monooxygenase reactions, as reported for chlorophyll *b* biosynthesis (*1*, *23*, *24*). Barros et al. (*20*, *25*) found in *Schizosaccharomyces pombe* that ferredoxin is fused at the C-terminus of CtaA and demonstrated that ferredoxin (Yah1p) and ferredoxin reductase (Arh1p) are required for heme A synthesis in yeast (*Saccharomyces cerevisiae*). These observations are consistent with the P450-like monooxygenase mechanism; however, hemes in CtaA are not coordinated by the sulphur atom of cysteines (*10*). The source of oxygen for the 8-formyl group has been considered as molecular oxygen, but Brown *et al*. (*26*) suggested that the dominant source of the oxygen atom in heme A is derived from H_2O.

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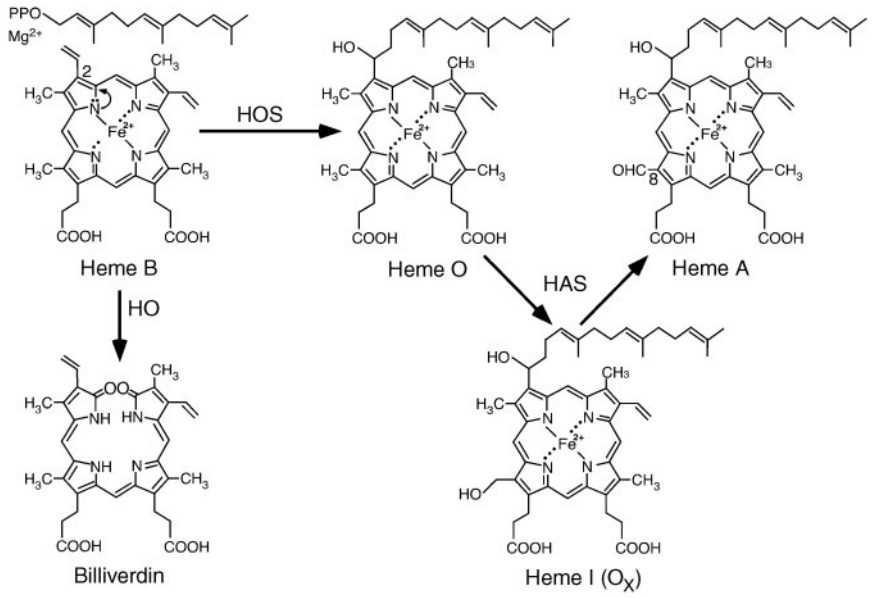

Fig. 1. **Biosynthesis of Heme O and Heme A.** Conversion of heme B to heme O is catalysed by HOS (heme O synthase), which is encoded by the *E. coli cyoE*, *Bacillus subtilis ctaB*, and mitochondrial *COX10* genes. HAS (heme A synthase), which is encoded by the *B. subtilis ctaA* and mitochondrial *COX15* genes, oxidizes heme O to heme A through heme I (O_X). PPO indicates a diphosphoryl group. Degradation of heme B to billiverdin is catalysed by heme oxygenase (HO).

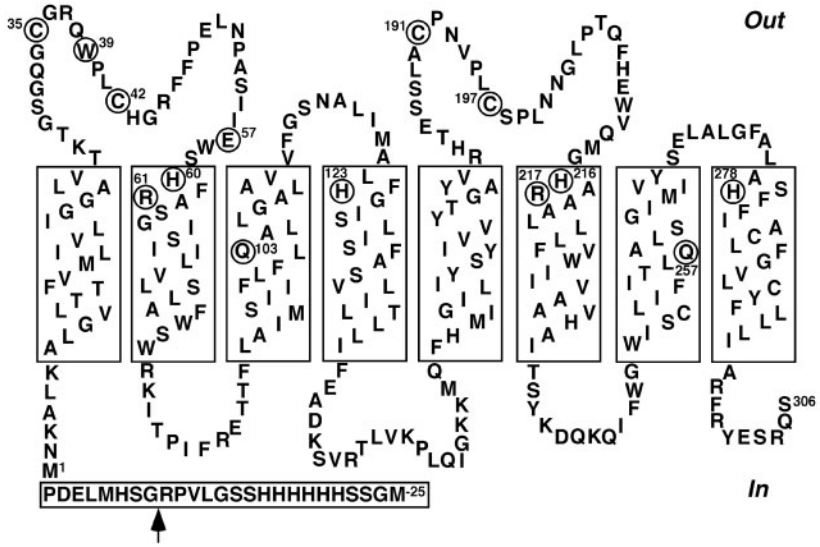

Fig. 2. **Topological model of heme A synthase from *B. subtilis* (taken from ref. 22).** Amino acid residues substituted in this study are 'encircled', and the vector-derived extra sequence at the N-terminus is shown by box. An arrow indicates the thrombin cleavage site. Among histidines in CtaA, His123, His216 and His278 are strictly conserved while His60 is substituted by Asn in the Actinomycetales (*Mycobacterium* and *Streptomyces*) (*1*). A '$Cx_{5-12}C$' motif in loops 1/2 and 5/6 is another conserved feature in Gram-positive bacteria. CtaA is fused to CtaB in *Thermus thermophilus* (GenBank accession no. Q5SLI3) while it lacks one-halves in the hyperthermophilic archaebacterium *Aeropyrum pernix* (GenBank accession no. B72551).

Svensson *et al*. (*10*, *22*) expressed the wild-type CtaA in *B. subtilis* and *E. coli* and found that the isolated CtaA binds only a substoichiometric amount (~0.4 mol) of low-spin heme b_{558}. It showed peaks at 428, 528 and 558 nm in the reduced state and at 414 nm in the oxidized state. No other redox metal centres were identified at a significant level. When co-expressed with heme O synthase (CtaB) in *B. subtilis*, they found that CtaA binds ~0.2 mol each of heme B and heme A and shows the α peaks at 558 and 585 nm, respectively, in the reduced state (*10*). It was assumed that heme A was unreleased product and that heme B was a cofactor for the activation of dioxygen. Svensson *et al*. (*10*, *22*) proposed that CtaA has originated by the result of a tandem gene duplication and that conserved His60, His123, His 216 and His278 in transmembrane helices

2, 4, 6 and 8 (Fig. 2) serve as axial ligands for low-spin heme b_{558} (E_m, +85 mV) and a_{585} (+242 mV). However, His60 is substituted by Asn in the Actinomycetales like *Streptomyces* and *Mycobacterium* (*1*), may not be essential for the heme binding and activity. Later, Sakamoto *et al*. (*27*) expressed the *Bacillus stearothermophilus* CtaA in *E. coli* (XL-1 blue/pUCctaA) and showed the presence of substoichiometric amounts of heme B and A in *ba*-type cytochrome expressed. They found also a single turnover of exogenous heme O to heme A probably within cytochrome b_{558} present in membranes isolated from XL-1 blue/pTrcctaA (*27*).

In this study, to explore structure of heme-binding site(s) in heme A synthase, we overproduced the *B. subtilis* His_6-CtaA in *E. coli* and characterized spectroscopic properties of the purified CtaA. On the contrary to previous reports by Svensson *et al*. (*10*, *22*), we found that two molecules of heme B were bound to CtaA. Further, we identified by site-directed mutagenesis that His216 and His278 in the carboxy-halves are essential for heme binding and that Ala substitutions of Cys191 and Cys197 in loop 5/6 reduced heme content to a half of the wild-type level. On the basis of our findings, we proposed a helical-wheel-projection model of CtaA.

EXPERIMENTAL PROCEDURES

Cloning, Mutagenesis and Expression of CtaA Gene—Heme A synthase gene (*ctaA*, 921 nt) was amplified by PCR from the chromosomal DNA of *B. subtilis* strain IAM12119 (Institute of Molecular and Cellular Biosciences, University of Tokyo) with primers CtaA43F and CtaA57R (Table S1), and cloned into the *Nde*I–*Eco*RI site of pET-3b (Novagen). Since the expression of CtaA with pET-3b-ctaA was poor, the *ctaA* gene was amplified as a *Bam*HI–*Bcl*I fragment by PCR with primers CtaAPet37F and CtaABcl44R (Table S1), and subcloned into the *Bam*HI site of pET-15b (Novagen). A resultant clone with the correct orientation was confirmed by DNA sequencing and named as pET-15b-ctaA. Amino acid substitutions were introduced with QuickChange XL (Stratagene) using synthetic oligonucleotides. Nucleotide sequence of the entire wild-type and mutant genes was confirmed by DNA sequencing with 3100 Genetic Analyzer (Applied Biosystems, CA, USA). For the expression of CtaA, *E. coli* BL21 (DE3)/pLysS (Novagen) was transformed with mutant plasmids, and then transformants were grown at 37°C in IM medium (*28*) supplemented with trace metals, 0.1 mg/ml Na-ampicillin, and 0.2 mM 5-aminolevulinic acid (Wako Pure Chemicals, Osaka, Japan). At OD_{600} of ~0.4, the expression of CtaA was induced for 4 h after addition of isopropyl-β-D-thiogalactopyranoside (Wako Pure Chemicals) to a final concentration of 0.5 mM.

Isolation of His_6-CtaA by Affinity Chromatography—Cells were suspended in 50 mM Tris–HCl (pH 7.4) containing 10 mM Na-EDTA (pH 8), 0.5 mM phenylmethanelsulphonyl fluoride (Sigma), 0.5 mM benzamidine (Sigma) and 0.5 mg/ml egg white lysozyme (Sigma), incubated on ice for 30 min, and disrupted by sonication. After removal of unbroken cells and spheroplasts, cytoplasmic membranes were isolated by isopicnic sucrose density centrifugation (*29*). Proteins were solubilized from the membrane vesicles with 2.5% sucrose monolaurate (SML, Mitsubishi-Kagaku Foods Co., Tokyo, Japan) in 50 mM Na-phosphate (pH 7.4) containing 1 mM phenylmethanelsulphonyl fluoride by stirring for 30 min at 4°C. The mixture was centrifuged for 30 min at 100,000×*g*. Supernatant was applied to a Ni–NTA His-Bind Superflow (Novagen) column (25 mm i. d. × 10 cm) equilibrated with buffer A [50 mM Na-phosphate (pH 7.4), 0.3 M NaCl and 0.1% SML] containing 40 mM imidazole (MP Biochemicals, Ohio), and His_6-CtaA was eluted with buffer A containing 200 mM imidazole. The eluate was concentrated and desalted by ultrafiltration with Amicon Ultra-15 (50,000 MWCO, Millipore). Then, the CtaA-enriched fraction was applied to a Toyopearl AF-Chelate-650M (Tosoh, Tokyo) column (15 mm i. d. × 12 cm), and CtaA was eluted with a 0–125 mM linear gradient of imidazole in buffer A at the flow rate of 3 ml/min. For removal of the His_6-tag at the *N*-terminus, the isolated CtaA in phosphate-buffered saline containing 0.05% SML was digested with thrombin (Amersham Biosciences; 10 U/mg CtaA) for 2 h at room temperature, and the His-tag-free CtaA was isolated as the flow through fraction from a Ni–NTA His-Bind Superflow column. Purified enzymes in buffer A were stored at –80°C until use.

Electrophoresis and Western Blotting Analysis—Forty micrograms of cytoplasmic membrane proteins were subjected to 5–20% SDS–polyacrylamide gel electrophoresis, followed by western blotting analysis with rabbit Anti-His-Tag antibody (Medical Biological Lab., Nagoya, Japan). His_6-CtaA was visualized by using alkaline phosphatase-conjugated sheep anti-rabbit IgG (Chemicon Int.). Two micrograms of the purified CtaA protein was subjected to 5–20% SDS–polyacrylamide gel electrophoresis, and visualized with GelCode Blue Stain Reagent (Pierce).

Determinations of Heme and Protein Content and Heme Analysis—Heme B content was determined by pyridine hemochromogen method, and protein concentration was determined with BCA Protein Assay Reagent (Pierce) (*29*). Hemes were extracted from the membranes or the purified CtaA with HCl-acetone and separated by reverse-phase high-performance liquid chromatography (HPLC) using an Altex Ultrashere ODS column (4.6 mm i. d. × 25 cm; Beckman Coulter) or Devolosil 300ODS-HG-5 (4.6 mm inner diameter × 15 cm; Nomura Chemical) (*5*). The solvent was 95% ethanol/acetic acid/water (70:17:7, vol/vol), and the flow rate was 0.5 ml/min. The elution profile was monitored at 396 and 400 nm with a SPD-M10A_{VP} photodiode array detector (Shimadzu Co., Kyoto Japan). Hemin (Sigma) and hemes B and O extracted from the *E. coli* cytochrome *bo*, heme A from bovine cytochrome *c* oxidase were used as standards.

Absorption Spectroscopy—Absorption spectra of the air-oxidized and Na-hydrosulphite reduced forms of CtaA in buffer A were determined with a V-550 UV/Vis spectrophotometer (JASCO, Tokyo, Japan) (*30*). Low-temperature absorption spectra were recorded with a UV-3000 double wavelength spectrophotometer (Shimadzu, Kyoto, Japan) (*29*).

RESULTS AND DISCUSSION

Over-expression of His_6-CtaA—For the over-expression of the *B. subtilis* CtaA in *E. coli*, we first constructed pET-3b-ctaA and expressed the *ctaA* gene in BL21(DE3)/pET-3b-ctaA. Western blotting analysis of cytoplasmic membranes with the anti-His-tag antibody showed that the expression level of His_6-CtaA was poor (data not shown). Then we subcloned the *ctaA* gene into the *Bam*HI site of pET-15b. Upon 4 h of the induction with 0.5 mM isopropyl-β-*D*-thiogalactopyranoside in BL21 (DE3)/pLysS/pET-15b-ctaA, a 29-kDa protein, which cross-reacted with the anti-His-tag antibody, was accumulated in cytoplasmic membranes (Fig. 3). Due to anomalous mobility of membrane proteins in SDS–polyacrylamide gels, an apparent molecular mass of His_6-CtaA was much smaller than 36,834 Da, expected from DNA sequence. Similarly, the molecular mass of the *E. coli* heme O synthase (CyoE, 32,249 Da) has been estimated to be 26 kDa in 12.5% SDS–polyacrylamide gel electrophoresis (*5*). On the basis of the heme B content, the expression level of His_6-CtaA was estimated to be 10–15% of membrane proteins (Table 1), which is much higher than that reported by Brown *et al*. (*31*).

Isolation and Characterization of Wild-Type His_6-CtaA by Affinity Chromatography—His_6-CtaA was solubilized from cytoplasmic membrane vesicles by 2.5% SML and partially purified by one-step elution from a Ni–NTA column with 200 mM imidazole. The yield was about 1 mg His_6-CtaA per litre culture (~5 g wet cells), and the purity was about 20% in SDS–polyacrylamide gel

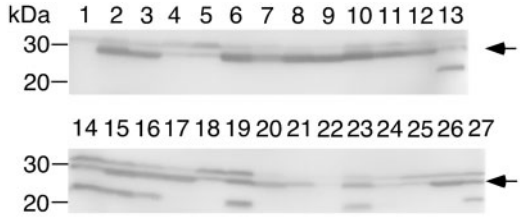

Fig. 3. **Western blot analysis of cytoplasmic membranes isolated from CtaA mutants with the Anti-His-Tag antibody.** Samples are: (i) vector control (BL21(DE3)/pLysS/pET-15b), (ii) wild type, (iii) W39A, (iv) E57A, (v) E57Q, (vi) H60A, (vii) H60Q, (viii) R61A, (ix) R61Q, (x) Q103A, (xi) H123A, (xii) H123Q, (xiii) H216A, (xiv) H216Q, (xv) R217A, (xvi) R217Q, (xvii) Q257A, (xviii) H278A, (xix) H278Q, (xx) H60A/H123A, (xxi) H60Q/H123Q, (xxii) H216A/H278A, (xxiii) H216Q/H278Q, (xxiv) E57A/H216A, (xxv) E57A/H278A, (xxvi) C35A/C42A and (xxvii) C191A/C197A. Forty micrograms of membrane proteins were loaded per lane on 5–20% SDS–polyacrylamide gel. His_6-CtaA was visualized with alkaline phosphatase-conjugated second antibody. Arrows indicate His_6-CtaA (29 kDa).

Table 1. **Heme content in cytoplasmic membranes and properties of CtaA mutants isolated by affinity chromatography**

Mutants	Heme content (nmol/mg protein)		Absorption maxima (nm)
	Membranes	Purified CtaA	Air-oxidized form/fully reduced form
PET-15b	2.4	ND[a]	ND
Wild-type	5.7	54.6 (556.0)[b]	413.5/427.0, 529.5. 558.5
W39A	5.9	45.8 (556.0)	413.5/427.0, 529.0. 558.5
E57A	2.9	33.6 (555.0)	413.0/425.5, 528.0. 557.5
E57Q	3.0	35.3 (555.3)	413.0/426.0, 528.5. 557.5
H60A	6.8	40.3 (556.0)	413.0/427.0, 529.5, 558.5
H60Q	5.8	35.7 (555.5)	413.0/427.0, 529.5, 558.5
R61A	6.8	42.1 (555.5)	413.5/427.0, 529.5, 558.5
R61Q	7.2	43.1 (556.0)	413.0/427.5, 529.5, 558.5
Q103A	6.1	44.8 (556.0)	413.5/427.0, 529.5, 558.5
H123A	4.5	40.3 (555.5)	413.0/426.5, 529.5, 558.5
H123Q	4.9	34.9 (555.5)	413.5/427.0, 529.5, 558.5
H216A	3.2	12.1 (555.0)	407.8, 671/424.0, 529.0, 558.0, 671
H216Q	3.2	18.2 (553.5)	403.0, 672/423.5, 530.5, 558.0, 671
H216M	4.6	20.2 (554.5)	404.5, 672/423.0, 532.3, 557.5, 671
E57A-H216A	3.3	18.2 (553.5)	411.0, 669/423.5, 527.0, 556.5, 671
R217A	5.4	45.7 (555.5)	410.5/426.0, 530.0, 560.0
R217Q	5.2	48.1 (556.0)	408.5/426.0, 530.0, 560.0
Q257A	5.8	42.6 (556.0)	413.5/427.5, 530.5, 560.5
H278A	2.5	30.5 (555.5)	408.5/424.0, 529.5, 559.5
H278Q	2.8	8.3 (552.5)	406.5/423.0, 528.0, 557.5
H278M	3.4	22.1 (555.5)	409.5/424.0, 530.0, 560.0
H278C	3.4	7.6 (555.0)	409.5/423.8, 528.0, 558.0
E57A-H278A	2.7	22.8 (552.5)	411.0/423.3, 527.0, 556.5
C35A-C42A	6.9	42.2 (556.0)	413.0/427.0, 529.0, 558.5
C191A-C197A	6.0	27.8 (556.0)	411.5/425.5, 530.5, 561.0
H60A-H123A	7.5	48.0 (556.0)	412.0/427.0, 529.5, 558.5
H60Q-H123Q	4.5	45.4 (555.5)	413.5/427.0, 529.5, 558.5
H216A-H278A	2.7	2.9 (552.5)	407.0/422.8, 525.8, 556.5
H216Q-H278Q	2.6	3.3 (553.5)	407.0/422.5, 526.5, 555.0

[a]Not determined. [b]Absorption maxima of pyridine ferrohemochrome.

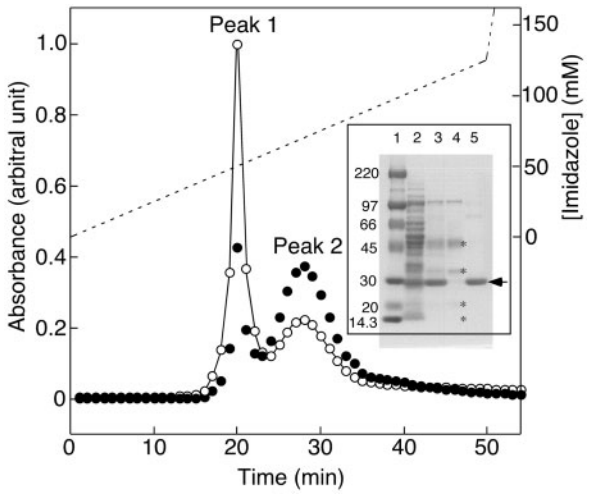

Fig. 4. **Elution profile of *b*-type cytochromes on affinity chromatography.** Wild-type CtaA fractions from Ni-NTA chromatography were applied on a Toyopearl AF-Chelate-650M column and *b*-type cytochromes were eluted with a 0–125 mM linear gradient of imidazole in 50 mM Na-phosphate (pH 7.4), 0.3 M NaCl and 0.1% SML. Elution profiles of proteins and cytochromes were monitored at 280 (open circle) and 413 (closed circle) nm, respectively. Inset: 5–20% SDS–polyacrylamide gel electrophoresis analysis. Lane 1, rainbow-coloured protein molecular weight markers (Amersham); lane 2, cytoplasmic membranes isolated from BL21(DE3)/pLysS/pET-15b-ctaA; lane 3, Ni–NTA chromatography fractions; lane 3, peak 1; lane 4, peak 2. Asterisks and an arrow indicate four subunits of cytochrome *bo* (CyoB, CyoA, CyoC and CyoD) and His_6-CtaA, respectively.

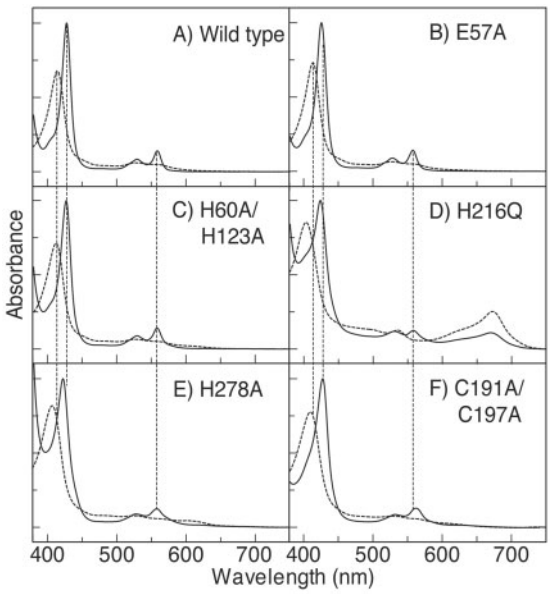

Fig. 5. **Absorption spectra of the air-oxidized (broken line) and fully reduced (solid line) forms of purified CtaA mutants.** Absolute spectra of purified CtaA mutants (10 μM heme B) were recorded in 50 mM Na-phosphate (pH 7.4) containing 0.1% SML before and after reduction with Na-hydrosulfite.

electrophoresis analysis (lane 3 in inset of Fig. 4). Then, His_6-CtaA was purified by affinity chromatography with a Toyopearl AF-Chelate 650M column. We found that two *b*-type cytochromes were eluted by a linear 0–125 mM imidazole gradient (Fig. 4). Peak 1 has an Abs413/Abs280 ratio of 0.42 (Fig. 4) and shows peaks at 427, 532 and 561 nm in the reduced state and at 411 nm in the air-oxidized state (data not shown). A broad α peak at around 561 nm is an indicative of low-spin heme *b* of cytochrome *bo*-type quinol oxidase (*32*) and splits into 559 and 565 nm at room temperature (data not shown) and 555.5 and 563.5 nm at 77 K (*29*) in the second-order finite difference spectra. Five to twenty percent SDS–polyacrylamide gel electrophoresis showed the presence of four subunits of cytochrome *bo* with apparent molecular mass of 55, 33.5, 21.5 and 14 kDa, corresponding to CyoB (74,367.9 Da), CyoA (34,911.3 Da), CyoC (22,622.6 Da) and CyoD (12,029.5 Da), respectively (lane 4 in inset of Fig. 4). Accordingly, we concluded that peak 1 was cytochrome *bo*.

Peak 2 contains a 29-kDa protein, which can be detected with anti-His-tag antibody (data not shown). Thus, we concluded that peak 2 was His_6-CtaA. It should be noted that the *E. coli* heme O synthase (CyoE) appears not to be co-purified with the *B. subtilis* CtaA, although Brown *et al*. (*31*) reported the co-purification of CtaA with CtaB by affinity chromatography. Such a heterodimer has not been reported for the co-expression of CtaA with CtaB in *B. subtilis* (*10*) and of Cox15p with Cox10p in yeast (*33*).

Purified His_6-CtaA has an Abs413/Abs280 ratio of 1.7 (Fig. 4) and shows peaks at 427, 529.5 and 558.5 nm in the reduced state and at 413.5 nm in the air-oxidized state (Fig. 5, Table 1), which are similar to spectroscopic properties described by Svensson *et al*. (*10*, *22*) for the *B. subtilis* CtaA which has not been co-expressed with the *B. subtilis* heme O synthase (CtaB). Removal of extra 17 amino acid residues at the *N*-terminus of His_6-CtaA (Fig. 2) by thrombin reduced apparent molecular mass from 29 kDa to 28 kDa (*cf*. 34,952 Da expected from DNA sequence) but did not affect spectroscopic properties (data not shown). At 77K, the redox difference spectrum showed peaks at 427, 527 and 553.5 nm with a shoulder peak at 556 nm and a broad feature at 473 nm (data not shown). In the second-order finite difference spectrum ($\Delta\lambda = 2.1$ nm), the α peak splits into 553 and 557.5 nm and the β peak into 527.5, 533 and 536.5 nm. The α peak splitting was similar to 553 and 558 nm, reported by Svensson *et al*. (*10*) and may be originated from interactions of the 2-vinyl group of heme B with the protein which cause the distortion in the electronic transitions in the heme plane (*34*). Alternatively, despite the similarity in structures between the *N*-terminal and *C*-terminal halves of CtaA (Fig. 2), there may be some differences in molecular environments of two heme-binding sites.

On the contrary to Svensson *et al*. (*10*, *22*), who found only substoichiometric amount of hemes (0.4 mol/mol CtaA), the heme B content of the wild-type His_6-CtaA (54.6 nmol/mg protein in Table 1) clearly indicates the

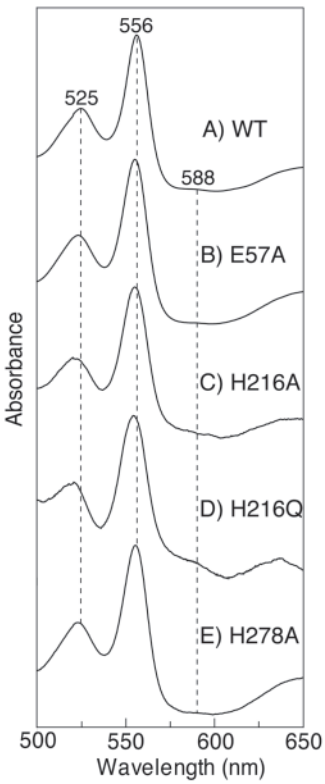

Fig. 6. **Redox difference spectra of pyridine hemochrome extracted from purified CtaA mutants.**

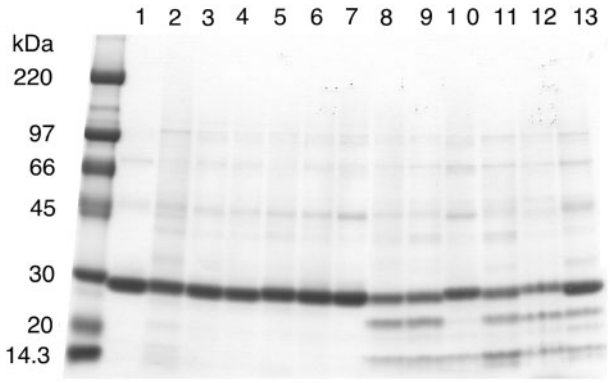

Fig. 7. **SDS–polyacrylamide analysis of mutant CtaA proteins isolated by affinity chromatography.** Samples are: (i) wild-type, (ii) E57A, (iii) H60A, (iv) H60Q, (v) H123A, (vi) H123Q, (vii) H60A/H123A, (viii) H216A, (ix) H216Q, (x) H278A, (xi) H278Q, (xii) H216A/H278A and xiii) C191A/C197A. Two micrograms of purified CtaA mutants were loaded per lane on 5–20% SDS–polyacrylamide gel, and proteins were visualized with GelCode.

presence of two heme-binding sites (2.01 mol heme B/mol His_6-CtaA). In the redox difference spectrum of pyridine ferrohemochrome, we found a major α peak at 556 nm, which is identical to that for heme B (*1*), and a very small peak at 588.5 nm (below 0.4% of the 556-nm peak) (Fig. 6). The latter peak can be also found for hemin, and the peak position was different from 585 nm for heme A (*1*). It is likely some degradation product of heme B, not heme A. Molar extinction coefficients for CtaA were estimated to be $\varepsilon_{427} = 364{,}000$, $\varepsilon_{558.5} = 52{,}000$ and $\varepsilon_{413.5} = 254{,}000$. These values are comparable to $\varepsilon_{428} = 362{,}000$, $\varepsilon_{562} = 43{,}400$ and $\varepsilon_{413.5} = 271{,}000$ of the *E. coli* cytochrome *bo*, where the contribution of high-spin heme *o* to the 562-nm peak is ~76% of that of low-spin heme *b* (*34*). We found that 74% of *b*-hemes in CtaA can be reduced by Na-ascorbate (E_m, +47 mV) in 30 min, not by ubiquinol-1 (E_m, +90 mV) (data not shown). This indicates that two *b*-hemes in His_6-CtaA have similar E_m values, which is comparable to +85 mV determined for low-spin heme b_{558} of the *B. subtilis* CtaA (*10*).

Construction and Expression of CtaA Mutants—To probe structure of the heme-binding sites, we substituted conserved Trp39, Gln103 and Gln257 by Ala, Glu57, His60, Arg61, His123, Arg217, Gln257 and His278 by Ala and Gln, His216 by Ala, Gln and Met, His278 by Ala, Gln, Met and Cys (Fig. 2). We also constructed double mutants: E57A/H216A, E57A/H278A, H60A/H123A, H60Q/H123Q, H216A/H278A, H216Q/H278Q, C35A/C42A and C191A/C197A. Four histidines are likely candidates for heme ligands (*10*, *22*). Two cysteines in loop 1/2 and 5/6 are conserved in Gram-positive bacteria and have been suggested to be functionally important (*22*). They may serve as ligands for unidentified metals to mediate electron transfer to the bound hemes (*1*).

Western blotting analysis of cytoplasmic membranes with anti-His-tag antibody showed that the expression level of CtaA mutants was reduced in Glu57, His216 and His278 mutants including their double mutants and C191A/C197A double mutant (Fig. 3). A 23-kDa cross-reacting protein seen in these mutants is likely a degradation product of His_6-CtaA. We purified CtaA mutants by two-step affinity chromatography and examined mutant proteins by 5–20% SDS–polyacrylamide gel electrophoresis (Fig. 7). We found possible degradation products of His_6-CtaA with apparent molecular mass of 23 and ~14 kDa in His216 and His278 mutants and C191A/C197A double mutant. This indicates that CtaA has two structural domains as suggested by Svensson *et al*. (*22*) and that mutations in the *C*-terminal halves destabilize the structure of the *C*-terminal halves leading to cleavage(s) by the *E. coli* protease(s).

Characterizations of Hemes Bound to CtaA Mutants—We characterized hemes bound to CtaA mutants by room temperature absorption spectroscopy and reverse-phase HPLC analysis of HCl-acetone extractable hemes. Reverse-phase HPLC analysis demonstrated that wild-type CtaA binds only heme B (Fig. 8, Table 2), expected from the α peak (*i.e.* 556 nm) of pyridine ferrohemochrome. Glu57 mutations reduced heme content to about two-third of the wild-type level and resulted in blue-shifts for the 427-, 529.5- and 558.5-nm peaks of the fully reduced form (Table 1, Fig. 4), probably due to the increased heme O content (Fig. 8, Table 2). His216 and His278 mutations reduced heme content to 14–56% of the wild-type level and showed blue-shifts for the Soret peak of the air-oxidized and fully reduced forms (Table 1, Fig. 5). Notably, His216 mutants showed a redox insensitive peak at around 671 nm, which is comparable to the peak position reported for billiverdin (Fig. 1), an open

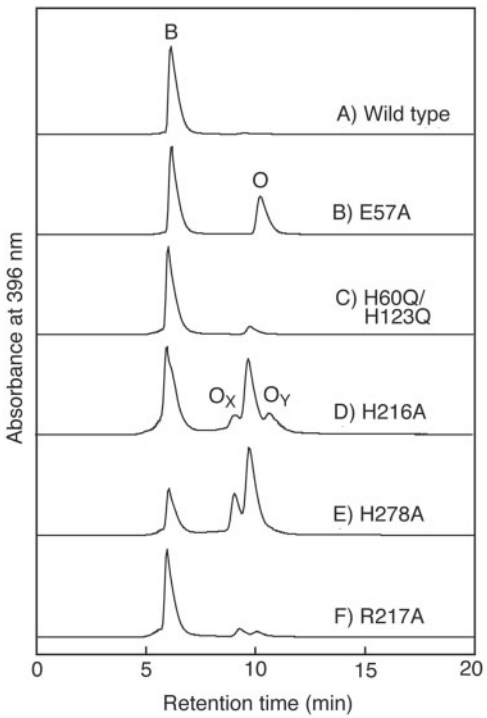

Fig. 8. **Reverse-phase HPLC analysis of hemes bound to the wild-type and mutant CtaA proteins.**

Table 2. **Composition of hemes bound to CtaA mutants**

Mutants	Heme composition $B:O:O_X:O_Y$
Wild-type	1.95:0.02:0.03:0
W39A	1.88:0.10:0.02:0
E57A	1.28:0.71:0:0
E57Q	1.50:0.50:0:0
H60A	1.88:0.12:0:0
H60Q	1.86:0.14: 0:0
R61A	1.92:0.08:0:0
R61Q	1.94:0.06:0:0
Q103A	1.79:0.09:0.13:0
H123A	1.88:0.12:0:0
H123Q	1.92:0.08:0:0
H216A	0.98:0.54:0.18:0.30
H216Q	1.18:0.70:0.10:0
R217A	1.80:0.08:0.12:0
R217Q	1.84:0.06:0.10:0
Q257A	1.90:0.04:0.06:0
H278A	1.25:0.61:0:0.14
H278Q	1.06:0.84:0.10:0
C35A-C42A	1.90:0.10:0:0
C191A-C197A	1.32:0.33:0:0.35
H60A-H123A	1.95:0.02:0.03:0
H60Q-H123Q	1.84:0:0.16:0

Bound hemes in isolated CtaA mutants were extracted by acid acetone and analysed by reverse pahse HPLC.

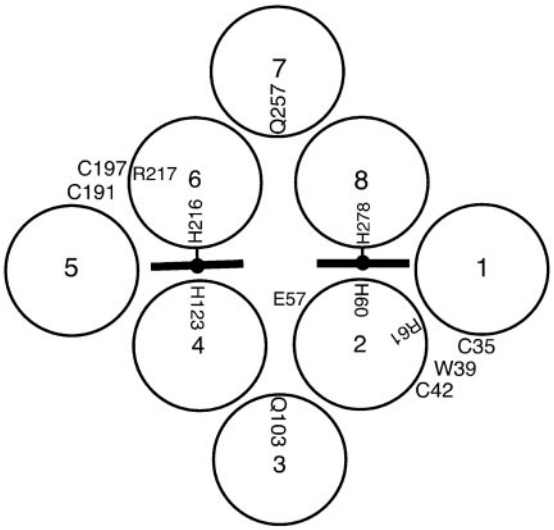

Fig. 9. **Helical-wheel-projection model for the *B. subtilis* heme A synthase.**

tetrapyrrole derivative of heme B (*35*). This pigment was unable to be extracted by acid-acetone. In addition, His216 and His278 mutations increased the relative heme O content and resulted in two new heme species, named heme O_X and O_Y (Fig. 8). Heme B and O were eluted at 6.0 and 9.7 min, respectively, and heme O_X and O_Y were found at 8.9 and 10.7 min, respectively. Their Soret peak maximum in the HPLC solvent was similar to that of heme O (396 nm); thus, we tentatively assumed that they are heme O derivatives. It should be noted that heme O_X is present at some extent in cytoplasmic membranes isolated from heme O-overproducing strains (BL21(DE3)/pLysS/pTTQ18-cyoE, BL21 CodonPlus(DE3)-RIL/pQE2-ctaB). Because of its peak position heme O_X is likely an 8-hydroxymethyl derivative of heme O (*24*). Arg217 retained *b*-hemes but showed blue-shift for the Soret peak. C35A/C42A mutation did not affect heme binding but C191A/C197A double mutations reduced heme content to a half of the wild-type level. H60A/H123A and H60Q/H123Q double mutants retained *b*-hemes while H216A/H278A and H216Q/H278Q double mutants severely reduced heme content to about 6% of the control level, indicating that His216 and His278 in the C-terminal halves are essential for heme binding (Fig. 9). Effects of mutations on the heme composition suggest that one of *b*-heme-binding sites has a higher affinity for heme O, but under our expression conditions this site is not competent to accept heme O. It could be due to the absence of physilogical electron donor(s) to CtaA or unidentified factor for heme A synthesis in *E. coli*. The other site may have a higher affinity for heme B and serves as the electron-accepting site (*10*), as reported for *bo*-type (*34*, *36*), ba_3-type (*37–39*) and *bd*-type (*32*) quinol oxidase. We noticed also that Arg217, Gln257, His278, Cys191/Cys197 mutations in the C-terminal halves caused red-shift of the 558.5-nm peak.

Hederstedt *et al*. (*40*) reported site-directed mutagenesis studies on four invariant histidines (His60, His123, His216 and His278) in *B. subtilis* CtaA, which are complementary to this work. They constructed Leu and Met mutants of *B. subtilis* CtaA-His_6 and found the bound heme A in H60M, H216M, H278L and H278M.

They postulated that His123 is directly involved in catalysis and that His60 and His216 in the *N*- and *C*-terminal halves, respectively, are axial ligands for bound hemes. However, the absence of His60 in CtaA from the Actinomycetales (*1*) and the heme binding by H60A (Table 1) do not support their proposal. Low amounts of bound hemes B and A in H278L and H278M mutants (*40*) are rather consistent with our assignment that His278 serves as one of the heme ligands. The presence of heme I (8-hydroxymethyl derivative of heme O) in H216M (*40*) and heme O_X and the 671-nm pigment in His216 and His278 mutants (this study) and the absence of heme A in H60L, H123L and H123M mutants (*40*) suggest that His60 and His123 are involved in catalysis.

Conclusion—We over-expressed heme A synthase in *E. coli* as cytochrome b_{558} and demonstrated that heme A synthase has two heme binding sites. This preparation binds the stoichiometric amount of two heme B molecules and thus suitable for future X-ray crystallographic studies. Site-directed mutagenesis studies suggest that His216 and His278 are likely axial ligands of hemes and that Cys191 and Cys197 are also important for heme binding. We hope that future *in vitro* studies with CtaA mutants will provide us a clue for understanding the catalytic and structural roles of His216 and His278 in heme A synthesis and identify structures of intermediates including the 671-nm pigment.

SUPPLEMENTARY DATA

Supplementary data are available at http://jb.oxfordjournals.org

CONFLICT OF INTEREST

None declared.

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