Heme A Synthase Enzyme Functions Dissected by Mutagenesis of *Bacillus subtilis* CtaA

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Heme A, as a prosthetic group, is found exclusively in respiratory oxidases of mitochondria and aerobic bacteria. *Bacillus subtilis* CtaA and other heme A synthases catalyze the conversion of a methyl side group on heme O into a formyl group. The catalytic mechanism of heme A synthase is not understood, and little is known about the composition and structure of the enzyme. In this work, we have: (i) constructed a ctaA deletion mutant and a system for overproduction of mutant variants of the CtaA protein in *B. subtilis*, (ii) developed an affinity purification procedure for isolation of preparative amounts of CtaA, and (iii) investigated the functional roles of four invariant histidine residues in heme A synthase by in vivo and in vitro analyses of the properties of mutant variants of CtaA. Our results show an important function of three histidine residues for heme A synthase activity. Several of the purified mutant enzyme proteins contained tightly bound heme O. One variant also contained trapped hydroxylated heme O, which is a postulated enzyme reaction intermediate. The findings indicate functional roles for the invariant histidine residues and provide strong evidence that the heme A synthase enzyme reaction includes two consecutive monoxygenations.

Heme A is a highly specialized variant of heme that is important for cellular aerobic respiration and energy conversion in animals, plants, and microorganisms. It is a prosthetic group in the mitochondrial cytochrome oxidase and in similar oxidases of bacterial aerobic respiratory chains. Heme A is synthesized from heme B (protopheme IX) with heme O as a stable intermediate (15). Heme O is derived from heme B by substitution of the vinyl group on carbon 2 of the porphyrin ring (Fisher nomenclature) with a hydroxethyl farnesyl side group. Heme A differs from heme O by containing a formyl group instead of a methyl group on carbon 8 of the porphyrin ring. All known heme A-containing oxidases belong to the heme copper superfamily of terminal oxidases (6). The gram-positive bacterium *Bacillus subtilis* has two heme A-containing terminal oxidases, cytochrome *aa*₃ and cytochrome *ca*₃. The polypeptide constituents of these two oxidases are encoded by the *qoxABCD* operon and the *ctaCDEF* gene cluster, respectively (30). Cytochrome *aa*₃ is the major type *a* cytochrome under most growth conditions (32). Expression of cytochrome *ca*₃ is catabolite repressed; cells grown in nutrient sporulation medium supplemented with phosphate (NSMP) contain 10-fold more of this oxidase compared to cells grown in NSMP supplemented with 0.5% glucose (1). Heme O and heme A synthase are encoded in *B. subtilis* by the *ctaB* and *ctaA* genes, respectively (16, 24). These genes are located immediately upstream of the *ctaCDEF* gene cluster at 133° on the circular chromosomal map. There is also a second heme *O* synthase gene, *ctaO* (*yjdK*), located at 109° on the chromosome, but this gene is not essential for heme *O* synthesis (28). The *ctaB* gene is cotranscribed with *ctaCDEF* (13), and *ctaA* is transcribed in the opposite direction (Fig. 1A). The promoter regions for *ctaA* and *ctaB* overlap (34). CtaA and CtaB are integral polytopic membrane proteins (15, 25). *B. subtilis* cells can grow aerobically in the absence of heme O and heme A. CtaA- and CtaB-deficient mutants are viable because of the presence of a cytochrome *bd* terminal respiratory oxidase (32). This property makes *B. subtilis* ideal for experimental studies of heme A synthesis and cytochrome *a* biogenesis.

From studies of *Escherichia coli* cells expressing *B. subtilis* *ctaA* or *Bacillus steatothrophus* *ctaA*, it has been demonstrated that the CtaA protein catalyzes heme A synthesis from heme O (3, 20, 26). Recently, Hegg and collaborators, using the *B. subtilis* *ctaA* gene expressed in *E. coli*, showed that heme A synthase activity is dependent on the presence of molecular oxygen and presented evidence that hydroxymethyl heme O (heme I) is a reaction intermediate (3). It is thought that the methyl side chain on carbon 8 of the porphyrin ring of heme O is subjected to two consecutive hydroxylations catalyzed by the heme A synthase. The second hydroxylation would result in an unstable dihydroxymethyl group, which spontaneously decomposes into a formyl group and a water molecule. Isotope labeling experiments suggest that the oxygen atom of the formyl group is not directly derived from molecular oxygen (4). This is unexpected, and notably, *B. subtilis* heme A synthase does not have the biochemical or amino acid sequence characteristics of a P450 type of enzyme, which contains heme as a prosthetic group and activates molecular oxygen (25). The molecular mechanism of catalysis by heme A synthase remains to be elucidated and might include unprecedented features. Also, little is known about the chemical composition and structure of heme A synthase. Furthermore, the CtaA protein might play a direct role in the assembly of cytochrome *a* by mediating heme A insertion into the QoxA and CtaD proteins in the *B. subtilis* membrane.

By using recombinant *B. subtilis* strains that overproduce CtaA, two types of preparations which differ in heme compo-
sition have been isolated (24), i.e., cyt b-CTA containing heme B and small amounts of heme A and cyt ba-CTA containing about equal amounts of heme B and heme A. The iron atoms of heme B and heme A in isolated oxidized CtaA are low spin (electron paramagnetic resonance $g_{\text{max}}$ signals at 3.7 and 3.5, respectively) (24). Heme A ($E_{\text{m,7}} = +242 \text{ mV}$) bound to isolated CtaA is thought to be the enzyme product waiting to be used in the synthesis of heme A-containing terminal oxidases. It has been suggested that bound heme B ($E_{\text{m,7}} = +85 \text{ mV}$) is a prosthetic group of CtaA with an electron transfer function within the enzyme. However, only substoichiometric amounts of heme B ($\leq 0.4 \text{ mol of heme per mol of CtaA polypeptide}$) have been found in isolated CtaA (25). The possibility cannot be ruled out that the presence of heme B, or the substoichiometric amount of heme, is an experimental artifact resulting from overproduction of CtaA polypeptide. Less than 0.02 mol of copper and non-heme iron atoms per mol of polypeptide has been found in isolated $B.\ subtilis$ CtaA (25). In the absence of cofactors other than heme, it is reasonable to assume that heme iron is part of the catalytic center of heme A synthase. Heme O can thus be both a substrate and a cofactor for the enzyme, similar to the situation with heme oxygenase, where heme B plays these two roles (19).

Based on the hydropathy profile (11), the positive-inside rule (29), and topology analysis by fusions to alkaline phosphatase (our own unpublished data), the CtaA polypeptide is predicted to have eight membrane-spanning $\alpha$-helical segments and the N terminus exposed on the negative side of the membrane (Fig. 2). Genes encoding proteins similar to CtaA are present in various organisms, such as eubacteria, archaea, yeast, and humans (18, 27). The CtaA orthologues contain nine highly invariant residues. Four of these are histidine residues, His-60, His-123, His-216, and His-278, in the $B.\ subtilis$ protein (Fig. 2). These residues are all predicted to be located close to the positive (extracytoplasmic) side of the membrane. Histidine residues often function as axial ligands to heme iron in proteins. To analyze the functional roles of the four invariant

**FIG. 1.** Maps of the genes and DNA constructs used in this work. (A) The cta region in the $B.\ subtilis$ chromosome. Two promoters present between ctaA and ctaB are indicated by bent arrows. (B) The DNA fragment used to delete the chromosomal ctaA gene by homologous recombination, resulting in strain LMT20R. EcoRI and BamHI restriction sites added via PCR are indicated. (C) The ctaA gene in pCTHI10. The position of an introduced HindIII restriction site and a sequence (seq.) resulting in a His$_{6}$ tag at the C terminus of the CtaA polypeptide are indicated.

**FIG. 2.** Topology model of the CtaA protein in the cytoplasmic membrane of $B.\ subtilis$. The eight predicted membrane-spanning $\alpha$-helices are numbered I to VIII. The outer side (+) and cytoplasmic side (−) of the membrane and the approximate location of four histidine residues that are invariant in CtaA orthologues are indicated.
TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>B. subtilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A1</td>
<td>trpC2</td>
<td>Bacillus Genetic</td>
</tr>
<tr>
<td>LMT20R</td>
<td>trpC2 ΔctaA Spe&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Stock Center&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>LOA10R</td>
<td>trpC2 ctaO::Spe ΔctaB Spe&lt;sup&gt;c&lt;/sup&gt; Cam&lt;sup&gt;e&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
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<tr>
<td>SURE</td>
<td>c14&lt;sup&gt;+&lt;/sup&gt; (McrA&lt;sup&gt;b&lt;/sup&gt;) Δ(mcrCD hsdSMR-mrr&lt;sup&gt;171&lt;/sup&gt; endA1 sup&lt;sup&gt;44&lt;/sup&gt; thi-1 grr96 relA1 lac recB1 recJ171 endA1 recA1 grr96 thi hsdR17 (r&lt;sub&gt;K&lt;/sub&gt; m&lt;sub&gt;K&lt;/sub&gt;) relA1 sup&lt;sup&gt;44&lt;/sup&gt; λ Δ(lac-proAB)</td>
<td>Stragagen</td>
</tr>
<tr>
<td>JM109</td>
<td>endA1 recA1 grr96 thi hsdR17 (r&lt;sub&gt;K&lt;/sub&gt; m&lt;sub&gt;K&lt;/sub&gt;) relA1 sup&lt;sup&gt;44&lt;/sup&gt; λ Δ(lac-proAB)</td>
<td>Promega</td>
</tr>
<tr>
<td>ES1301</td>
<td>lacZ33 mutS201::Tn5 thiA36 rha-5 medB1 deoC VNM(rMD-rmE)</td>
<td>Promega</td>
</tr>
</tbody>
</table>

Plasmids

| pH13Es             | pH13 derivative; Cam<sup>e</sup> | 5  |
| pBluescript II SK<sup>−</sup> | Amp<sup>a</sup> | Stragagen |
| pHPKS              | pH13 derivative; Cam<sup>e</sup> Ery<sup>a</sup> | 10  |
| pDG1726            | Spe<sup>c</sup> Amp<sup>a</sup> | 7  |
| pALTER-1           | Amp<sup>a</sup> Tet<sup>b</sup> | Promega |
| pCTALT10           | pALTER-1 with part of ctaA on a 0.9-kb KpnI-BamHI fragment from pCTAHHIND1; Amp<sup>a</sup> Tet<sup>b</sup> | This work |
| pCTA1302           | pH13 with ctaA; Cam<sup>e</sup> Ery<sup>a</sup> | 25  |
| pCTA1305           | pH13 with ctaABC on a 0.9-kb fragment; Cam<sup>e</sup> Ery<sup>a</sup> | 24  |
| pDCTA1             | pBluescript II SK<sup>−</sup> with part of the pyc-A-ctaA intercistronic region on a 0.4-kb fragment; Amp<sup>a</sup> Spe<sup>c</sup> | This work |
| pDCTA2             | pDCTA1 with a 1.2-kb EcoRI-BamHI fragment containing the spe<sup>c</sup> gene from pSPC1<sup>−</sup>; Amp<sup>a</sup> Spe<sup>c</sup> | This work |
| pCTAAHIND1         | pHPKS with part of ctaA on a 0.9-kb EcoRI-HindIII fragment; Cam<sup>e</sup> Ery<sup>a</sup> | This work |
| pCTAAHIND2         | pHPKS with the promoter region of ctaA on a 0.2-kb BamHI-HindIII fragment; Cam<sup>e</sup> Ery<sup>a</sup> | This work |
| pCTH10             | pHPKS derivative identical to the pHxxL<sup>c</sup> and pHxxH<sup>c</sup> plasmids with the wild-type ctaA sequence | This work |
| pSPC1<sup>−</sup>/+(-) | pBluescript II SK<sup>−</sup> with spe from pDG1726 on a 1.2-kb fragment; Amp<sup>a</sup> Spe<sup>c</sup> | This work |
| pHxxL              | pCTAAHIND2 with a 0.9-kb HindIII-Sall fragment containing ctaA encoding a His<sup>b</sup>-to-Leu mutation (xxx indicates amino acid residue number in B. subtilis CtaA) | This work |
| pHxxM              | pCTAAHIND2 with a 0.9-kb HindIII-Sall fragment containing ctaA encoding a His<sup>b</sup>-to-Met mutation (xxx indicates amino acid residue number in B. subtilis CtaA) | This work |

<sup>a</sup> Spe<sup>c</sup>, Kan<sup>e</sup>, Tet<sup>b</sup>, Cam<sup>e</sup>, Ery<sup>a</sup>, and Amp<sup>a</sup> indicate resistance to spectinomycin, kanamycin, tetracycline, chloramphenicol, erythromycin, and ampicillin, respectively.

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**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and oligonucleotides.** The strains and plasmids used in this work are listed in Table 1. The oligonucleotides used for PCR, mutagenesis, and DNA sequence analysis are presented in Table 2.

**Media and general growth of bacteria.** B. subtilis strains were kept on tryptose blood agar base (TBAB; Difco Co.) plates supplemented with 0.5% (wt/vol) glucose. For the preparation of membranes, B. subtilis strains were grown in NSMP, pH 7.0, containing 0.5% (wt/vol) glucose. Membranes for cytochrome c oxidase activity measurements were prepared from cells grown in NSMP without glucose. Antibiotics were used at the following concentrations when appropriate: B. subtilis, chloramphenicol at 3 to 5 mg/liter and spectinomycin at 150 mg/liter; E. coli, ampicillin at 125 mg/liter and tetracycline, kanamycin, and chloramphenicol at 12.5 mg/liter.

**General methods.** B. subtilis was grown to competence essentially as described by Hoch (9). N,N,N′,N″-Tetramethyl-p-phenylenediamine (TMPD) oxidation by colonies on TBAB and NSMP plates was assayed as described before (1, 12). Membranes were isolated from B. subtilis strains as described previously (8) and stored at −80°C. Light absorption spectroscopy (22) and cytochrome c oxidase activity measurements (5) were performed as previously described. Protein concentrations were determined by the biocinchoninic acid protein assay (Pierce Chemical Co.) with bovine serum albumin as the standard. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was done by the method of Schagger and von Jagow (21). Immunoblotting (Western blotting) was done as described before (1), with mouse anti-hexahistidyl monoclonal antibodies (Amersham Pharmacia), diluted 3,000-fold, and anti-mouse immunoglobulin G peroxidase-linked sheep antisera (Amersham Pharmacia) diluted 1,500-fold.

**Construction of B. subtilis strain LMT20R.** The ctaA gene in B. subtilis strain 1A1 was deleted by homologous double-crossover recombination with a DNA fragment spanning the ctaA chromosomal region but carrying a gene for spectinomycin resistance, spe, in place of ctaA. First, plasmid pSPC1<sup>−</sup>/+(-) was constructed to obtain appropriate restriction sites on each side of the spe gene. This plasmid was obtained by moving a 1.2-kb PsI fragment from pDG1726 into pBluescript SK<sup>−</sup>(-). The resulting two variant plasmids were named pSPC1<sup>−</sup> and pSPC<sup>−</sup>, respectively, depending on the relative orientation of the spe gene. The orientation of the spe gene in pSPC1<sup>−</sup> is the opposite of that of the bla gene in pBluescript II SK<sup>−</sup>(-). A 0.4-kb fragment containing the pycA-ctaA intergenic region was amplified with strain 1A1 chromosomal DNA as a template...
Construction of plasmids

- EcoRI
- KpnI
- His-216 to Met
- BamHI
- His-123 to Met
- HindIII
- EcoRI, His6 tag
- HindIII
- His-278 to Leu
- His-278 to Met
- His-123 to Leu
- His-216 to Leu
- His-123 to Met

**TABLE 2. Oligonucleotides used as primers in this work**

<table>
<thead>
<tr>
<th>Primer use and name</th>
<th>Sequence, 5’ to 3’ (features underlined)</th>
<th>Feature(s)</th>
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<tr>
<td>pycA1</td>
<td>CGTTATGATTTCCAGGCAATCTCATAAGGA</td>
<td>EcoRI</td>
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<tr>
<td>pycA3</td>
<td>GAGAGGATCCGAGCTCTTTGAATTTGAAAGGCGCA</td>
<td>KpnI</td>
</tr>
<tr>
<td>ctaA1</td>
<td>GAGAGGATCCGAGCTCTTTGAATTCACTAAGGGC</td>
<td>BamHI</td>
</tr>
<tr>
<td>ctaB6</td>
<td>AGCACGGTTAGGCCTGGA</td>
<td>None</td>
</tr>
<tr>
<td>ctaB7</td>
<td>GAGAGGATCCGAGCTCTTTGAATTCACTAAGGGC</td>
<td>BamHI</td>
</tr>
<tr>
<td>CTAHIA6*</td>
<td>TARGAATTTAGCTTAAGCTGGGA</td>
<td>HindIII</td>
</tr>
<tr>
<td>CTAHIND1*</td>
<td>AGCGTCAAAGCTTAAAGCTGGGA</td>
<td>HindIII</td>
</tr>
</tbody>
</table>

**Site-directed mutagenesis**

- H60L
- H60M
- H123L
- H123M
- H123L
- H216L
- H278L
- H278L
- H278M
- H278M

Primers CTAHIA and CTAHIND1 where used both for the construction of plasmids and in overlap extension PCR site-directed mutagenesis.

Extension PCR in a PCR with primers pycA1 and pycA3 (Table 2). This introduced an EcoRI and a KpnI restriction site at each end of the fragment, respectively. The amplified fragment was cut with KpnI and ligated into pBluescript II SK(-) cleaved with KpnI and HinClI, resulting in plasmid pDCTA1. An EcoRI-BamHI fragment of pSPC1(+) containing the spe gene was ligated into pDCTA1, resulting in plasmid pDCTA2. A 1.5-kb fragment containing the ctaB gene was amplified by using pCTA1305 as a template in a PCR with primers ctaB6 and ctaB7 (Table 2). A BamHI restriction site was added via the ctaB7 primer. The amplified fragment was cut with BamHI and HindIII and ligated into pHPKS, resulting in plasmid pCTALT10, which was used as the template in site-directed mutagenesis.

Construction of plasmids for site-directed mutagenesis and expression of His6-tagged CtaA. Transforming E. coli cells with plasmids carrying the entire B. subtilis ctaA gene, including the promoter region, resulted in accumulation of mutations in ctaA, most likely due to a toxic effect of the CtaA protein in E. coli. Therefore, the ctaA gene was cloned as two separate fragments in pCTAHHIND1 and pCTAAHIND2. The obtained two fragments, a HindIII site at bp 8 to 13 of the coding sequence (Fig. 1C) was introduced by changing bp 12 from an A to a T. This change in the DNA sequence does not affect the amino acid sequence of the CtaA protein but enables cloning of the major part of ctaA unexpressed in E. coli.

For the construction of pCTAHHIND1, a 0.9-kb fragment containing the ctaA gene was amplified by using pCTA1305 as the template in a PCR with primers CTAHIA and CTAHIND1 (Table 2), introducing an EcoRI and a HindIII restriction site at each end of the fragment. The CTAHIA primer also introduced a sequence resulting in a His tag at the C terminus of CtaA. The amplified fragment was cut with EcoRI and HindIII and ligated into pHPKS, resulting in plasmid pCTAHHIND1. A 0.9-kb KpnI-BamHI fragment from pCTAAHIND1 (the KpnI site is in the vector sequence) was cloned in pALTER-1, resulting in plasmid pCTALT10, which was used as the template in site-directed mutagenesis.

For construction of pCTAHHIND2, carrying the native ctaA promoter region, a Shine-Dalgarno sequence, and the first 12 bp of the coding sequence of the ctaA gene, a 164-bp BamHI-HindIII fragment was first amplified by using pCTA1305 as a template in a PCR with primers ctaA1 and CTAHIND2 (Table 2). The BamHI and HindIII restriction sites were added via the primers. The amplified fragment was cut with BamHI and HindIII and ligated into pHPKS, resulting in plasmid pCTAHHIND2. A 0.9-kb HindIII-Sall fragment from pCTAAHIND1 (the Sall site is in the vector sequence) was moved into pCTAHHIND2, resulting in plasmid pCTH110.

Site-directed mutagenesis. Site-directed mutagenesis of the ctaA gene was carried out with the Altered Sites II mutagenesis kit (Promega) or by overlap extension PCR (17). In both procedures, pCTALT10 was used as a template. The different oligonucleotides used for mutagenesis are presented in Table 2. To minimize the risk of reversion, each mutation included the change of two nucleotides in the codon. Mutant ctaA DNA fragments were moved, as 0.9-kb HindIII-Sall fragments, from pCTALT10 derivatives into pCTAHHIND2, resulting in the pHxexd and pHexxM series of plasmids (xxx indicates the corresponding CtaA amino acid residue number; Fig. 2), which were used to transform B. subtilis LMT20R. DNA sequence analysis of the entire ctaA fragment in the pCTALT10 derivatives confirmed the desired mutations and excluded the presence of unwanted mutations.

**RESULTS**

Construction and properties of a B. subtilis ctaA deletion mutant, strain LMT20R. CtaA not only catalyzes the conversion of heme O into heme A in B. subtilis, it also physically interacts with the Ctb protein (2) and possibly functions in the assembly of cytochrome aa3 and cytochrome caa3. To be able to assay these functions, we decided to produce and analyze mutant CtaA variants in B. subtilis. By this approach, we...
also circumvent problems associated with heterologous expression of CtaA in, e.g., *E. coli* such as toxicity of the protein and possible assembly defects. To have a robust experimental system, excluding problems with genetic recombination between chromosomal and plasmid-borne ctaA sequence variants, a *B. subtilis* strain, LMT20R, with the entire ctaA coding region deleted and replaced with a spectinomycin resistance gene (Fig. 1B) but with the rest of the cta gene cluster intact was constructed as described in Materials and Methods.

When grown under aerobic conditions, strain LMT20R was found to lack cytochromes of type *a* but to contain cytochrome *bd*, as judged by light absorption spectroscopy of isolated membranes. As expected from the absence of a functional cytochrome *ca*$_3$, colonies of *B. subtilis* LMT20R grown on TBAB or NSMP agar plates were unable to oxidize TMPD. Membranes isolated from LMT20R grown in NSMP without glucose showed less than 0.5% cytochrome *c* oxidase activity compared to that of membranes from the parental *B. subtilis* strain 1A1 grown under the same conditions. These properties of strain LMT20R are as expected from the lack of a functional CtaA.

![FIG. 3. Difference (dithionite-reduced minus ferricyanide-oxidized) light absorption spectra of membranes from *B. subtilis* LMT20R carrying plasmids pH60L (A), pH60M (C), pH123L (D), pH123M (E), pH216L (F), pH216M (G), pH278L (H), pH278M (I), and pHPKS (J). The absorbance scale is indicated by the bar. The cuvettes contained 4 mg of membrane protein per ml, except in the case of the LMT20R/pH60L sample, which contained 2 mg of membrane protein per ml. The spectra were recorded at room temperature.](image)

Complementation of LMT20R by plasmid-borne ctaA. Plasmid pCTHI10 was constructed for the production of *B. subtilis* CtaA polypeptide containing a hexahistidyl sequence (His$_6$ tag) at the C-terminal end. The ctaA gene in the plasmid has its natural promoter region (Fig. 1C). Strain LMT20R containing plasmid pCTHI10 or plasmid pCTA1302, which encodes untagged CtaA, showed normal TMPD oxidation activity, cytochrome *c* oxidase activity, and cytochrome *a* content, as indicated by the absorption band at 600 nm in the difference (reduced-minus-oxidized) spectrum of membranes from cells grown in NSMP supplemented with 0.5% glucose (Fig. 3, trace A). LMT20R containing the empty plasmid vector pHPKS did not complement the cytochrome *a* deficiency (Fig. 3, trace J). The results confirmed that heme O synthase, CtaB, is produced in strain LMT20R and demonstrated that the His$_6$-tagged variant of CtaA encoded by pCTHI10 is functional.

**Purification and analysis of His$_6$-tagged CtaA.** By Western blot analysis with antibodies directed against the His$_6$ tag, CtaA was detected in isolated membranes from LMT20R/pCTHI10 (Fig. 4). As expected, this antigen was not found in membranes from LMT20R/pHPKS.

The His$_6$-tagged CtaA protein was solubilized from
LMT20R/pCTHI10 membranes with the nonionic detergent Thesit and purified by affinity chromatography as described in Materials and Methods. Membranes of LMT20R/pHPKS, which do not contain CtaA, and LMT20R/pCTA1302, which contain untagged CtaA, were subjected to the same purification procedure, and the samples obtained were used as controls to estimate the presence of contaminating proteins and hemes in the final preparations of purified CtaA. The His6-tagged CtaA protein purified from LMT20R/pCTHI10 is referred to in the following text as CtaA-wt, whereas the preparation from LMT20R/pHPKS is referred to as the negative control. SDS-PAGE and staining for protein showed that CtaA was the major protein in the CtaA-wt preparation (Fig. 5, lane 1).

This demonstrated that His6-tagged CtaA protein can be isolated from detergent-solubilized B. subtilis membranes by the use of the affinity tag at the C terminus.

HPLC analysis of hemes extracted from purified CtaA-wt showed heme B and a small amount of heme A (Fig. 6). The light absorption spectrum of CtaA-wt reduced with dithionite is shown in Fig. 7A. This spectrum is very similar to that of untagged CtaA (cyt b-CTA) previously purified with a combination of chromatographic methods (24), except for the weak broad absorption in the 580- to 600-nm region. Reduced heme B and heme A bound to CtaA show α-band absorption maxima at 559 nm and 585 nm, respectively (24). It has previously been shown that heme A in CtaA is fully reducible with ascorbate, whereas the heme B component is only partly reducible with ascorbate (24). CtaA-wt showed absorption maxima at 559 nm and 585 nm when reduced with 8 mM ascorbate (spectrum not shown).

Properties of CtaA produced in a heme O-deficient strain.
To determine if the weak broad absorption in the 585- to 600-nm region in spectra of dithionite-reduced samples of CtaA-wt is due to compounds derived from heme O, we analyzed the protein after production in B. subtilis strain LOA10R, which lacks the two heme O synthases CtaB and CtaO. CtaA purified from LOA10R/pCTHI10 contained only heme B, as determined by HPLC analysis (data not shown), and lacked absorption in the 585- to 600-nm spectral region (Fig. 7A). This result and the observation that samples obtained from membranes with untagged CtaA (LMT20R/pCTA1302 membranes) lacked absorption in the 500- to 600-nm region (Fig. 7A) showed that the material responsible for the absorption in the 585- to 600-nm region is bound to CtaA-wt and derived from heme O. Mutant CtaA variants lacking heme A synthase activity but containing bound heme O (see below) did not show absorption in the 585- to 600-nm region (see, e.g., CtaA-H60L in Fig. 7B). We conclude that the absorption in the 585- to 600-nm region in spectra of dithionite-reduced CtaA-wt is due
TABLE 3. Phenotype of *B. subtilis* LMT20R containing different plasmids and types of heme present in CtaA isolated from the respective strain

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Functional cytochrome <em>a</em> synthesized&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Types of heme found in CtaA&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>pCTHI10 (CtaA-wt)</td>
<td>+</td>
<td>B, (A)</td>
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<td>pHPS (negative control)</td>
<td>−</td>
<td>Not applicable&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>pH60L (CtaA-H60L)</td>
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<td>B, O</td>
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<tr>
<td>pH123M (CtaA-H123M)</td>
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<td>B, O</td>
</tr>
<tr>
<td>pH216L (CtaA-H216L)</td>
<td>−</td>
<td>B, O</td>
</tr>
<tr>
<td>pH216M (CtaA-H216M)</td>
<td>+&lt;sup&gt;d&lt;/sup&gt;</td>
<td>B, O, I, (A)</td>
</tr>
<tr>
<td>pH278L (CtaA-H278L)</td>
<td>+</td>
<td>(B), (A)</td>
</tr>
<tr>
<td>pH278M (CtaA-H278M)</td>
<td>+</td>
<td>(B), (A)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined as TMPD oxidation activity of colonies grown on NSMP plates. +, TMPD positive; −, TMPD negative. The TMPD oxidation phenotype of the strains was completely consistent with the presence of cytochrome *a* as determined by light absorption spectroscopy of membranes isolated from cells grown in NSMP supplemented with 0.5% glucose (Fig. 3).

<sup>b</sup> Determined by HPLC of heme extracted from purified CtaA. B, heme B; A, heme A; O, heme O; I, heme I (monohydroxyemethyl heme O). Parentheses indicate small amounts of this type of heme.

<sup>c</sup> Membranes of LMT20R/pHPKS and LMT20R/pH216L do not contain CtaA polypeptide (Fig. 4).

<sup>d</sup> Membranes isolated from LMT20R/pH216M grown in NSMP medium without glucose showed 20% cytochrome *c* oxidase activity compared to that of LMT20R/pCTHI10, which oxidized 28 nmol of cytochrome *c* per min per mg of protein.

to products originating from heme O and the activity of CtaA synthase.

**Site-directed mutagenesis of the ctaA gene and analysis of mutant CtaA.** To analyze the roles of the four invariant histidine residues in *B. subtilis* CtaA, His-60, His-123, His-216, and His-278 (Fig. 2), we individually changed each of these residues to a methionine and a leucine. Plasmids encoding mutant CtaA were named pH60L for a mutation changing His-60 into a Met, and so on. These plasmids (Table 1) are identical to pCTHI10 (encoding CtaA-wt), except for the introduced base pair substitutions in *ctaA*.

*B. subtilis* strain LMT20R was transformed with plasmids pH60L, pH60M, pH123L, pH123M, pH216L, pH216M, pH278L, and pH278M. Membranes isolated from all of the different LMT20R transformants, except LMT20R/pH216L, contained His<sub>x</sub>-tagged CtaA (Fig. 4). Colonies of LMT20R carrying plasmid pH60M, pH278L, or pH278M were TMPD oxidation positive on NSMP plates, showing that the CtaA variants encoded by these plasmids are functional. Plasmid pH216M weakly complemented the TMPD oxidation deficiency of strain LMT20R (Table 3). The TMPD oxidation-negative phenotype of strain LMT20R was not complemented by pH60L, pH123L, or pH123M (Table 3), indicating that the CtaA variants encoded by these plasmids are not enzymatically active. As expected from the lack of CtaA protein, LMT20R/pH216L cells were TMPD oxidation negative.

Membranes from LMT20R carrying pH60M, pH278L, or pH278M contained normal amounts of cytochrome *a*, as judged from the absorption peak at 600 nm in reduced-minus-oxidized difference spectra (Fig. 3, traces C, H, and I). In contrast, membranes from LMT20R containing pH60L, pH123L, pH123M, pH216L, or pH216M (Fig. 3, traces B, D, E, F, and G) were deficient in cytochrome *a*. Cytochrome *bd* terminal oxidase was present in membranes of all of the strains, as indicated by the absorption at about 597 nm and 627 nm in the difference spectra.

**Properties of isolated mutant CtaA variants.** The seven mutant variants of CtaA were purified from membranes by the same protocol as for the purification of CtaA-wt from LMT20R/pCTHI10 membranes. SDS-PAGE analysis of the preparations and staining for protein showed that they contained CtaA and little contaminating polypeptides (Fig. 5). Purified preparations of the mutant variants of CtaA are referred to in the following text as CtaA-H60L for CtaA purified from LMT20R/pH60L and so on.

Hemes were present in all samples that contained purified mutant CtaA protein. CtaA-H216M, CtaA-H278L, and CtaA-H278M contained smaller amounts of heme per CtaA protein than the other variants, as determined by the pyridine hemochromogen (data not shown). To determine which types of heme were bound to the various purified CtaA variants, heme was extracted and analyzed by HPLC (Fig. 6 and Table 3). CtaA-H60M contained heme B and heme A at levels comparable to that of CtaA-wt. Small amounts of heme B and heme A were found in CtaA-H278L and CtaA-278 M. CtaA-H216M contained heme B, heme O, and heme I (see below), as well as trace amounts of heme A. CtaA-H60L, CtaA-H123L, and CtaA-H123M contained heme B and heme O.

The light absorption spectrum of CtaA-H60M reduced with dithionite was very similar to that of CtaA-wt, with a peak at 559 nm from heme B and the broad absorption in the 585- to 600-nm region indicative of bound heme A (Fig. 7B). The spectra of reduced CtaA-H60L, CtaA-H123L, and CtaA-H123M were very similar to each other and showed the α-band absorption peak at 556 nm (Fig. 7B and spectra not shown). A peak at 556 nm correlated with the presence of mainly heme O bound to CtaA, and we therefore conclude that it is due to heme O. The observed 3-nm spectral difference between heme O and heme B bound to CtaA is congruent with the pyridine hemochromogen spectrum of heme O, which is blue shifted 4 nm in comparison to that of heme B (33). The tight binding of heme O to the protein and the distinct spectra typical of low-spin cytochrome indicated that the overall structure of the mutant enzymes was not much disturbed compared to that of the wild-type protein.

Reduced CtaA-H278L and CtaA-H278M showed little absorption in the 500- to 700-nm region (spectra not shown), which agreed with the low heme content of these mutant variants.

The H216M variant of CtaA binds a heme A synthase reaction intermediate. CtaA-H216M showed features that were unique among the mutants analyzed. This CtaA variant has a decreased heme A synthase activity. Membranes of LMT20R/pH216M contained wild-type levels of CtaA protein (Fig. 4) but contained small amounts of cytochrome *a* (Fig. 3, trace G) and showed a fivefold reduction in cytochrome *c* oxidase activity compared to that of membranes from LMT20R/pCTHI10 (Table 3). The CtaA-H216M variant contained heme B, heme O, and a third heme compound which we identified as heme I based on the HPLC elution pattern (Fig. 6) and the light absorption spectrum in acidic acetonitrile with an absorption maximum at 394 nm. Hegg et al. (3) have isolated heme I from membranes...
of recombinant *E. coli* strains containing *B. subtilis* CtaA and identified it as monohydroxymethyl heme O, which is a proposed heme A synthase reaction intermediate (Fig. 8). The light absorption spectrum of the dithionite-reduced CtaA-H216M protein showed a broader α-band absorption peak compared to that of CtaA containing only heme B or heme O (Fig. 7). This indicates that heme B (peak at 559 nm) and heme O (peak at 556 nm), and possibly also heme I, contribute to the α-band absorption peak in the spectrum of dithionite-reduced CtaA-H216M.

**DISCUSSION**

Heme A synthase catalyzes the synthesis of heme A from heme O, which involves the conversion of a methyl side group on the heme O porphyrin ring into a formyl group (Fig. 8). We have in this work analyzed the role of four invariant histidine residues in heme A synthases by using the *B. subtilis* heme A synthase, CtaA, as a model enzyme. We first established a system for production and easy isolation of wild-type and mutant variants of CtaA in *B. subtilis*. To our knowledge, *B. subtilis* CtaA is the only heme A synthase that has been isolated in preparative amounts and analyzed in any detail (reference 24 and this work).

The functions of the four invariant histidine residues in heme A synthases, located at positions 60, 123, 216, and 278 in the *B. subtilis* CtaA protein sequence (Fig. 2), were analyzed by changing each residue individually into a leucine and a methionine by site-directed mutagenesis. Our results show that His-60, -123, and -216 are important for heme A synthase activity but are not required for binding of the enzyme substrate, heme O, or heme B, which might function as a prosthetic group in CtaA. The results also indicate that His-278, located in the most C-terminal transmembrane segment of CtaA (Fig. 2), is not critical for heme A synthase activity but seems to play a role in the tight binding of heme to the protein since the His-278 mutant variants CtaA-H278L and CtaA-H278M both contained little bound heme compared to the wild type.

The N- and C-terminal halves of CtaA, containing transmembrane segments I to IV and V to IX, respectively, are homologous and might each constitute a four-helix bundle protein domain (25). Residues His-60 and His-216 have equivalent positions in the two halves (Fig. 2). Substitution of His-60 and His-216 with a leucine or a methionine affected the heme A synthase activity and heme content of CtaA in ways which are indicative of a role for these two histidine residues in heme coordination. A methionine at position 60 results in active enzyme with the same heme composition and light absorption spectrum as wild-type CtaA. In contrast, a leucine residue in position 60 of CtaA resulted in an inactive enzyme protein containing tightly bound heme O. Methionine, but not leucine, can function as an axial heme ligand and could substitute for a histidine residue in this respect. This can explain why the CtaA-H60M, but not the CtaA-H60L, enzyme is catalytically active, assuming that His-60 plays a role in heme coordination. CtaA protein with a leucine in position 216 could not be detected in membranes or be purified. Defective heme ligation could result in improper folding or decreased stability of the CtaA polypeptide in the membrane, which in turn could lead to degradation of the polypeptide. A methionine at position 216 in CtaA resulted in a stable enzyme protein with decreased heme A synthase activity. This CtaA variant accumulated bound heme I (monohydroxymethyl heme O), indicating a deficiency in step 2 of the proposed enzyme reaction (Fig. 8). The presence of heme O and heme I in purified mutant CtaA proteins supports the view that CtaA catalyzes the entire heme O-to-heme A synthesis reaction. A methionine residue instead of a histidine as the axial ligand to heme iron is expected to increase the midpoint redox potential of that heme. For example, a histidine-to-methionine substitution of an axial heme ligand in the cytochrome *b* subunit of *B. subtilis* succinate-quinone oxidoreductase increases the midpoint potential of that heme by more than 100 mV (14). Such a large change in redox potential is expected to have a significant effect on the reactivity of the heme and can explain the deficiency of the

![FIG. 8. Proposed three-step reaction for the heme O-to-heme A synthesis catalyzed by heme A synthase. Mutations in *B. subtilis* CtaA that have been used in this work and negatively affect reaction steps are indicated (see text for details); a solid line indicates a block and a dashed line indicates decreased activity of heme A synthase.](http://jb.asm.org/content/83/9/8368/F1)
CtaA-H216M variant. It is not known if CtaA has one or two heme-binding sites and if axial ligand switching to heme iron occurs during the catalytic cycle, as has been observed, for example, in Thiobacillus pantotropha cytochrome cd$_1$ nitrite reductase (31) and Nitrosomonas europaea cytochrome c peroxidase (23). We can therefore only speculate on whether His-60 and His-216 in CtaA would be ligands to the same heme or to two different heme groups.

Residue His-123 in CtaA seems to be crucial for step 1 in the proposed heme A synthase reaction (Fig. 8). Both CtaA-H123L and CtaA-H123M were found to be inactive but formed stable proteins containing heme O and heme B and showed spectra similar to that of the CtaA-H60L variant. We suggest that His-123 functions directly in catalysis.

In conclusion, we have established an experimental platform for functional analysis of heme A synthase in Bacillus subtilis. Mutant variants of the enzyme have for the first time been purified and analyzed with respect to in vivo activity and heme content. Analysis of the mutant heme A synthase variants allowed dissection of the biosynthetic reaction performed by the enzyme and provided strong support for the proposed enzyme reaction pathway (Fig. 8).

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REFERENCES


