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The CcmFH complex is the system I holocytochrome c synthetase: engineering cytochrome c maturation independent of CcmABCDE

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SUMMARY

Cytochrome c maturation (ccm) in many bacteria, archaea, and plant mitochondria requires eight membrane proteins, CcmABCDEFGH, called system I. This pathway delivers and attaches heme covalently to two cysteines (of Cys-Xxx-Xxx-Cys-His) in the cytochrome c. All models propose that CcmFH facilitates covalent attachment of heme to the apocytochrome; namely, that it is the synthetase. However, holocytochrome c synthetase activity has not been directly demonstrated for CcmFH. We report formation of holocytochromes c by CcmFH and CcmG, a periplasmic thioredoxin, independent of CcmABCDE (we term this activity CcmFGH-only). Cytochrome c produced in the absence of CcmABCDE is indistinguishable from cytochrome c produced by the full system I, with a cleaved signal sequence and two covalent bonds to heme. We engineered increased cytochrome c production by CcmFGH-only, with yields approaching those from the full system I. Three conserved histidines in CcmF (TM-His1, TM-His2, and P-His1) are required for activity, as are the conserved cysteine pairs in CcmG and CcmH. Our findings establish that CcmFH is the system I holocytochrome c synthetase. Although we discuss why this engineering would likely not replace the need for CcmABCDE in nature, these results provide unique mechanistic and evolutionary insights into cytochrome c biosynthesis.

Keywords

cytochrome c maturation; synthetase; heme trafficking; biosynthesis; pathway

INTRODUCTION

C-type cytochromes are heme proteins that carry out essential electron transfer reactions in organisms from every kingdom of life. These cytochromes function outside of the cytoplasmic membrane in prokaryotes, in the lumen of chloroplasts, and in the intermembrane space of mitochondria [for example, reviewed in (Allen, 2011; Hamel *et al.*, 2009; Kranz *et al.*, 2009; Mavridou *et al.*, 2013; Sanders *et al.*, 2010; Sawyer and Barker, 2012)]. Cytochromes c are characterized by covalent attachment between the heme and the apoprotein (via thioether linkages between the 2- and 4-vinyls of heme and two thiols of a conserved Cys-Xxx-Xxx-Cys-His motif in the apoprotein). Because c-type cytochromes are assembled at their site of action (separated from heme biosynthesis by a lipid bilayer), holocytochrome c formation poses unique challenges to heme trafficking and post-translational modification. Three major pathways (called systems I, II, and III) exist in nature to direct the covalent attachment of heme to cytochrome c (Kranz *et al.*, 1998; Page *et al.*, 1998). In many bacteria, plant and protozoal mitochondria, and archaea,

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holocytochrome c formation is carried out by the cytochrome c maturation (*ccm*) pathway, called system I, which comprises eight membrane proteins (in *E. coli*, CcmABCDEFGH) (Hamel *et al.*, 2009; Kranz *et al.*, 2009; Mavridou *et al.*, 2013; Sanders *et al.*, 2010; Sawyer and Barker, 2012).

At the center of heme trafficking in system I is the periplasmic heme chaperone protein, CcmE. CcmE forms a unique and well-studied covalent intermediate with heme, between a conserved histidine (His130, in *E. coli* CcmE) and the β carbon of the heme 2-vinyl (Lee *et al.*, 2005; Stevens *et al.*, 2003; Uchida *et al.*, 2004). Heme in "holo" (heme bound) CcmE is transferred to the apocytochrome (putatively, by the action of CcmFH), but details of this aspect of system I are just emerging (San Francisco and Kranz, in press). CcmABCD (an ABC transporter complex) are involved in formation and release of holoCcmE, presumably now free to associate with CcmF (Christensen *et al.*, 2007; Feissner *et al.*, 2006a; Goldman *et al.*, 1997; Schulz *et al.*, 1999). CcmF forms an integral membrane complex with CcmH (Rayapuram *et al.*, 2008; Ren *et al.*, 2002; Richard-Fogal *et al.*, 2009; Sanders *et al.*, 2008), and is believed to be the site of thioether formation between the heme and the apocytochrome. CcmF contains a separate, stably bound non-covalent heme b (Richard-Fogal *et al.*, 2009; San Francisco *et al.*, 2011), which we have proposed is involved in reducing the incoming heme from holoCcmE (Kranz *et al.*, 2009; Richard-Fogal *et al.*, 2009; San Francisco *et al.*, 2011). Reduced heme (Fe^{2+}) is required for thioether formation with the apocytochrome (Barker *et al.*, 1993; Nicholson and Neupert, 1989), and reduction of the heme in holoCcmE would also favor discharge of heme from the covalent His130 intermediate (Kranz *et al.*, 2009; Richard-Fogal and Kranz, 2010; San Francisco *et al.*, 2011). CcmG (Beckman and Kranz, 1993; Di Matteo *et al.*, 2010; Ouyang *et al.*, 2006) and CcmH (Di Matteo *et al.*, 2007; Zheng *et al.*, 2012) are membrane-tethered thioredox-active proteins that likely maintain the thiol groups of the apocytochrome (in the conserved Cys-Xxx-Xxx-Cys-His motif) in the reduced state (Meyer *et al.*, 2005; Monika *et al.*, 1997; Setterdahl *et al.*, 2000; Turkarslan *et al.*, 2008).

CcmF (in complex with CcmH) has been referred to extensively as the "cytochrome c heme lyase" or "cytochrome c synthetase" of system I, although the assignment of this function to CcmF is largely circumstantial. It is well-established that CcmF forms a complex with CcmH (Rayapuram *et al.*, 2008; Ren *et al.*, 2002; Richard-Fogal *et al.*, 2009; Sanders *et al.*, 2008), and there is evidence that CcmH interacts directly with the apocytochrome (Di Matteo *et al.*, 2007; Di Silvio *et al.*, 2013; Meyer *et al.*, 2005; Verissimo *et al.*, 2011). However, interaction(s) between holoCcmE (the protein assumed to deliver heme to site of thioether formation with the apocytochrome) and CcmFH have only recently been demonstrated (San Francisco and Kranz, in press). The absence of direct experimental evidence demonstrating that CcmFH is the cytochrome c synthetase thus constitutes a major gap in our understanding of the system I pathway.

Here, we report maturation of holocytochrome c by CcmFH and CcmG, in the absence of CcmABCDE. The cytochrome c produced by the CcmABCDE-independent pathway (here termed "CcmFGH-only") is biochemically and spectroscopically indistinguishable from cytochrome c produced by the full system I. We engineer production of holocytochrome c by the CcmFGH-only pathway (by increasing cellular heme concentrations and expression of the apocytochrome and *ccmFGH*, by gene dosage) to levels approaching those of the full system I. Three of the conserved histidines in CcmF (TM-His1, TM-His2, and P-His1), as well as the conserved cysteine pairs in the thioredox proteins CcmG and CcmH, are required for holocytochrome formation by CcmFGH-only. These findings establish that CcmFH is the holocytochrome c synthetase for system I (with thioreduction mediated by CcmG). We discuss evolutionary and mechanistic implications, comparing CcmFGH-only with the system II cytochrome c synthetase, CcsBA.

RESULTS

CcmFH and CcmG can attach heme to cytochrome c in the absence of CcmABCDE

Conceptually, system I occurs in two steps: i) formation and release of holoCcmE (by CcmABCD), and ii) heme delivery to CcmFH (by holoCcmE), the putative site of holocytochrome c formation (hence, the "cytochrome c synthetase"). Studies in our lab (Feissner *et al.*, 2006a; Goldman *et al.*, 1997; Richard-Fogal and Kranz, 2010; Richard-Fogal *et al.*, 2009) and others (Christensen *et al.*, 2007; Ren and Thony-Meyer, 2001) have addressed step one, analyzing intermediates during formation of holoCcmE (such as the stable CcmCDE complex) and its release. However, less is known about the second step. To study the two steps independently, we engineered the IPTG-inducible pGEX with the genes encoding step one of the pathway, *ccmABCDE*, and an arabinose-inducible pBAD-based plasmid with the genes encoding step two, *ccmFGH*. Heme staining of BPER cell extracts revealed that expression of all Ccm proteins with the arabinose-inducible chromosmally-integrated cytochrome c4 (San Francisco *et al.*, 2011), led to robust production of holocytochrome c4 (Fig 1A, lane 5). Note that, typically, only heme that is covalently bound remains with the cytochrome c after denaturing SDS-PAGE, and this heme is readily detectable by heme stain (Feissner *et al.*, 2003). Surprisingly, we noticed that cells containing only CcmFGH (i.e., lacking *ccmABCDE*), also produced holocytochrome c4 (Fig 1A, lane 4). Thus, it appeared that holocytochrome formation could proceed in the absence of CcmABCDE, albeit, under these conditions, at levels less than one-tenth those when CcmABCDE were present. We confirmed chromosomal deletion of the *ccmA-H* operon in our *E. coli* Δ*ccm* strains (Feissner *et al.*, 2006b; San Francisco *et al.*, 2011) by genomic PCR (Fig S1).

The holocytochrome c synthetase activity of CcmFGH-only is not limited to the di-heme cytochrome c4, but also extends to the mono-heme cytochrome c2 (Fig 1B and C, lane 4). The cytochrome c2 plasmid used here has an in-frame C-terminal fusion to *E. coli* alkaline phosphatase (Pho), expressed from an IPTG-inducible promoter (Beckman *et al.*, 1992). We conclude that CcmFGH, in the absence of other Ccm components, can attach heme to two unrelated (other than the Cys-Xxx-Xxx-Cys-His motif) cytochrome c substrates. Furthermore, since alkaline phosphatase is secreted to the periplasm, covalent heme attachment to the cytochrome c2:Pho fusion protein must occur in the periplasm (i.e., where CcmFGH function).

Cytochrome c produced by CcmFGH-only is identical to cytochrome c produced by full system I

We wanted to further characterize the di-heme holocytochrome c4 produced by CcmFGH-only. To confirm proper periplasmic secretion (and cleavage of the periplasmic signal sequence), and to analyze the spectral properties of the cytochrome c produced by CcmFGH-only, we grew 1L cultures and purified hexahistidine-tagged holocytochrome c4 from the soluble fraction (Fig 2A–C). The full-length 24 kDa holocytochrome c4 and the proteolyzed 12 kDa holocytochrome c4′ were detectable by Coomassie blue staining of SDS-PAGE (Fig 2A, lane 8 "E1"). Note that it is common for endogeneous proteolysis of the 24 kDa cytochrome to occur, yielding two 12 kDa mono-heme forms (Feissner *et al.*, 2006b); however, only the C-terminal of these products is purified (along with the full-length 24 kDa protein) since the hexahistidine tag is at the C-terminus of the protein. The purified 24 and 12 kDa forms contained covalent heme (Fig 2B, lane 8), and reacted with cytochrome c4 antisera (Fig 2C, lane 8). The UV-Vis absorption spectrum (Fig 2D, red line) and reduced pyridine hemochrome (Fig 2D, inset, red line) of the purified cytochrome c4 are consistent with two covalent attachments between the thiols of cytochrome c (in Cys-Xxx-Xxx-Cys-His) and the vinyls of heme. The spectral features of cytochrome c4 produced by

CcmFGH-only (e.g., 552-nm absorption maximum in the spectrum of the reduced sample, and 550-nm absorption maximum in the reduced pyridine hemochrome spectrum) are indistinguishable from those of the cytochrome c4 produced when all system I proteins are present (CcmABCDEFGH; Fig 2D, blue line; Fig 2D, inset, blue line). Mass spectral analysis of cytochrome c4 purified from cells expressing CcmFGH-only or the full system I identified species of the expected molecular weights in each preparation (i.e., within 2 Daltons of the published molecular weights for the full-length holocytochrome c4 and proteolyzed holocytochrome c4′ (Feissner *et al.*, 2006b); Fig S2). This further confirmed covalent attachment of heme and proper cleavage of the periplasmic secretion signal (Fig S2B). In these large-scale cultures (under the growth and induction conditions described in experimental procedures), yields of holocytochrome c via CcmFGH-only were approximately one-sixth those of the full system I.

Engineering optimal biosynthesis of holocytochrome c produced by CcmFGH-only

To engineer increased cytochrome c maturation in the absence of CcmABCDE, we piloted the following strategies: i) increasing the concentration of inducer (arabinose; "↑ arab" condition), ii) raising intracellular heme levels by addition of the heme biosynthetic precursor δ-aminolevulinic acid (ALA; "ALA" condition), iii) decreasing oxygen tension by reduced shaking during growth ("↓ rpm" condition), and iv) engineering the cytochrome c4 gene for increased expression (by gene dosage) from a single pBAD-based vector, downstream of *ccmF*:His6*GH* ("p-c4" condition). Addition of ALA and increasing cytochrome c4 gene dosage led to the largest increases in yields of holocytochrome c (Fig 3A, lanes 4 and 6, respectively; Fig 3B, red and orange lines; quantified in Fig 3C). Additively, with each of the four modifications together, levels of holocytochrome c4 produced by CcmFGH-only increased approximately 6-fold (up to 0.3 mg per L culture), similar to yields of holocytochrome c4 when CcmABCDE are also present with the chromosomally integrated cytochrome c4 gene (Fig 3A, compare lanes 7 and 9; Fig 3B, compare light blue and brown lines; quantified in Fig 3C). In cells carrying CcmABCDE in the absence of CcmFGH with the chromosomally integrated cytochrome c4 gene, no holocytochrome c formation was detected (Fig 3A, lane 8). In the presence of CcmABCDE and CcmFGH (i.e., full system I) and each of the four modifications described above, yields of cytochrome c4 were approximately 1 mg per L culture (Fig 3A, lane 10; Fig 3B, top blue line; quantified in Fig 3C). Analysis of membrane fractions by immunoblot confirmed the presence of CcmF and CcmH, respectively (except in the strain carrying only pGEX CcmABCDE; Fig 3D and 3E, lane 8), and the absence of CcmE (except for those cells carrying pGEX CcmABCDE; Fig 3F). These results demonstrate that, under optimized conditions, CcmFGH-only is a robust synthetase capable of producing substantial levels of periplasmic holocytochrome c.

To establish that the cytochrome c4 was not self-assembling (i.e., non-enzymatically binding and attaching heme) under the optimized conditions, *E. coli* Δ*ccm* containing only the cytochrome c4 gene (in the pBAD plasmid) was induced with arabinose in the presence or absence of ALA, and the hexahistidine-tagged cytochrome c4 was purified and analyzed (Fig 3G and Fig S3). No covalently bound heme was detected by heme stain (Fig 3G, lanes 2 and 3) or by absorption spectroscopy (Fig S3). To test whether CcmABCDE could act as a synthetase under the optimized conditions, we co-expressed the cytochrome c4 (in pBAD) and CcmABCDE (in pGEX) in the presence or absence of ALA. Again, holocytochrome c4 was not produced at detectable levels, as determined by heme stain (Fig 3G, lanes 4 and 5) and absorption spectroscopy (Fig S3), while the CcmFGH-only yielded readily-detectable holocytochrome c4 (Fig 3G, lanes 6 and 7; Fig S3).

The role of conserved P-His and TM-His residues in CcmF for holocytochrome c assembly via CcmFGH-only

CcmF contains four conserved histidine residues: TM-His1 and TM-His2 in transmembrane domains 5 and 12, respectively, and P-His1 and P-His2, located in periplasmic loops flanking the WWD domain (see Fig 4A). P-His1 and P-His2 are proposed to ligate heme from holoCcmE when it is bound in the WWD domain, en route to covalent attachment to apocytochrome c; TM-His1 and TM-His2 are ligands to the stable heme b (San Francisco *et al.*, 2011), which may play a role in reducing the holoCcmE heme (bound in the WWD domain) prior to covalent attachment. In the context of the full system I, each of the four histidines is essential for holocytochrome c formation (Richard-Fogal *et al.*, 2009; San Francisco *et al.*, 2011). To test whether holocytochrome c formation via CcmFGH-only required these four histidines, we engineered substitutions at each residue (in pRGK388) and assayed for heme attachment to cytochrome c4. Substitutions at TM-His1, TM-His2, and P-His1 abolished holocytochrome formation to undetectable levels, indicating that these residues are absolutely required for heme attachment to cytochrome c via the CcmABCDE-independent pathway (Fig 5A, lanes 3, 4, and 6; quantified in Fig 5B). Substitution of P-His2 with glycine supported holocytochrome formation at approximately 20 % levels of WT (Fig 5A, lane 5; quantified in Fig 5B). This contrasts with the absolute requirement for P-His2 in the context of the full system I (i.e., in the presence of CcmABCDE), and may be suggestive of a holoCcmE-specific role for P-His2 in cytochrome c formation (see Discussion).

To test whether other substitutions could support holocytochrome formation at P-His1 or P-His2, we engineered alanine, cysteine, methionine, or tyrosine substitutions at P-His1 or P-His2. While no P-His1 substitution supported holocytochrome c formation (Fig 5C, upper panel), we discovered that P-His2Cys supported cytochrome c assembly at approximately 60 % WT levels (Fig 5C, lower panel, lane 4; quantified in Fig 5D). P-His2Ala was similar to substitution with glycine (20 % function relative to WT; Fig 5C, lower panel, lane 3; quantified in Fig 5D) while methionine and tyrosine substitutions were 3 % and 10 % WT levels, respectively (Fig 5C, lower panel, lanes 5 and 6; quantified in Fig 5D). The inability for any engineered substitution at P-His1 to support holocytochrome formation indicates that this residue cannot vary from the natural histidine, similar to results with full system I.

Conserved cysteines in CcmG and CcmH are required for holocytochrome formation via CcmFGH-only

CcmG and CcmH each contain a conserved pair of thioredox-active cysteines that are required for cytochrome c synthesis in the context of the full system I (Fabianek *et al.*, 1998; Fabianek *et al.*, 1999; Robertson *et al.*, 2008). To test whether holocytochrome c formation by CcmFGH-only required these thioredoxin functions, we engineered serine substitutions at the conserved cysteine pairs in CcmG or CcmH and assayed for cytochrome c formation. Similar to the full system I, in the absence of the conserved cysteine pair in either CcmG or CcmH, no holocytochrome formation was observed (Fig 6A, lanes 3 and 4; quantified in Fig 6B). Thus, the redox-active cysteine pairs of CcmG and CcmH are required for holocytochrome c formation via CcmFGH-only.

DISCUSSION

CcmF has been referred to as the “cytochrome c heme lyase” or the “cytochrome c synthetase” in nearly every review on system I in the last decade [for recent reviews, see (Hamel *et al.*, 2009; Kranz *et al.*, 2009; Mavridou *et al.*, 2013; Sanders *et al.*, 2010; Sawyer and Barker, 2012)]. However, direct experimental evidence demonstrating this activity for CcmF has been lacking. Here, we demonstrate that holocytochrome c formation can occur

completely (and robustly) in the absence of the CcmABCDE components of the system I pathway, thus establishing that the cytochrome c synthetase activity of system I inheres in CcmFH (and CcmG). We suspect that the synthetase activity of CcmFH (with CcmG) has gone unnoticed thus far due to challenges in expressing these membrane proteins and engineering conditions for optimal biogenesis. Only of late have we been able to express and purify CcmF in sufficient quantities to begin biochemical characterization of this protein (Richard-Fogal *et al.*, 2009; San Francisco *et al.*, 2011). Indeed, the discovery that CcmF contained a stable heme b (coordinated by TM-His1 and TM-His2) was made only recently (Richard-Fogal *et al.*, 2009). Thus, we attribute our observation of this "new" CcmFGH-only synthetase activity to our recent ability to successfully modulate expression of stable, functional CcmFH and CcmG in *E. coli*, and control the expression of substrate cytochromes c (e.g., the di-heme cytochrome c4 and mono-heme cytochrome c2 used here).

It is important to state that the CcmFGH-only synthetase activity we describe here would not substitute for the natural complete system I that is present in a wide range of organisms. Indeed, many mutations in CcmABCDE in different organisms have been shown to result in deficiencies in cytochrome c maturation [for example, (Beckman *et al.*, 1992; Kranz, 1989; Page *et al.*, 1997; Ramseier *et al.*, 1991; Schulz *et al.*, 1998)]. The CcmFGH-only activity is a ramification of engineering and optimization to establish the minimal components of system I that possess synthetase activity. This metabolic engineering, which included i) increasing expression by gene dosage (for both ccm and apocytochrome genes), ii) promoter and ribosome binding site changes (i.e., inducible promoters in pBAD and pGEX-based vectors), and iii) increasing endogenous synthesis of heme (by ALA addition), indicated that CcmFH, along with CcmG (but not CcmABCDE—i.e., holoCcmE) functions as the synthetase; that is, CcmFH attaches heme to the CXXCH motif of the apocytochrome. Even for organisms that depend on a single cytochrome c for respiration (which are rare), the above engineering approaches would likely be much less "beneficial" for holocytochrome c assembly than delivery of heme by holoCcmE (i.e., the natural CcmABCDE step of system I). Consider also that the full system I is able to use heme at low levels and can rely on holoCcmE as a heme reservoir, which are certainly important in the natural "lifestyles" of many organisms. Nevertheless, the results described here are the most compelling thus far to designate CcmF/H as the cytochrome c synthetase of system I.

Prior in vitro studies by Ferguson and colleagues showed that holoCcmE* (lacking its TMD) could transfer its heme directly to apocytochrome c (Daltrop *et al.*, 2002). The conditions for the in vitro transfer involved 18 hours of incubation and employed the stable apocytochrome c552 from a thermophile as an acceptor. The authors indicate that their results "mimic the molecular pathway in vivo". To test whether we could engineer this transfer in vivo, we over-expressed CcmABCDE (thus, holoCcmE) and the cytochrome c4 in the presence of increased heme (i.e., added ALA). No detectable holocytochrome c4 was formed in the absence of CcmFGH (Fig 3G, lane 8 and Fig S3). We conclude that in vivo, holoCcmE does not act as a "synthetase" itself. Although there is little doubt that in system I the heme from holoCcmE is transferred to apocytochrome c, and that the heme in holoCcmE binds to the CcmF WWD domain (San Francisco and Kranz, in press), the synthetase activity is ultimately provided by the CcmF/H complex.

Requirements for holocytochrome c formation by CcmFGH-only versus the full system I

Many of the requirements for cytochrome c assembly by the full system I extend to CcmFGH-only. In system I, the thio-active proteins CcmG and CcmH each contain a conserved pair of cysteines that are required for holocytochrome formation (Fabianek *et al.*, 1998; Fabianek *et al.*, 1999; Robertson *et al.*, 2008). CcmG and CcmH are involved in maintaining the thiols of the apocytochrome (at Cys-Xxx-Xxx-Cys-His) in the reduced state,

which is a requirement for thioether formation with the heme vinyls. The conserved cysteines in CcmG (which are reduced by the membrane protein DsbD) likely function to reduce the thiols of CcmH (Meyer *et al.*, 2005; Monika *et al.*, 1997; Setterdahl *et al.*, 2000; Turkarslan *et al.*, 2008). CcmH also has a direct apocytochrome binding function (Di Matteo *et al.*, 2007; Di Silvio *et al.*, 2013; Meyer *et al.*, 2005; Verissimo *et al.*, 2011), and it is proposed that, via interaction with CcmF, CcmH physically positions the apocytochrome (with reduced cysteine thiols) for covalent heme attachment (Meyer *et al.*, 2005; Verissimo *et al.*, 2011). We discovered that mutation of either of the cysteine pairs in CcmG or CcmH abolished holocytochrome formation by CcmFGH-only (Fig 6). The requirement for the conserved redox-active cysteine pairs in CcmG and CcmH demonstrates that the CcmFGH-only pathway is "intact" with regard to handling of the apocytochrome. Although other explanations are possible, we favor the hypothesis that the lower cytochrome c maturation activity of CcmFGH-only (relative to full system I) is likely due to less efficient heme delivery (i.e., the absence of the holoCcmE "heme reservoir" (Feissner *et al.*, 2006b); see below). This finding also highlights the significant evolutionary advantage of the full system I.

Holocytochrome c formation by the full system I requires conserved histidines in CcmF: mutation of any of the four (Fig 4A) renders the system non-functional (Ren *et al.*, 2002; Richard-Fogal *et al.*, 2009; San Francisco *et al.*, 2011). We have shown experimentally that TM-His1 and TM-His2 are ligands to the b-heme (San Francisco *et al.*, 2011), while P-His1 and P-His2 are proposed to ligate the incoming heme from holoCcmE when it is bound in the WWD domain (Kranz *et al.*, 2009; Richard-Fogal *et al.*, 2009; San Francisco *et al.*, 2011). For CcmFGH-only, we found that TM-His1, TM-His2, and P-His1 were each indispensable for holocytochrome formation. However, CcmFGH-only was only partially dependent on P-His2 (Ala or Gly substitutions at this position retained approximately 20 % activity, and Cys, 60 %, relative to WT). Since these same substitutions show no activity in the context of the full system I, we propose that P-His2 may have a function that is specific to the system I heme chaperone, holoCcmE. The lack of a requirement for P-His2 reflects, possibly, a different mechanism of heme trafficking to the WWD domain in CcmFGH-only. Below, we suggest two possible trafficking routes for the endogeneous heme that is attached to cytochrome c by CcmFGH-only.

Similarities between CcmFGH-only and CcsBA: evolutionary insights

The assignment of the synthetase (heme lyase) activity of system I to CcmF has been based in part on its topological similarity to the cytochrome c synthetase of system II, CcsBA (see Fig 4). System II is present in many gram positive (and other) bacteria, cyanobacteria, and the chloroplasts of plants and algae (Ahuja *et al.*, 2009; Simon and Hederstedt, 2011; Xie and Merchant, 1998). CcsBA, when expressed heterologously in *E. coli* Δ*ccm* is sufficient for assembly of cytochrome c (Feissner *et al.*, 2006b; Frawley and Kranz, 2009; Kern *et al.*, 2010; Richard-Fogal *et al.*, 2012); thus, it is the system II holocytochrome c synthetase. CcsBA is proposed to traffick heme across the cytoplasmic membrane, in addition to facilitating thioether formation between the heme and apocytochrome (Frawley and Kranz, 2009; Hamel *et al.*, 2003; Kranz *et al.*, 2009; Merchant, 2009). Thus, it is a heme transporter and a holocytochrome c synthetase. Comparison of the membrane topologies of CcmF and CcsBA reveals several similar features (see Fig 4): each contains four completely conserved His residues (two in transmembrane domains, here called TM-His1 and TM-His2; and two in extra-cytoplasmic loops, here called P-His1 and P-His2) and the conserved WWD domain, a hydrophobic, extra-cytoplasmic feature that has been shown (in the system I protein CcmC) to interact directly with heme (Richard-Fogal and Kranz, 2010). Additionally, both CcsBA (Frawley and Kranz, 2009) and CcmF (Richard-Fogal *et al.*, 2009) bind a non-covalent heme b (see Fig 4). However, in CcsBA, this heme b is ultimately

attached to cytochrome c, whereas the stable heme b in CcmF (in the context of the full system I) may play a role in reducing heme bound in the WWD domain (from holoCcmE).

The topological similarities between CcmF and CcsBA, coupled with the finding here that CcmF (together with CcmH and CcmG) is a cytochrome c synthetase, raise several interesting questions. First, how is heme trafficked to the site of thioether formation with the apocytochrome (presumably, at the CcmF WWD domain with axial ligation by P-His1) in CcmFGH-only synthesis? While we cannot rule out other hypotheses, we envision two possible scenarios: i) heme enters the WWD domain of CcmF directly from the cytoplasmic membrane outer leaflet, possibly, (where it is subsequently reduced by the transmembrane heme b), or ii) similar to the proposed mechanism for CcsBA, the transmembrane heme b may be channeled to the external WWD domain for attachment to the apocytochrome. While experiments addressing these hypotheses will be challenging, the possibility that the CcmFGH-only synthetase may be CcsBA-like (with respect to the channeling of heme from the transmembrane binding site to the WWD domain) is intriguing from an evolutionary perspective.

The complex phylogenetic distribution of systems I and II suggest significant lateral transfer (Goldman and Kranz, 1998), making it difficult to discern clear evolutionary patterns. We have shown previously that system I can mature cytochromes c at 5-fold lower heme concentrations than can system II (Richard-Fogal *et al.*, 2007), and that holoCcmE can function as a “heme reservoir” (i.e., when heme synthesis is completely inhibited) (Feissner *et al.*, 2006a). On the basis of these results, we have speculated that the holoCcmE reservoir aspect of system I may have evolved for cytochrome c synthesis under conditions of iron (or heme) scarcity. The ability to use heme at very low levels, and as a reservoir when no iron is present, might represent a possible selection advantage. Since the CcmFGH-only synthetase lacks the holoCcmE reservoir, it could be considered a “prototype” of an intermediate, or a “transition,” on the evolutionary path from system II to the full system I. CcmFGH-only synthesis thus provides a unique opportunity to directly compare holocytochrome c formation (under low heme conditions) by system I with and without the holoCcmE reservoir.

CcmFGH-only: a viable candidate for in vitro reconstitution of holocytochrome c assembly

One of the remaining challenges in the field of cytochrome c biogenesis is in vitro reconstitution of each of the systems for holocytochrome formation. Reconstitution of the synthetase reactions would be especially informative, as they are common to all three major pathways. To date, system II has been a more attractive candidate for in vitro reconstitution than system I, since it consists of a single protein complex. Our findings here demonstrate that the system I synthetase (CcmFGH-only) is now a viable candidate for in vitro reconstitution as well. The CcmFGH-only synthetase activity reported here thus represents a significant advance for the field, in terms of the proof of its function, the unique evolutionary and mechanistic insights it provides, and the potential for in vitro reconstitution.

EXPERIMENTAL PROCEDURES

Bacterial Growth Conditions

Unless otherwise noted, *Escherichia coli* strains (Table S1) were grown at 37°C by shaking at 230 rpm in Luria-Bertani broth (LB; Difco) supplemented with the appropriate antibiotics (Sigma-Aldrich) and other media additives at the following concentrations: carbenicillin, 50 μg mL^{-1}; chloramphenicol, 20 μg mL^{-1}; gentamicin, 10 μg mL^{-1}; kanamycin, 20 μg mL^{-1}; Isopropyl β-D-1-thiogalactopyranoside (IPTG; Gold Biotechnology), 1 mM; arabinose

(Gold Biotechnology), 0.2 % (wt/vol); δ-aminolevulinic acid (ALA; Sigma-Aldrich), 50 μg ml^{-1}.

Protein Expression and Purification

E. coli Δ*ccm* strains RK103 (Feissner *et al.*, 2006b) or RK111 (Δ*ccm* carrying the arabinose-inducible chromosomal integrate of the *cyt* c_4:His6 gene) (San Francisco *et al.*, 2011) (Table S1) were used for expression. Starter cultures were inoculated from a single colony and grown overnight in 10 mL of LB with the appropriate antibiotics. 1 L of LB was inoculated with the 10 mL starter culture and grown to an OD_{600} of 1.8, then induced with IPTG and/or arabinose for 14–16 hr. Cells were harvested at 5,000 × g and frozen at −80°C. Cell pellets were thawed and resuspended in phosphate-buffered saline (PBS; 100 mM NaCl, 7.5 mM Na_2HPO_4, 2 mM NaH_2PO_4, pH 7) and treated with 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich) and 100 μg ml^{-1} egg white lysozyme (Sigma-Aldrich) for 30 min while shaking on ice. Cells were disrupted by repeated sonication on ice for 30 sec bursts (5 × 30 sec on, 30 sec off) on a Branson 250 sonicator (50% duty, 8 output). Crude sonicate was centrifuged at 24,000 × g for 20 min to clear cell debris, and the soluble and membrane fractions were separated by centrifugation at 100,000 × g for 45 min. Soluble fractions (L; load) were passed over TALON resin per the manufacturer's recommendations and washed in 1 x modified TALON buffer with increasing concentrations of imidazole (wash 1 (W1), 0 mM imidazole; wash 2 (W2), 2 mM imidazole; wash 3 (W3), 5 mM imidazole). Bound hexahistidine-tagged protein was eluted in 1 x modified TALON buffer containing 150 mM imidazole (E; elution). Total protein concentration was determined using the Nanodrop 1000 spectrophotometer (Thermo Scientific). For growth by shaking at 120 rpm ("↓ rpm" condition), starter cultures were initiated from a single colony and grown overnight in 100 mL of LB with the appropriate antibiotics. 1 L of LB was inoculated with the 100 mL starter culture and grown to an OD_{600} of 1.8, then induced with IPTG and/or arabinose for 14–16 hr. For the "↑ arabinose" condition, expression was induced with 0.4 % arabinose rather than 0.2 %. For the "ALA" condition, 50 μg mL^{-1} ALA was added to culture at the time of induction. For analysis of crude membrane fractions, membrane pellets (isolated by centrifugation at 100,000 × g) were solubilized in a modified 1x TALON (Clontech) buffer (50 mM Tris-HCL, pH 7; 300 mM NaCl) with 1 % (wt/vol) dodecyl maltoside (DDM, Anatrace) by agitation on ice for 1 hr, and then centrifuged at 24,000 × g for 20 min to remove unsolubilized material.

Heme stains and other methods

Heme stains and immunoblots were performed as described previously (Feissner *et al.*, 2003; Feissner *et al.*, 2006b). Proteins were separated by 12.5 % SDS-PAGE and transferred to Hybond C nitrocellulose membranes (GE Healthcare). For immunoblots, anti-CcmF antibodies were used at a dilution of 1:10000, anti-CcmE antibodies at 1:10000, anti-cytochrome c4 antibodies at 1:10000, and anti-CcmH antibodies at 1:5000. Protein A peroxidase (Sigma-Aldrich) was used as the secondary label. The chemiluminescent signal for heme stains was developed using the SuperSignal Femto kit (Thermo Scientific) or, for immunoblots, the Immobilon Western kit (Millipore), and detected with an LAS-1000 Plus detection system (Fujifilm-GE Healthcare). The abundance of holocytochrome c4 was determined by pyridine extraction as described in (Berry and Trumpower, 1987), or by densitometry analysis of the chemiluminescent signal from heme staining using the Science Lab 99-Image Gauge version 3.4 software (Fujifilm-GE Healthcare) as described in (Richard-Fogal *et al.*, 2007). Protein purity was assessed by Coomassie Blue staining of SDS-PAGE.

UV/Vis absorption spectroscopy

UV-visible absorption spectra were recorded with a Shimadzu UV-2101 PC UV-Vis scanning spectrophotometer at room temperature as described previously (Frawley and Kranz, 2009). All spectra were recorded in the same buffer (modified 1x TALON buffer) in which the proteins were purified. Chemically reduced spectra were generated by addition of a few grains of sodium dithionite (sodium hydrosulfite).

Construction of plasmids

All oligonucleotide primer sequences, plasmids, and strains are given in Table S1. The single pBAD-based plasmid containing *ccmF*:His6*GH* and *cyt* c_4:His6 was constructed by polymerase chain reaction (PCR) amplification of the *cyt* c_4:His6 gene (and the upstream ribosome binding site; RBS) from pRGK332 (Feissner *et al.*, 2006b) and insertion of the resulting fragment into the single PstI site downstream of *ccmF*:His6*GH* in pRGK388 (Richard-Fogal *et al.*, 2009) to create pRGK448. The resulting plasmids were screened by restriction digest to confirm correct orientation of the insert, and then sequenced. All substitutions in pRGK388 (i.e., CcmG (Cys80Ser/Cys83Ser), CcmH (Cys43Ser/Cys46Ser), CcmF(His261Gly), and CcmF(His491Ala)) were engineered using the QuikChange I Site-Directed Mutagenesis Kit (Agilent Technologies) per the manufacturer's recommendations. All oligonucleotides were synthesized by Sigma-Aldrich. Each of the final constructs was sequenced to verify the mutation(s).

Proteomics analysis

Proteomics analyses were carried out by the Washington University Resource for Biomedical and Bio-organic Mass Spectrometry Facility. Briefly, TALON-purified holocytochrome c4:His6 samples (produced by the full system I or by CcmFGH-only) were loaded onto an Agilent 2.1 X 15 mm C8 Zorbax Cartridge-Column and eluted using a 9.5 min gradient with a flow rate of 200 μL/min. A positive ion mass spectrum was acquired using a Bruker Maxis Q-ToF mass spectrometer equipped with an electrospray ionization source. Protein molecular ions were identified with multiple charge states and deconvoluted to identify the molecular weight(s) of the main species.

Genomic PCR

E. coli strains (WT *E. coli* K-12 MG1655; Δ*ccm* (RK103); Δ*ccm cyt* c_4:His6 chromosomal integrate (RK111); and RK111 carrying pBAD *ccmF*:His6*GH*) were streaked onto LB plates containing the appropriate antibiotics and incubated at 37°C overnight. Single colonies were resuspended in 100 μL H_2O by vortexing, placed at 90°C for 10 min to lyse cells, and centrifuged at 16 000 × g for 5 min to remove cell debris. 1 μL of the supernatant (containing cellular DNA) was used as the template in each of four PCRs with the following primer sets: A) delCcmF-right + delCcmH-left, B) delCcmC-right + delCcmF-left, C) delCcmB-right + delCcmE-left, D) menBflank-right + menBflank-left. Oligonucleotide primer sequences are given in Table S1. PCRs were performed using GoTaq Green Master Mix (Promega) for 25 cycles, per the manufacturer's recommendations. PCR products were visualized by ethidium bromide staining of agarose gels (0.8 %).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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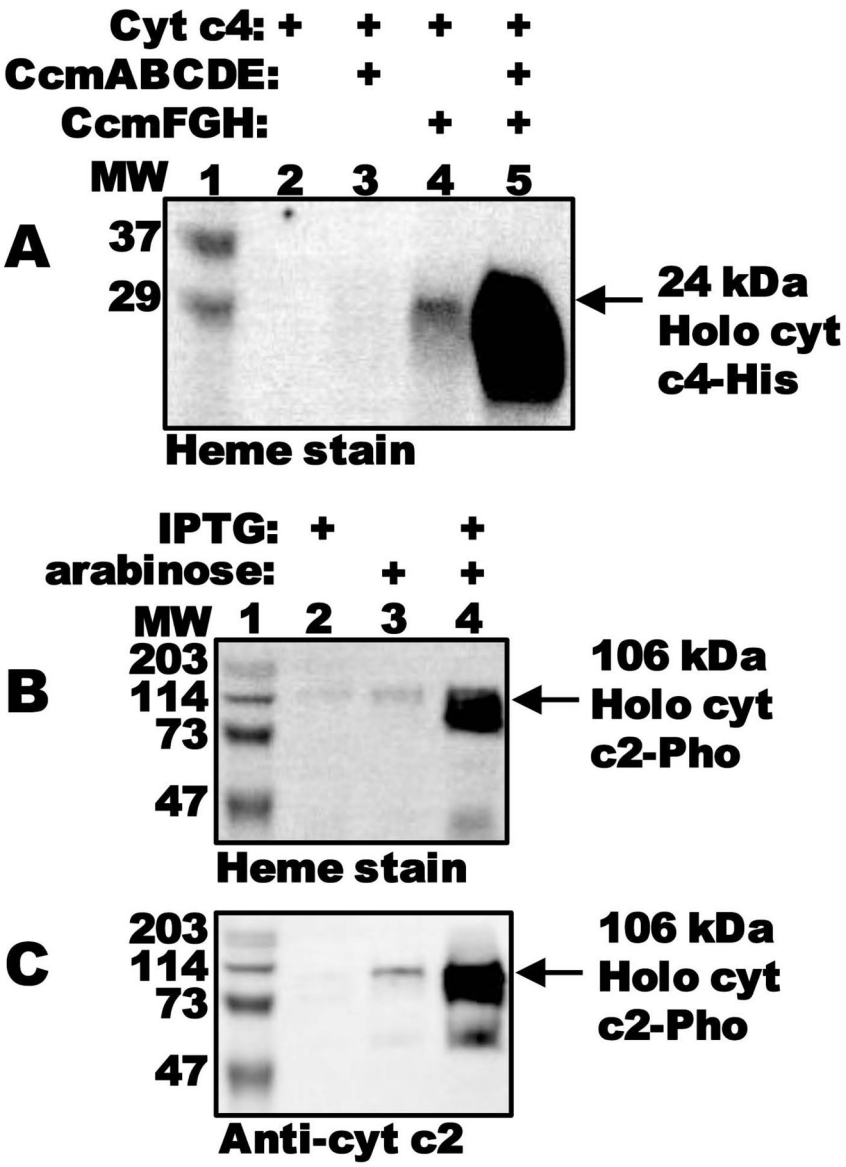

Fig. 1.

CcmFGH attaches heme to cytochrome c in the absence of CcmABCDE. (A) Heme staining of BPER cell extracts showing synthesis of 24-kDa holocytochrome c4:His6 as a function of expression of separate Ccm components, as indicated. (B) Heme staining and (C) anti-cytochrome c2 immunoblot of BPER cell extracts showing synthesis of 106-kDa holocytochrome c2:Pho by CcmFGH as a function of induction with IPTG and/or arabinose. One hundred μg total protein was loaded in each lane. MW, molecular weight standards (lanes 1).

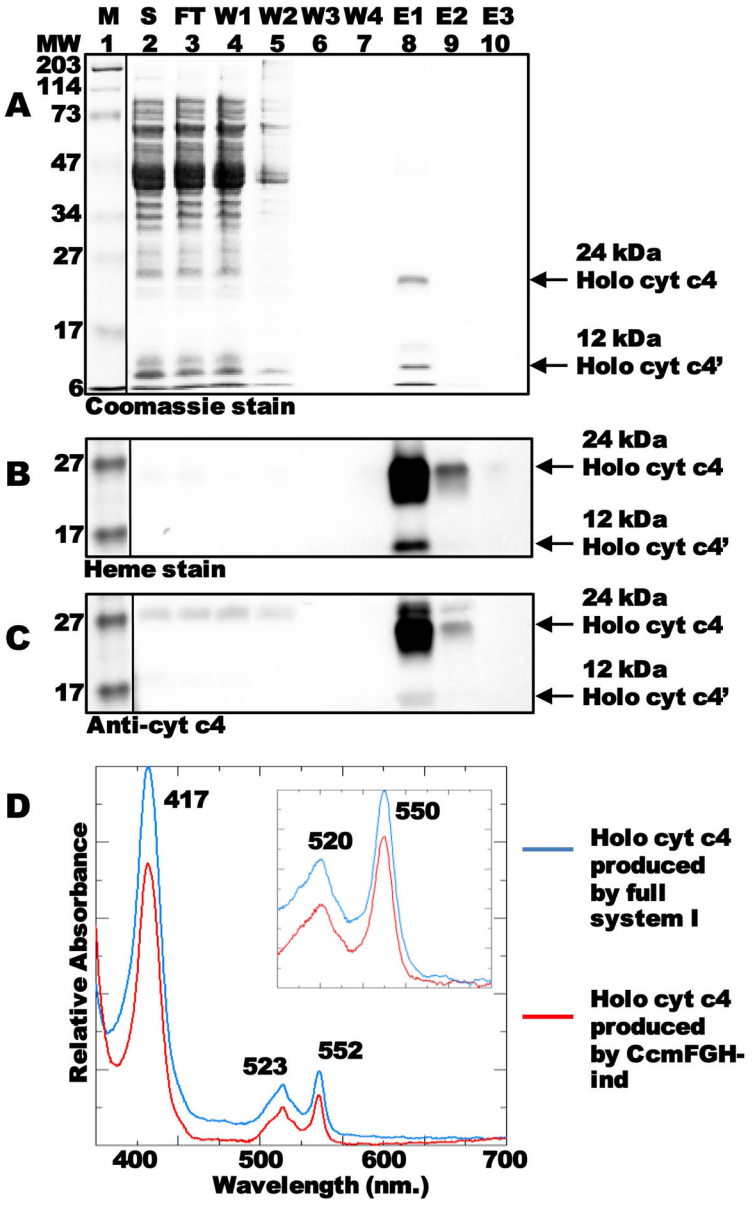

Fig. 2.

Purification and characterization of cytochrome c produced by CcmFGH in the absence of CcmABCDE. (A) Coomassie blue staining of purified cytochrome c4:His6 showing full length 24-kDa holocytochrome c4 and proteolyzed 12-kDa holocytochrome c4′. (B) Heme staining of purified cytochrome c4 showing 24-kDa and 12-kDa forms. (C) Anti-cytochrome c4 immunoblot showing 24-kDa and 12-kDa holocytochrome c4. For (A)–(C), abbreviations are M, molecular weight standards; S, soluble fraction; FT, flow through; W1, wash 1; W2, wash 2; W3, wash 3; W4, wash 4; E1, elution 1; E2, elution 2; E3, elution 3. (D) UV-Vis absorption spectra of purified, sodium dithionite-reduced holocytochrome c4 produced by the full system I (blue line) or in the absence of CcmABCDE (red line). (Inset) Sodium dithionite-reduced pyridine hemochrome spectrum of purified holocytochrome c4 produced by the full system I (blue line) or in the absence of CcmABCDE (red line) from 500–600 nm. Absorption maxima are indicated.

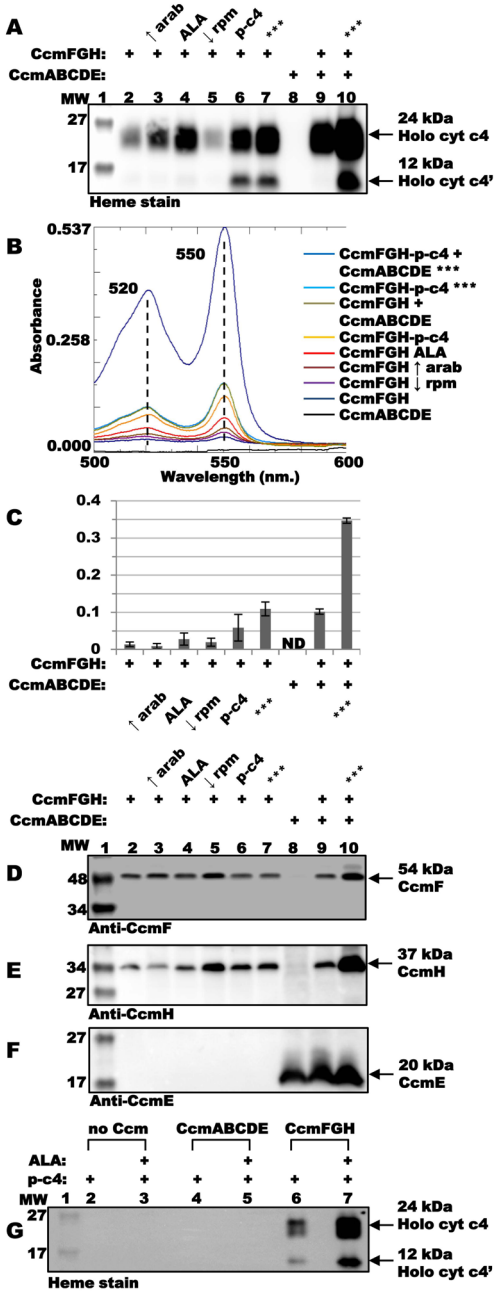

Fig. 3.

Optimization of holocytochrome c4 produced by CcmFGH in the absence of CcmABCDE. (A) Heme staining of TALON-purified proteins showing relative levels of holocytochrome c4 synthesized by CcmFGH-only (lanes 2–7), CcmABCDE (lane 8), and CcmFGH + CcmABCDE (lanes 9 and 10) for each indicated condition/additive. "↑ arab," increase in final arabinose inducer concentration from 0.2 % to 0.4 %; "ALA," addition of ALA to 50 μg mL^{-1}; "↓ rpm," decrease in shaking during growth from 230 rpm to 120 rpm; "p-c4," plasmid-borne cytochrome c4 engineered into pBAD CcmFGH; "***," each of the above four additives/conditions together. Arrows denote full-length 24-kDa and proteolyzed 12-kDa holocytochrome c4. (B) Sodium dithionite-reduced pyridine hemochrome spectra of TALON-purified proteins for each condition, from 500–600 nm. Absorption maxima are

indicated. (C) Quantification of yields of holocytochrome c4 (based on 550 nm. absorption in reduced pyridine hemochrome) for each additive/condition from three independent experiments. Error bars denote standard deviation. ND, none detected. (D) Anti-CcmF immunoblot of DDM-solubilized membrane fractions showing 54-kDa CcmF. (E) Anti-CcmH immunoblot of DDM-solubilized membrane fractions showing 37-kDa CcmH. (F) Anti-CcmE immunoblot of DDM-solubilized membrane fractions showing 20-kDa CcmE. Labels for (D) – (F) are as in (A). (G) Heme staining of TALON-purified proteins showing relative levels of plasmid-borne holocytochrome c4 ("p-c4") synthesized in the absence of Ccm proteins (lanes 2 and 3), by CcmABCDE (lanes 4 and 5), and by CcmFGH-only (lanes 6 and 7) in the presence or absence of ALA (50 $\mu g\ mL^{-1}$). Arrows denote full-length 24-kDa and proteolyzed 12-kDa holocytochrome c4.

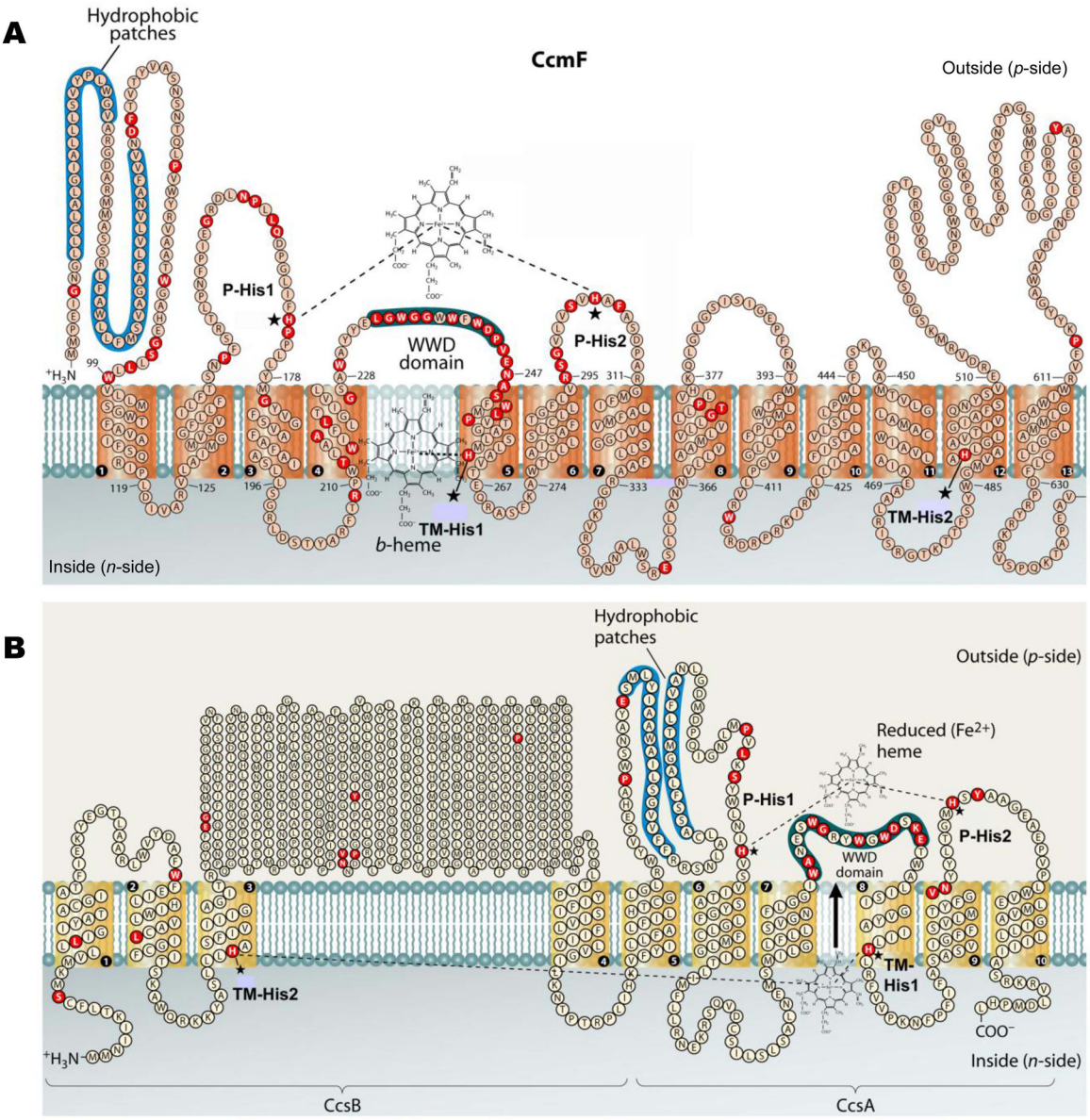

Fig. 4.

Topology of the system I CcmF protein from *Escherichia coli* (A) and the system II CcsBA fusion protein from *Helicobacter hepaticus* (B). Possible histidine axial ligands to heme are starred, and are designated P-His1, P-His2, TM-His1, and TM-His2. The highly conserved WWD domain and the hydrophobic patches are shaded. Completely conserved amino acids (red) were identified by individual protein alignments using CcmF or the CcsB and CcsA ORFs from selected organisms, as described in (Kranz *et al.*, 2009). Diagram is modified from (Kranz *et al.*, 2009).

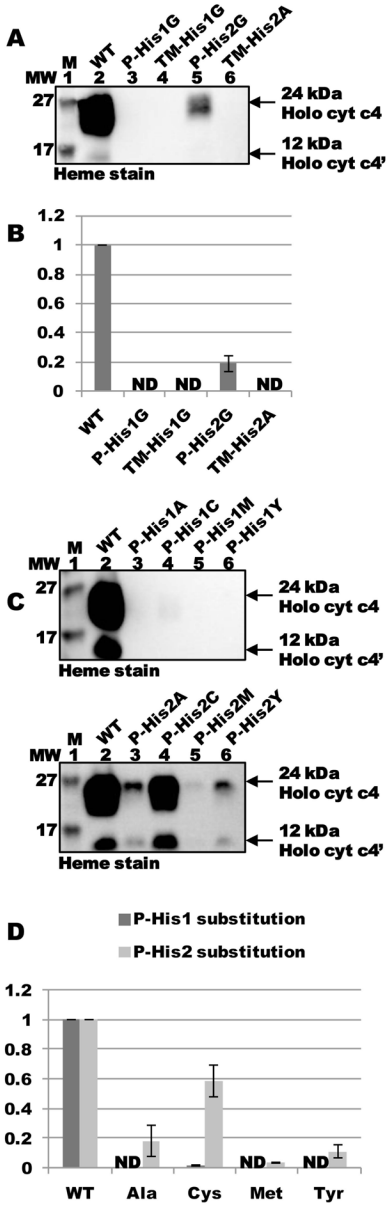

Fig. 5.

Effect of substitutions at conserved His residues in CcmF on holocytochrome c formation in the absence of CcmABCDE. (A) Heme staining of purified holocytochrome c4 assembled by CcmFGH-only ("WT") and site-directed variants of CcmF. (B) Quantification of the results of heme staining (24 kDa holocytochrome c4) from purified fractions from three independent experiments. (C) Heme staining of purified holocytochrome c4 assembled by CcmFGH-only ("WT") and the indicated site-directed variants at CcmF P-His1 (upper panel) or P-His2 (lower panel). (D) Quantification of the results of heme staining from purified fractions from three independent experiments. For (B) and (D), synthesis of holocytochrome c4 is relative to CcmFGH-only ("WT"), which has been set at 100 %. Error bars denote standard deviation. ND, none detected.

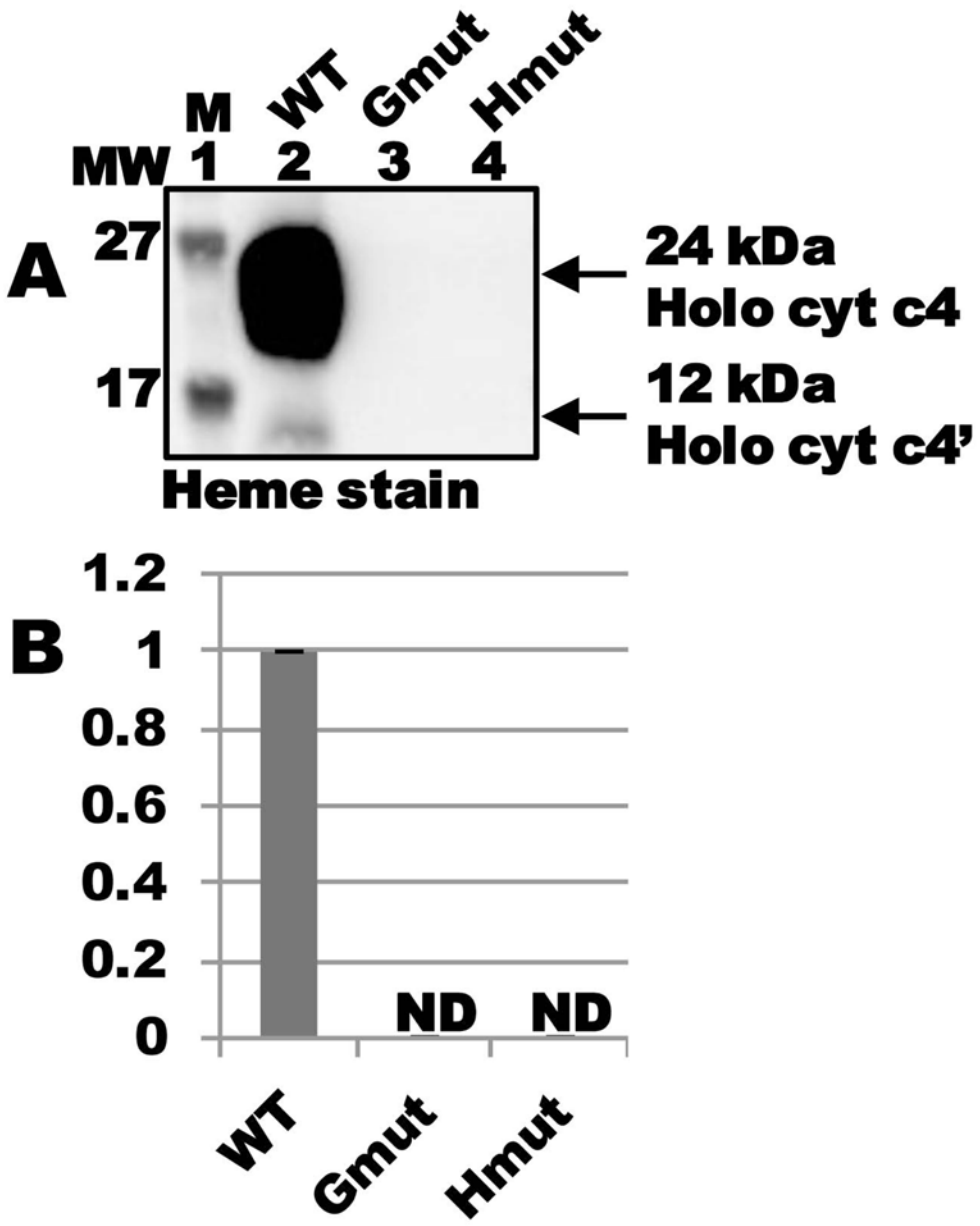

Fig. 6.

Conserved cysteines in CcmG and CcmH are required for holocytochrome c formation by CcmFGH in the absence of CcmABCDE. (A) Heme staining of purified holocytochrome c4 assembled by CcmFGH-only ("WT") and the indicated site-directed variants at CcmG and CcmH. "Gmut" is a Cys80Ser/Cys83Ser double mutant, and "Hmut" is a Cys43Ser/Cys46Ser double mutant. (B) Quantification of the results of heme staining (24 kDa holocytochrome c4) from purified fractions from three independent experiments. Synthesis of holocytochrome c4 is relative to CcmFGH-only ("WT"), which has been set at 100 %. ND, none detected.