CcsBA is a cytochrome *c* synthetase that also functions in heme transport

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Little is known about trafficking of heme from its sites of synthesis to sites of heme-protein assembly. We describe an integral membrane protein that allows trapping of endogenous heme to elucidate trafficking mechanisms. We show that CcsBA, a representative of a superfamily of integral membrane proteins involved in cytochrome *c* biosynthesis, exports and protects heme from oxidation. CcsBA has 10 transmembrane domains (TMDs) and reconstitutes cytochrome *c* synthesis in the *Escherichia coli* periplasm; thus, CcsBA is a cytochrome *c* synthetase. Purified CcsBA contains heme in an "external heme binding domain" for which two external histidines are shown to serve as axial ligands that protect the heme iron from oxidation. This is likely the active site of the synthetase. Furthermore, two conserved histidines in TMDs are required for heme to travel to the external heme binding domain. Remarkably, the function of CcsBA with mutations in these TMD histidines is corrected by exogenous imidazole, a result analogous to correction of heme binding by myoglobin when its proximal histidine is mutated. These data suggest that CcsBA has a heme binding site within the bilayer and that CcsBA is a heme channel.

channel | cofactor | imidazole complementation | biogenesis | assembly

Heme is the cofactor for proteins such as hemoglobins and myoglobins that carry oxygen and for the cytochromes, which are involved in energy conversion. The transmembrane trafficking of heme from its site of synthesis, inside the mitochondrion, chloroplast, or bacterium, to these proteins is poorly understood (1). There is emerging evidence that organisms use specific transporters to traffic heme (1–4) and porphyrins (5) into cells and intracellularly, although trapping of heme in such integral membrane proteins has not yet been accomplished. Because all cytochromes *c* function and are synthesized in the prokaryotic periplasm, mitochondrial intermembrane space, or chloroplast lumen, heme must be delivered to these sites, yet the molecular mechanisms of delivery are unknown. Cytochrome *c* synthesis is carried out by one of three pathways (systems I, II, and III) (6–10) and requires the formation of two thioether bonds between the cysteines of a CXXCH motif in the apoprotein and the vinyl groups of heme. For this covalent ligation to occur, the heme must be reduced (Fe^{2+}) (11, 12), but the mechanism of heme reduction in vivo is unknown. One pathway for this synthesis (system II), originally discovered from genetic work in *Chlamydomonas reinhardtii* (13–17), *Bacillus subtilis* (18–20), and *Bordetella pertussis* (21, 22), is present in plant chloroplasts and many bacteria (Fig. 1) (6). We reconstituted the function of this pathway in recombinant *Escherichia coli*, demonstrating that in an appropriate thiol reducing environment only the CcsB and CcsA proteins are required for periplasmic cytochromes *c* biogenesis (23) (see Fig. S1 for model). CcsA has a highly conserved, tryptophan-rich region called the WWD domain that is proposed to be involved in heme binding, but no member of this WWD superfamily has been purified (16, 24–26). Each WWD superfamily member is an integral membrane protein with two conserved histidine residues in periplasmic loops flanking the WWD domain that have been proposed to act as axial ligands to heme iron (16, 24, 26, 27). Mutations in these two conserved histidines in CcsA and the putative system I synthetase CcmF result in a loss of function (16, 27). Due to a lack of biochemical analysis of this family, important questions remain unanswered, including whether WWD superfamily members indeed bind heme and, if so, the location of the binding site or sites and whether these proteins export heme to the external surface, and, if so, by what mechanism.

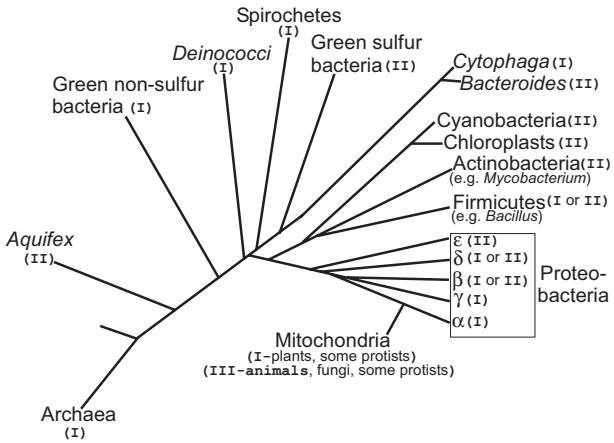

Fig. 1. Representative distribution of systems I, II, and III among the bacteria and archaea. The chloroplasts and mitochondria are also included. The system number is noted in parentheses after each group name. Some classifications contain organisms that do not make cytochromes *c* and thus do not have system proteins. Relative relationships between groups are based on 16S rRNA sequences. Several groups include organisms that may have either system I or system II; this scenario may become more common as more genome sequences become available.

Although most bacterial and plant genomes encode individual *ccsB* and *ccsA* genes, in some organisms, including *Helicobacter hepaticus*, the CcsB and CcsA proteins are naturally fused into a single large ORF, *ccsBA*. We show that CcsBA from *H. hepaticus* has 10 transmembrane domains (TMDs) and reconstitutes cytochrome *c* synthesis in the *Escherichia coli* periplasm; thus, CcsBA is a cytochrome *c* synthetase. Use of this CcsBA recombinant fused protein has facilitated purification, trapping of endogenous heme, and characterization of heme export. We demonstrate that CcsBA binds reduced (Fe^{2+}) heme in an "external heme binding domain" composed of the two external histidines flanking the WWD domain. When either of these histidines is mutated, the absorption spectrum of the heme in CcsBA is dramatically perturbed, and unlike WT, the heme iron is oxidized (Fe^{3+}). These perturbations show that these histi-

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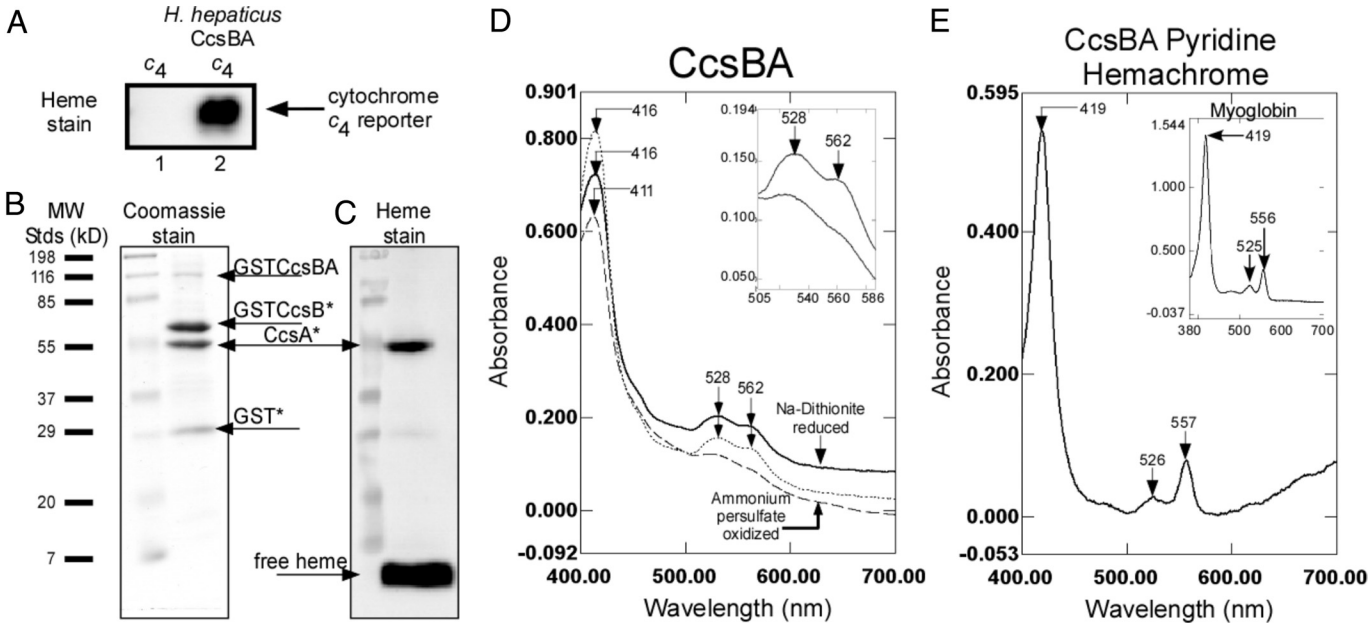

Fig. 2. Purified CcsBA is a heme protein. (*A*) Cytochrome c_4 is synthesized by *Escherichia coli* expressing the *Helicobacter hepaticus* GSTCcsBA as detected by chemiluminescence (heme stain). Only covalently bound heme in a cytochrome is detected. Full-length blots are shown in Fig. S3. (*B*) Coomassie blue stain of purified GST-tagged CcsBA showing 133-kDa full-length GSTCcsBA, 70-kDa GSTCcsB*, 55-kDa CcsA*, and 26-kDa GST*. (*C*) Heme stain of purified CcsBA protein separated by SDS/PAGE. (*D*) UV-visible spectra of purified WT CcsBA as indicated: as-purified (dotted line), reduced with sodium dithionite (solid line), and oxidized with ammonium persulfate (dashed line) and with magnification of the α and β regions of the as-purified and oxidized spectra (*Inset*). Maxima are indicated by arrows. (*E*) A pyridine hemochrome spectrum of CcsBA and the *b*-type heme from myoglobin (*Inset*).

dines are the axial ligands to the heme iron and that they play a critical role in protecting the heme from oxidation. We also show that two conserved TMD histidines in CcsBA are required for translocation of reduced heme from its site of synthesis in the cytoplasm to the external heme binding domain. The function of CcsBA with mutations in these TMD histidines is corrected by exogenous imidazole, a histidine side chain mimic. This result is similar to the correction of a proximal histidine mutant of myoglobin (H93G) where imidazole binds in the H93G "cavity" to restore heme binding (28). These data suggest a low-affinity heme binding site inside CcsBA and that CcsBA forms a channel for the translocation of reduced heme across the membrane.

Results

Purification of the CcsBA System II Cytochrome *c* Synthetase. We compared the abilities of four recombinant CcsBA proteins (*B. pertussis*, *Helicobacter pylori*, *H. hepaticus*, and *Bacteroides thetaiotaomicron*) to synthesize cytochrome c_4 in *E. coli*. The *H. hepaticus* CcsBA exhibited high cytochrome c_4 synthetase activity (Fig. 2*A*), and we were able to purify significant amounts of recombinant protein (see below). CcsBA was expressed as a functional N-terminal fusion to GST, solubilized in *n*-dodecyl β-D-maltoside (DDM), and affinity-purified to >95% purity. In addition to the full-length protein (133 kDa), three polypeptides are observed (Fig. 2*B*) whose identities were determined by N-terminal sequencing and anti-GST immunoblot. The smallest polypeptide (≈26 kDa) is GST. The two major polypeptides (labeled GSTCcsB* and CcsA* in Fig. 2*B*) are the products of a single proteolytic event in the periplasmic domain of CcsB (Fig. 3*A*). The larger (≈70 kDa) is GST-tagged CcsB*, and the smaller (≈55 kDa) is called CcsA*. The CcsA* polypeptide copurifies with GSTCcsB* with equimolar stoichiometry, certainly due to the tight complex that CcsB and CcsA form, as shown by immunological methods in *B. pertussis* and *C. reinhardtii* (16, 21). The natural proteolytic susceptibility of CcsB has facilitated analysis and localization of heme within CcsA*, and we later use this susceptibility as a diagnostic tool for studies on mutant CcsBA proteins. Purified CcsBA preparations are red, suggesting a heme protein. A chemiluminescent stain to detect heme (Fig. 2*C*) of the CcsBA preparation shows that free heme is present, dissociated by SDS, in addition to some heme associated with the CcsA* polypeptide that disappears upon boiling. This suggests that the CcsA portion of CcsBA binds heme noncovalently.

Heme in CcsBA Is Reduced *b*-Type Heme. Using UV-visible absorption spectroscopy on purified CcsBA, we demonstrate that a member of the WWD superfamily binds heme and that the heme is reduced (Fe^{2+}). CcsBA exhibits the spectrum of a reduced, *b*-type heme protein with an α peak at 562 nm, a β peak at 528 nm, and a Soret peak at 416 nm, which did not change after reduction with sodium dithionite (Fig. 2*D*). The heme in CcsBA is oxidized (to Fe^{3+}) by ammonium persulfate, diminishing the α and β absorptions and blue-shifting the Soret peak by 5 nm to 411 nm. The spectrum of the heme from CcsBA extracted in pyridine (Fig. 2*E*) shows that it is *b*-type (noncovalently bound) heme (29), as is present, for example, in myoglobin (Fig. 2*E*, *Inset*).

Characterization of the External Heme Binding Domain: His-761 and His-897 Are Axial Ligands. We next determined the features in CcsBA that constitute the heme binding domain. We experimentally established the membrane topology of the *H. hepaticus* CcsBA protein, identifying the locations of conserved residues (Fig. 3*A*, red). For topology data and analysis, see Fig. S2 and legend. The topology is consistent with previous experimentally determined topologies for CcsA from *Mycobacterium leprae* and *C. reinhardtii* (16, 24). Of particular interest are the conserved WWD domain and four absolutely conserved histidines (starred) that could serve as axial ligands to a heme iron. His-761 and His-897 flank the WWD domain in the periplasm. To determine which residues of CcsBA are involved in heme binding (i.e., where the heme is trapped), we generated a mutant for each of these and a triple mutant for three conserved residues in the

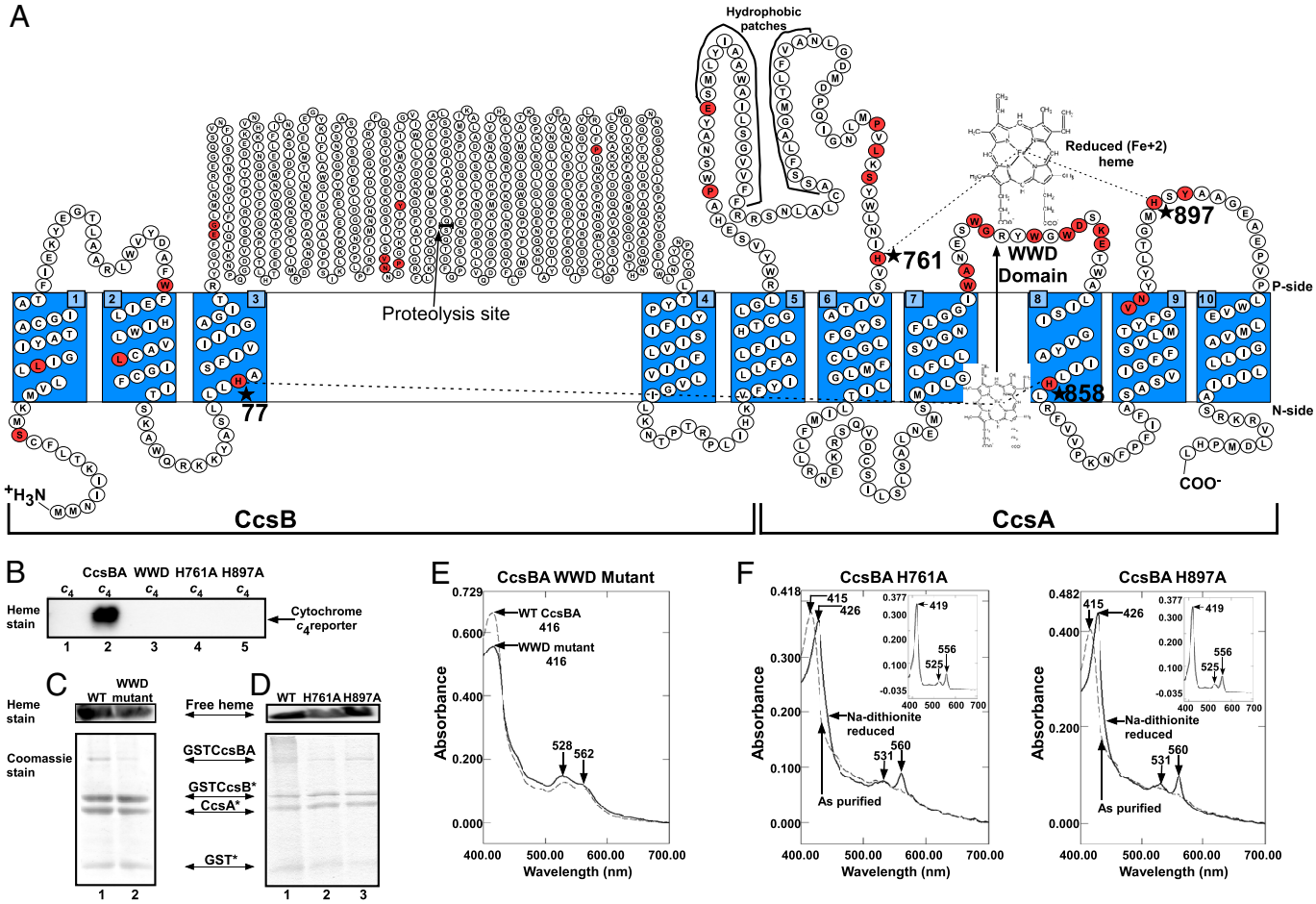

Fig. 3. Topology of CcsBA and determination of the external heme binding site. (*A*) Topology and completely conserved residues (red) of the CcsBA fusion protein from *Helicobacter hepaticus*. Data for topology determination are described in Fig. S2. Absolutely conserved (identical) residues in CcsB and CcsA proteins (in red) were determined using the organisms listed in Table S1. The sites of proteolytic susceptibility in CcsB and hydrophobic patches in CcsA are noted. Four conserved histidines that are potential axial ligands to the heme iron are starred, and the periplasmic WWD domain in CcsA is labelled. (*B*) Heme stain of cytochrome c_4:His reporter synthesized by the indicated strains after SDS/PAGE. Full-length blots are shown in Fig. S3. (*C* and *D*) Free heme stains and Coomassie blue stains of the indicated purified CcsBA proteins after SDS/PAGE. (*E*) UV-visible spectra of the CcsBA(WWD) protein (solid line) and WT CcsBA (dashed line) as-purified. (*F*) UV-visible spectra of as-purified (dashed lines) and reduced with sodium dithionite (solid lines) CcsBA(H761A) (*Left*) and CcsBA(H897A) (*Right*), with pyridine hemochrome spectra (*Insets*).

WWD domain (-WGWD- in Fig. 3*A*, with WWD changed to alanines). The mutants, designated CcsBA(H761A), CcsBA(H897A), and CcsBA(WWD), are not functional for cytochromes *c* biogenesis (Fig. 3*B*). The CcsBA(WWD), CcsBA(H761A), and CcsBA(H897A) proteins purify with similar amounts of heme to WT (Fig. 3 *C* and *D*, *Top*) and similar stabilities, with the same GSTCcsB* to CcsA* stoichiometry (Fig. 3 *C* and *D Lower*). The CcsBA(WWD) protein has a spectrum identical to that of WT, with reduced (Fe^{2+}) heme (Fig. 3*E*). The CcsBA(H761A) and CcsBA(H897A) proteins, however, purify in the oxidized (Fe^{3+}) state (Fig. 3*F*); no α or β peaks are detectable, and the Soret maximum is at 415 nm. Upon addition of sodium dithionite, the heme is reduced, with sharp peaks at 560 and 531 nm, and the Soret peak red-shifted to 426 nm, a maximum wavelength nearly 10 nm longer than that of WT. Spectra of pyridine-extracted CcsBA(H761A) and CcsBA(H897A) (Fig. 3*F Insets*) show that *b*-type heme is present, like WT. These results show that, although CcsBA(H761A) and CcsBA(H897A) still bind heme, the environment of that heme is altered when one of the histidine ligands is missing.

Transmembrane Histidines His-77 and His-858 Are Involved in Channeling Heme to the External Heme Binding Domain. We hypothesized that the conserved His-77 and His-858 residues, located in TMDs close to the cytoplasmic surface (Fig. 3*A*), may bind heme in a channel for export. Neither CcsBA(H77A) nor CcsBA(H858A) is functional for cytochromes *c* biogenesis (Fig. 4*A*). We determined whether mutations in His-77 and His-858 might affect the ability of CcsBA to deliver heme to the external heme binding domain. The CcsBA(H77A) and CcsBA(H858A) proteins are stable (Fig. 4 *B* and *C Lower*), but strikingly, the CcsBA(H858A) protein has no heme detectable by chemiluminescent heme stain (Fig. 4*B Upper*) or spectral detection, indicating at least 100-fold less heme is present than in the WT CcsBA (30). The CcsBA(H77A) protein has approximately 4-fold less heme than WT by heme stain (Fig. 4*C Upper*) and pyridine extraction analysis (Fig. 4*D*). Spectral characteristics of CcsBA(H77A) are similar to those of WT except that more heme is in the oxidized state (Fig. 4*E*). We propose that the TMDs that include the conserved His-77 and His-858 associate in the membrane with both histidines participating in the formation of a low-affinity heme binding site (see *Discussion*).

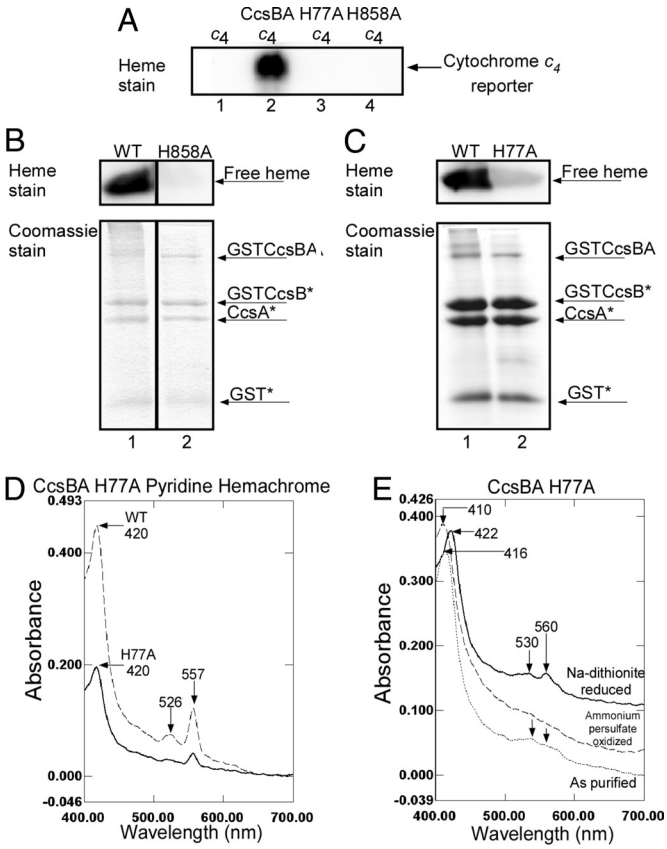

Fig. 4. Analysis of CcsBA(H77A) and CcsBA(H858A). (*A*) Heme stain of cytochrome c_4:His reporter synthesized by the indicated strains after SDS/PAGE. Full-length blots are shown in Fig. S3. (*B* and *C*) Free heme stains and Coomassie blue stains of the indicated purified CcsBA proteins after SDS/PAGE. (*D*) Pyridine hemochrome spectra of purified CcsBA(H77A) (solid line) and WT CcsBA (dashed line). (*E*) UV-visible absorption spectra of as-purified (dotted line), reduced with sodium dithionite (solid line), and oxidized with ammonium persulfate (dashed line) purified CcsBA(H77A).

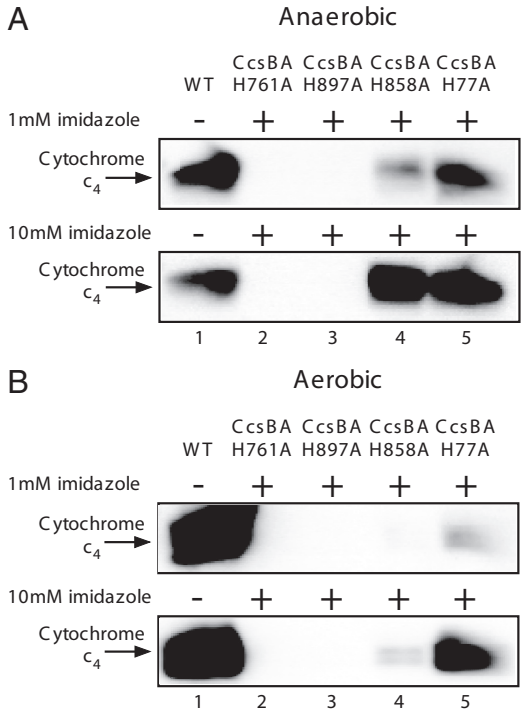

Fig. 5. Complementation of CcsBA(H77A) and CcsBA(H858A) function by imidazole. (*A*) Heme stain of cytochrome c_4 synthesized in vivo by the indicated CcsBA mutants complemented with 1 or 10 mM imidazole under anaerobic growth conditions. (*B*) Heme stain of cytochrome c_4 synthesized in vivo by the indicated CcsBA mutants complemented with 1 or 10 mM imidazole under aerobic growth conditions. Full-length blots are shown in Fig. S3.

Imidazole Complementation of TMD Histidine Mutants. In a second approach to investigate the presence of a TMD heme binding site, we tested our recombinant CcsBA histidine mutants for restoration of function by exogenous imidazole. A recombinant form of myoglobin that is mutated at its proximal axial ligand, His-93, does not bind heme (28). The myoglobin H93G cavity mutant is complemented intermolecularly by growth in the presence of 10 mM imidazole (28). The imidazole-reconstituted cavity mutant has an absorption spectrum like that of WT myoglobin, and crystal structures show imidazole bound in the H93G cavity. Although the CcsBA external heme binding site mutants are not corrected, both weak binding site TMD histidine mutants are fully restored with 10 mM imidazole (Fig. 5). The TMD histidine mutants are corrected more dramatically under anaerobic (Fig. 5*A*) than aerobic (Fig. 5*B*) growth, suggesting that heme must be protected from oxidation as it traverses the membrane, consistent with the fact that some of the heme in CcsBA(H77A) is oxidized.

Discussion

Here, we present the CcsBA cytochrome *c* synthetase as a paradigm of heme transport, processing, and cytochrome *c* synthesis. This purification of a WWD domain protein provides proof that this superfamily binds heme. The function of two pairs of conserved histidine residues was also determined. These four histidines are present in all CcsB/A proteins examined (Fig. 3*A* and Table S1), including three fused CcsBA proteins from *Wolinella succinogenes* that recognize different apocytochrome *c* motifs (i.e., CXXCH, CXXCK, and $CX_{15}CH$) (31, 32) and CcsB/A from eukaryotes such as *C. reinhardtii*, where they have been shown to be required for function (13, 16). Spectra of the *H. hepaticus* CcsBA(H761A) and CcsBA(H897A) proteins have dramatically different Soret maxima compared with those of WT for both the reduced (427 and 416 nm) and the oxidized (415 and 411 nm) forms, indicating a change in axial ligands to the heme iron, as noted for other heme proteins (33, 34). The α maxima of the mutant proteins (560 nm) is also blue-shifted by 2 nm, consistent with the results of mutating a histidine axial ligand to alanine in cytochrome b_{562} (35). We conclude that His-761 and His-897 compose the external heme binding domain in what likely constitutes the active site for the CcsBA cytochrome *c* synthetase. Purification of these mutant proteins in the oxidized rather than the reduced form demonstrates that these histidines also protect the heme iron from the oxidizing environment of the periplasm. This protection is critical because only reduced (Fe^{2+}) heme can form correct covalent bonds with an apocytochrome *c* (11, 12). Moreover, the compartments where cytochrome *c* synthesis occurs (the bacterial periplasm or chloroplast lumen) can be quite oxidizing environments for heme compared with the sites of heme biosynthesis.

Two conserved histidines in CcsBA, His-77 and His-858, are in TMDs. A pair of conserved TMD histidines has been shown to be required for the function of an ABC transporter for heme uptake from *Shigella dysenteriae* (3). His-77 and His-858 in CcsBA are required not only for synthetase function but also for translocation of heme to the external heme binding domain. Our data also suggest that CcsBA TMD3 (with His-77) is positioned close to TMD8 (with His-858); therefore, TMDs in CcsB may associate with TMDs in CcsA. We suggest that His-77 and

His-858 compose an initial low-affinity binding site for reduced (Fe^{2+}) heme at the cytoplasmic face of a channel formed by CcsBA. In this respect, crystal structures of various channels and transporters indicate that the presence of a low-affinity binding site near a channel opening, in addition to the expected high-affinity binding site, might be more common than once thought (36). Intermolecular complementation of CcsBA(H77A) and CcsBA(H858A) function with exogenous imidazole was used as a second approach to establish that His-77 and His-858 are axial ligands to heme. Inability to correct the CcsBA(H761A) and CcsBA(H897A) mutants in the external heme binding domain with imidazole might be due to ligand switching that occurs at this active site (see below) or the inability of free imidazole to bind in these cavities. There are at least three examples of recombinant, soluble heme proteins (myoglobin, horseradish peroxidase, and guanylate cyclase) corrected for heme binding by imidazole when their histidine ligands are mutated (28, 37, 38). CcsBA is an example of a membrane protein or putative channel that is chemically complemented for function by imidazole when histidine ligands are mutated. The myoglobin H93G cavity mutant has become a model for heme and heme-iron interaction with ligands, in part due to exogenous complementation with small molecules that expand the genetic code (e.g., refs. 28 and 39–41). We envision the CcsBA(H77A) and CcsBA(H858A) mutants as similar models for channel functions and interactions with heme. Enhanced imidazole complementation under anaerobic growth conditions implicates these TMD histidines in redox protection of heme as well.

On the basis of the results of this study, we propose a mechanistic model for cytochromes *c* biogenesis by CcsBA (Fig. 3*A* and Fig. S1). The CcsBA TMDs associate in the membrane to form a channel for translocation of heme. Reduced heme from the cytoplasm binds in a low-affinity binding site, liganded by His-77 (TMD3) and His-858 (TMD8), and then translocates to the high-affinity external heme binding domain, with iron liganded and protected from oxidation by His-761 and His-897. We expect that a heme concentration gradient drives passive translocation but cannot rule out active translocation. The WWD domain orients the heme, positioning the vinyl groups in close proximity to the reduced thiols of the apocytochrome *c* CXXCH motif (perhaps positioned by the periplasmic domain of CcsB) for spontaneous thioether bond formation. Ligand switching occurs, with the histidine of the CXXCH motif replacing either His-761 or His-897 as the fifth axial heme ligand. The sixth axial ligand of cytochrome *c* subsequently forms upon folding into its mature, native form, which could provide the energy for heme release from the high-affinity binding site.

Materials and Methods

Bacterial Growth Conditions. *E. coli* were grown aerobically in LB media (Difco) at 22 °C with shaking at 300 rpm. Antibiotic concentrations used are as follows: 50 μg mL^{-1} carbenicillin (Carb), 20 μg mL^{-1} chloramphenicol (Cm), and 100 μg mL^{-1} kanamycin (Kan).

Plasmid Construction. *E. coli* strains TB1 and HB101 were used as the host strains for cloning. Construction of pRGK332 was described in ref. 23.

pGEX-4T-1-derived vectors (GE Healthcare) have an N-terminal fusion to GST. Expression is under the control of the isopropyl β-D-thiogalactopyranoside (IPTG)-inducible *tac* promoter. Construction of pRGK368 was described in ref. 42.

CcsBA:PhoA fusions were generated by PCR amplification of pRGK368 using oligonucleotide primer pGEXNotI and the individual CcsBA:PhoA fusion oligonucleotide primers listed in Table S2. In-frame fusions to alkaline phosphatase A (PhoA) were created at the amino acid numbers indicated in the primer names. The *phoA* sequence from pRGK323 (22) was amplified using oligonucleotide primers PhoALI XhoI and PhoAR NotI. Both products were digested with XhoI and NotI and ligated.

CcsBA:GFP fusions were generated as described in ref. 43. The *ccsBA* coding region of pRGK368 was amplified by PCR using oligonucleotide primer Hh-CcsBA fwd NdeI and the CcsBA:GFP fusion primers listed in Table S2 to generate fusions at the amino acid numbers indicated in the primer names. PCR products were digested with NdeI and BamHI and ligated to pWALDO digested with NdeI and BamHI (43, 44).

The H761A, H897A, WWD, and H858A mutant versions of pRGK368 were generated by first amplifying with the appropriate mismatch primer (listed in Table S2) and HH ccsBA Rv2 to generate a short PCR product. This product was gel-purified and then used in a second amplification with HH ccsBA Lv2. The H77A mutant was generated by first amplifying with the CcsBA H77A mismatch primer and HH ccsBA Lv2. The gel-purified product was used with HH ccsBA Rv2 for the final amplification. The final, full-length products were digested with BamHI and XhoI and inserted into pGEX-4T-1 as for pRGK368. pRGK368 was the template for all amplifications. Constructs were sequenced to confirm the mutation.

Cytochrome Reporter Assays. Constructs were transformed into RK103 (Δ*ccm*) containing pRGK332 (cytochrome c_4, di-heme, *B. pertussis*). After induction with 1 mM IPTG and 0.2% arabinose, cells were harvested by centrifugation, and the pellet was resuspended in 200 μL of B-PER (Pierce) to lyse the cells and extract protein. Total protein concentration was determined by bicinchoninic acid assay (Pierce), and 20 μg was analyzed using chemiluminescence (heme stain).

Protein Expression, Purification, and Analysis. Heme stains, immunoblots, and protein purification were performed as described in refs. 23 and 45. The free heme stain assay is exposure of the SDS/PAGE dye front (free heme is ≈616 Da) with chemiluminescent reagent. For expression, C43 *E. coli* (46) containing the CodonPlus RIL plasmid (Stratagene) grown for 8 h at 37 °C in 5 mL of LB-Carb-Cm were used to inoculate 1-L cultures that were grown overnight (12–15 h) at 22 °C and induced with 1 mM IPTG for 5.5 h. Cells were harvested and frozen at −80 °C for at least 30 min. Cells were thawed, resuspended in 10 mM Tris·HCl, pH 7.5, with 1 mM phenylmethylsulfonyl fluoride, and sonicated to disrupt membranes. The crude sonicate was centrifuged to clear cell debris, and then membranes were harvested from the supernatant by centrifugation at 310,000 × *g* for 1 h 15 min. Membrane pellets were suspended in GST bind/wash buffer (4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.3) and solubilized with 1% DDM. Solubilized membranes were purified over GST resin (Novagen), washed 3 times with 15 mL of GST bind/wash buffer, and eluted with 20 mM reduced glutathione in 50 mM Tris·HCl pH 8, 0.1% DDM. Yields were 0.3–0.5 mg of >95% pure protein per liter of culture.

Spectral Analysis. UV-visible absorption spectra were recorded using a Shimadzu UV-2101PC UV-VIS scanning spectrophotometer at room temperature. Resolution is ±1 nm based on the manufacturer's specifications and empirical testing with standard proteins such as myoglobin. Chemically reduced spectra were generated by adding a few grains of sodium dithionite (sodium hydrosulfite) to the sample. Chemically oxidized spectra were generated by adding a few grains of ammonium persulfate. Pyridine hemochrome analysis was performed as described in ref. 29 with the following volume modifications: 50 μL of sample was combined in a 100-μL cuvette with 50 μL of stock solution containing 200 mM NaOH, 40% pyridine (by volume), and 0.6 mM $K_3Fe(CN)_6$. Sodium dithionite was added to generate reduced spectra. Heme concentrations were determined as described, yielding heme-to-protein ratios of 0.25–0.4 heme per CcsBA.

Alkaline Phosphatase and GFP Activity Assays. CcsBA:PhoA and CcsBA:GFP fusion constructs were transformed into BL21(DE3) cells. Alkaline phosphatase assays were performed as described in ref. 24 using a SpectraMax Plus 384 plate reader (Molecular Devices). GFP assays were performed as described in ref. 43. GFP fluorescence emission was detected using a SpectraMax Gemini XS microtitre plate reader (Molecular Devices) with the excitation filter at 485 nm and emission filter at 525 nm. Mean activities for both assays were determined from at least three independent experiments.

Imidazole Complementation. Aerobic imidazole complementation assays were performed as described above for cytochrome c_4 reporter assays with a few modifications. Cells were grown in the presence of 1 or 10 mM imidazole, pH 7, in LB-Carb-Cm media. After induction with 1 mM IPTG, cells were allowed to express for 1 h before the reporter was induced with 0.2% arabinose. Heme stain analysis was performed on 40 μg of total protein (B-PER fraction) subjected to SDS/PAGE. Anaerobic imidazole complementation assays were performed as described above using 12.5-mL cultures (inoculated with 1 mL of aerobically grown overnight culture) that were allowed to grow for 3 h before IPTG induction and 4 h after arabinose induction.

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1. Rajagopal A, et al. (2008) Haem homeostasis is regulated by the conserved and concerted functions of HRG-1 proteins. *Nature* 453:1127–1131.
2. Quigley JG, et al. (2004) Identification of a human heme exporter that is essential for erythropoiesis. *Cell* 118:757–766.
3. Burkhard KA, Wilks A (2008) Functional characterization of the *Shigella dysenteriae* heme ABC transporter. *Biochemistry* 47:7977–7979.
4. Shayeghi M, et al. (2005) Identification of an intestinal heme transporter. *Cell* 122:789–801.
5. Krishnamurthy PC, et al. (2006) Identification of a mammalian mitochondrial porphyrin transporter. *Nature* 443:586–589.
6. Kranz R, Lill R, Goldman B, Bonnard G, Merchant S (1998) Molecular mechanisms of cytochrome *c* biogenesis: Three distinct systems. *Mol Microbiol* 29:383–396.
7. Ferguson SJ, Stevens JM, Allen JW, Robertson IB (2008) Cytochrome *c* assembly: A tale of ever increasing variation and mystery? *Biochim Biophys Acta* 1777:980–984.
8. Hamel P, Corvest V, Giegé P, Bonnard G (2009) Biochemical requirements for the maturation of mitochondrial *c*-type cytochromes. *Biochim Biophys Acta* 1793:125–138.
9. Thöny-Meyer L (2002) Cytochrome *c* maturation: A complex pathway for a simple task? *Biochem Soc Trans* 30:633–638.
10. Turkarslan S, Sanders C, Daldal F (2006) Extracytoplasmic prosthetic group ligation to apoproteins: Maturation of *c*-type cytochromes. *Mol Microbiol* 60:537–541.
11. Barker PD, et al. (1993) Transmutation of a heme protein. *Proc Natl Acad Sci USA* 90:6542–6546.
12. Nicholson DW, Neupert W (1989) Import of cytochrome *c* into mitochondria: Reduction of heme, mediated by NADH and flavin nucleotides, is obligatory for its covalent linkage to apocytochrome *c*. *Proc Natl Acad Sci USA* 86:4340–4344.
13. Dreyfuss BW, Hamel PP, Nakamoto SS, Merchant S (2003) Functional analysis of a divergent system II protein, Ccs1, involved in *c*-type cytochrome biogenesis. *J Biol Chem* 278:2604–2613.
14. Xie Z, et al. (1998) Genetic analysis of chloroplast *c*-type cytochrome assembly in *Chlamydomonas reinhardtii*: One chloroplast locus and at least four nuclear loci are required for heme attachment. *Genetics* 148:681–692.
15. Xie Z, Merchant S (1996) The plastid-encoded *ccsA* gene is required for heme attachment to chloroplast *c*-type cytochromes. *J Biol Chem* 271:4632–4639.
16. Hamel PP, Dreyfuss BW, Xie Z, Gabilly ST, Merchant S (2003) Essential histidine and tryptophan residues in CcsA, a system II polytopic cytochrome *c* biogenesis protein. *J Biol Chem* 278:2593–2603.
17. Inoue K, et al. (1997) Ccs1, a nuclear gene required for the post-translational assembly of chloroplast *c*-type cytochromes. *J Biol Chem* 272:31747–31754.
18. Le Brun NE, Bengtsson J, Hederstedt L (2000) Genes required for cytochrome *c* synthesis in *Bacillus subtilis. Mol Microbiol* 36:638–650.
19. Schiött T, Throne-Holst M, Hederstedt L (1997) *Bacillus subtilis* CcdA-defective mutants are blocked in a late step of cytochrome *c* biogenesis. *J Bacteriol* 179:4523–4529.
20. Schiött T, von Wachenfeldt C, Hederstedt L (1997) Identification and characterization of the *ccdA* gene, required for cytochrome *c* synthesis in *Bacillus subtilis*. *J Bacteriol* 179:1962–1973.
21. Feissner RE, Beckett CS, Loughman JA, Kranz RG (2005) Mutations in cytochrome assembly and periplasmic redox pathways in *Bordetella pertussis*. *J Bacteriol* 187:3941–3949.
22. Beckett CS, et al. (2000) Four genes are required for the system II cytochrome *c* biogenesis pathway in *Bordetella pertussis*, a unique bacterial model. *Mol Microbiol* 38:465–481.
23. Feissner RE, et al. (2006) Recombinant cytochromes *c* biogenesis systems I and II and analysis of haem delivery pathways in *Escherichia coli*. *Mol Microbiol* 60:563–577.
24. Goldman BS, Beck DL, Monika EM, Kranz RG (1998) Transmembrane heme delivery systems. *Proc Natl Acad Sci USA* 95:5003–5008.
25. Beckman DL, Trawick DR, Kranz RG (1992) Bacterial cytochromes *c* biogenesis. *Genes Dev* 6:268–283.
26. Lee JH, Harvat EM, Stevens JM, Ferguson SJ, Saier MH, Jr (2007) Evolutionary origins of members of a superfamily of integral membrane cytochrome *c* biogenesis proteins. *Biochim Biophys Acta* 1768:2164–2181.
27. Ren Q, Ahuja U, Thöny-Meyer L (2002) A bacterial cytochrome *c* heme lyase. CcmF forms a complex with the heme chaperone CcmE and CcmH but not with apocytochrome *c*. *J Biol Chem* 277:7657–7663.
28. Barrick D (1994) Replacement of the proximal ligand of sperm whale myoglobin with free imidazole in the mutant His-93→Gly. *Biochemistry* 33:6546–6554.
29. Berry EA, Trumpower BL (1987) Simultaneous determination of hemes *a*, *b*, and *c* from pyridine hemochrome spectra. *Anal Biochem* 161:1–15.
30. Feissner R, Xiang Y, Kranz RG (2003) Chemiluminescent-based methods to detect subpicomole levels of *c*-type cytochromes. *Anal Biochem* 315:90–94.
31. Hartshorne RS, et al. (2007) A dedicated haem lyase is required for the maturation of a novel bacterial cytochrome *c* with unconventional covalent haem binding. *Mol Microbiol* 64:1049–1060.
32. Pisa R, Stein T, Eichler R, Gross R, Simon J (2002) The *nrfI* gene is essential for the attachment of the active site haem group of *Wolinella succinogenes* cytochrome *c* nitrite reductase. *Mol Microbiol* 43:763–770.
33. Ito-Maki M, et al. (1995) Demonstration that histidine 25, but not 132, is the axial heme ligand in rat heme oxygenase-1. *Arch Biochem Biophys* 317:253–258.
34. Wang WH, Lu JX, Yao P, Xie Y, Huang ZX (2003) The distinct heme coordination environments and heme-binding stabilities of His39Ser and His39Cys mutants of cytochrome b_5. *Protein Eng* 16:1047–1054.
35. Oyedotun KS, Yau PF, Lemire BD (2004) Identification of the heme axial ligands in the cytochrome b_{562} of the *Saccharomyces cerevisiae* succinate dehydrogenase. *J Biol Chem* 279:9432–9439.
36. Diallinas G (2008) Biochemistry: An almost-complete movie. *Science* 322:1644–1645.
37. Zhao Y, Schelvis JP, Babcock GT, Marletta MA (1998) Identification of histidine 105 in the β1 subunit of soluble guanylate cyclase as the heme proximal ligand. *Biochemistry* 37:4502–4509.
38. Newmyer SL, de Montellano PR (1996) Rescue of the catalytic activity of an H42A mutant of horseradish peroxidase by exogenous imidazoles. *J Biol Chem* 271:14891–14896.
39. Roach MP, Ozaki S, Watanabe Y (2000) Investigations of the myoglobin cavity mutant H93G with unnatural imidazole proximal ligands as a modular peroxide O–O bond cleavage model system. *Biochemistry* 39:1446–1454.
40. Decatur SM, Boxer SG (1995) ^{1}H NMR characterization of myoglobins where exogenous ligands replace the proximal histidine. *Biochemistry* 34:2122–2129.
41. Qin J, Perera R, Lovelace LL, Dawson JH, Lebioda L (2006) Structures of thiolate- and carboxylate-ligated ferric H93G myoglobin: Models for cytochrome P450 and for oxyanion-bound heme proteins. *Biochemistry* 45:3170–3177.
42. Richard-Fogal CL, Frawley ER, Feissner RE, Kranz RG (2007) Heme concentration dependence and metalloporphyrin inhibition of the system I and II cytochrome *c* assembly pathways. *J Bacteriol* 189:455–463.
43. Rapp M, et al. (2004) Experimentally based topology models for *E. coli* inner membrane proteins. *Protein Sci* 13:937–945.
44. Waldo GS, Standish BM, Berendzen J, Terwilliger TC (1999) Rapid protein-folding assay using green fluorescent protein. *Nat Biotechnol* 17:691–695.
45. Feissner RE, Richard-Fogal CL, Frawley ER, Kranz RG (2006) ABC transporter-mediated release of a haem chaperone allows cytochrome *c* biogenesis. *Mol Microbiol* 61:219–231.
46. Miroux B, Walker JE (1996) Over-production of proteins in *Escherichia coli*: Mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J Mol Biol* 260:289–298.