

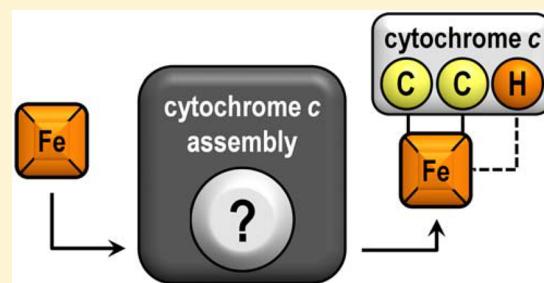
# Probing Heme Delivery Processes in Cytochrome *c* Biogenesis System I

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 Supporting Information

**ABSTRACT:** Cytochromes *c* comprise a diverse and widespread family of proteins containing covalently bound heme that are central to the life of most organisms. In many bacteria and in certain mitochondria, the synthesis of cytochromes *c* is performed by a complex post-translational modification apparatus called System I (or cytochrome *c* maturation, Ccm, system). In *Escherichia coli*, there are eight maturation proteins, several of which are involved in heme handling, but the mechanism of heme transfer from one protein to the next is not known. Attachment of the heme to the apocytochrome occurs via a novel covalent bond to a histidine residue of the heme chaperone CcmE. The discovery of a variant maturation system (System I\*) has provided a new tool for studying cytochrome *c* assembly because the variant CcmE functions via a cysteine residue in the place of the histidine of System I. In this work, we use site-directed mutagenesis on both maturation systems to probe the function of the individual component proteins as well as their concerted action in transferring heme to the cytochrome *c* substrate. The roles of CcmA, CcmC, CcmE, and CcmF in the heme delivery process are compared between Systems I and I\*. We show that a previously proposed quinone-binding site on CcmF is not essential for either system. Significant differences in the heme chemistry involved in the formation of cytochromes *c* in the variant system add new pieces to the cytochrome *c* biogenesis puzzle.



Nature has employed a surprisingly diverse array of proteins to perform the post-translational modification process of covalently attaching heme to two cysteine side chains in *c*-type cytochromes. These different maturation systems, named Systems I–VI, and their distributions have been recently reviewed.<sup>2–5</sup> None of the systems are fully understood on a mechanistic level. System I, found in many Gram-negative bacteria as well as plant and protozoal mitochondria, is the most complex and was identified more than 20 years ago.<sup>6–9</sup> It has been well established that the constituent proteins are CcmABCDEFGHI (Cytochrome *c* maturation (Ccm), shown in the top panel of Figure 1). In Gram-negative bacteria, holocytochromes *c* are matured in the periplasm, and the maturation proteins are all membranous or have periplasmically orientated domains, with the exception of CcmA, which is located on the cytoplasmic side of the membrane. The mechanism of how heme is transported from its cytoplasmic site of synthesis across the inner membrane is not known.

After the arrival of heme in the periplasm, the proteins CcmABCDE are involved in heme handling and delivery to the cytochrome substrate. CcmAB has features of an ATP-binding cassette (ABC) protein and forms a complex with the membrane protein CcmC.<sup>10,11</sup> CcmC, with the assistance of CcmD, interacts with the heme chaperone CcmE, a membrane-anchored globular protein, and transfers heme to it in a process involving CcmAB; when CcmA is inactivated, heme remains trapped in a complex with CcmC and CcmE.<sup>12,13</sup> It was thought that CcmABC form a ATP-driven heme transporter,<sup>14</sup> but this has since been disproven.<sup>15</sup> CcmE was found to have

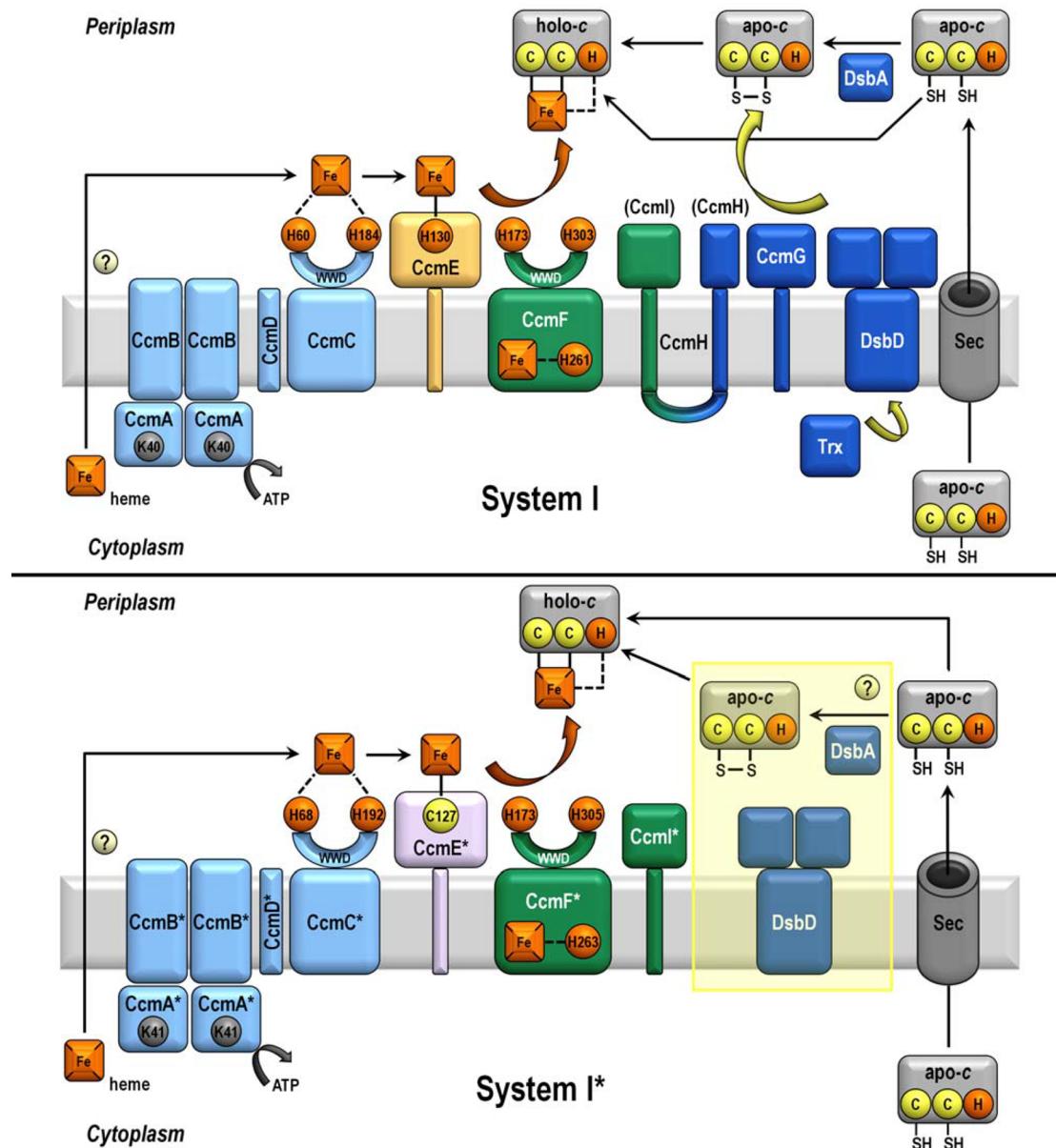
the surprising property of binding heme covalently, via a conserved histidine residue, before transferring the heme to the apocytochrome.<sup>16</sup> The large membrane protein CcmF is thought to transfer the heme from CcmE to the apocytochrome and to be involved in the formation of the thioether bonds between the heme and the cytochrome cysteines;<sup>17–19</sup> the mechanism is also not known. CcmG is proposed to provide reductant to the apocytochrome CXXCH heme-binding motif<sup>20,21</sup> should the latter become oxidized to form a disulfide in what is considered to be an oxidizing cellular compartment. In some organisms, including *Escherichia coli*, the last two proteins produced from the operon are a single polypeptide, referred to as CcmH (as indicated in Figure 1). CcmHI contains a pair of conserved cysteines and a protein–protein interaction domain, suggesting a chaperone-like activity and a possible role in thiol–disulfide isomerization.<sup>22</sup>

As essential roles for all of the Ccm proteins have been established,<sup>23</sup> it was a surprise to find a variant system that had significant differences. A bioinformatics analysis identified a form of System I in the genomes of sulfate-reducing bacteria and in several archaea.<sup>24</sup> This system is known as System I\* and is illustrated for comparison in the lower panel of Figure 1. (In this Article, the symbol \* is used to indicate a protein or gene from System I\*, and where it is absent the protein referred to is

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**Figure 1.** Scheme illustrating the main components of cytochrome *c* biogenesis Systems I (top) and I\* (bottom). The representations of Systems I and I\* are based on the model organisms *E. coli* and *D. alaskensis*, respectively. In both cases, apocytochrome *c* is synthesized in the cytoplasm and transported across the cytoplasmic membrane by the Sec apparatus. Heme is also synthesized in the cytoplasm, but its transport route to the point of assembly is unknown (shown by a question mark). Proteins responsible for sourcing and providing the heme (CcmA–D) to the heme chaperone (CcmE) are in light blue. CcmC ligates the heme through two histidine residues (shown in orange). The ATPase CcmA promotes the release of the heme from CcmC via a conformational change, presumably through CcmB. CcmE (yellow in System I and lilac in System I\*) is the pivotal protein for cytochrome *c* biogenesis and is one of the major points of difference between the two apparatuses. In System I, it binds heme covalently via a histidine residue (shown in orange), whereas in System I\* the key residue is a cysteine (shown in yellow). In both cases, the covalent bond between the heme and CcmE needs to break for the heme to covalently attach to the CXXCH motif of the apocytochrome. CcmF and CcmI (shown in green) are thought to facilitate the heme transfer to cytochrome *c*; histidine residues (shown in orange) in CcmF are involved in heme ligation. Proteins affecting the oxidation state of the cysteines of the apocytochrome are in dark blue. In System I, the interplay between DsbA and DsbD/CcmG/CcmH is well studied.<sup>48</sup> Oxidation of the CXXCH motif by DsbA is reversed through the action of DsbD, CcmG, and CcmH. In System I\*, the equivalents of CcmG and CcmH are absent, and the need for reduction of the CXXCH motif is still unclear (shown by the light-yellow box in the bottom panel). It is not possible to eliminate the possibility of the presence of an extracytoplasmic reductant, like CcmG, by bioinformatics methods. In most System I-containing organisms, CcmH and CcmI are two different membrane-anchored proteins; in *E. coli*, CcmH and CcmI are fused into one protein referred to as CcmH.

from System I). The Ccm operon from *Desulfovibrio alaskensis* has been used as a model for the variant System I\*.<sup>1</sup> Between the publication of ref 1 and the realization of this work, this species has been reclassified. It was formerly *Desulfovibrio desulfuricans*. The main differences compared to System I are

the absence of the protein CcmH (the N-terminal domain of the fused protein found in *E. coli*), the absence of CcmG, and a CcmE protein that binds heme covalently via a cysteine residue rather than the histidine of System I. The overall sequence similarity between representatives of proteins common to the

two systems (*E. coli* vs *D. alaskensis*) is about 60% (identity ~30%).

We have demonstrated that System I\* can perform cytochrome *c* maturation in *E. coli* lacking its endogenous Ccm proteins.<sup>1</sup> However, replacement of the crucial cysteine residue of CcmE\* by a histidine within the System I\* apparatus results in the absence of any *c*-type cytochrome. It is currently unclear how the two assembly systems function differently, but one can assume that complementary differences must exist to balance the replacement of a histidine with a cysteine on the heme chaperone and the lack of any specific thiol-disulfide oxidoreductase components in System I\*. It is not axiomatic that identified functional features of System I proteins will also be important in System I\*. Any similarities or differences between the two systems can be expected to provide complementary clues about events that lead to *c*-type cytochrome synthesis. In this work, we have replaced amino acids in CcmA\*, CcmC\*, CcmE\*, CcmF\*, and CcmF; the observed effects give new perspectives into the function of the more widely distributed System I, particularly regarding how the heme is chaperoned between the biogenesis proteins and finally to the apocytochrome.

## EXPERIMENTAL PROCEDURES

**Construction of Plasmids.** The plasmids used in this study are listed in Table 1. pCC12–15 were produced by site-directed mutagenesis (QuikChange, Stratagene) using plasmid pEC86 as a template. pMC01–08 and pMC1/3 were also produced by site-directed mutagenesis of plasmid pDD86.

**Table 1. Plasmids Used in This Work**

name	description	source
pbS62R98CY101C	<i>E. coli</i> cytochrome <i>b</i> <sub>562</sub> carrying the R98C/Y101C mutations in <i>b</i> <sub>562</sub> , Amp <sup>R</sup>	ref 45
pKPD1	<i>P. denitrificans</i> cytochrome <i>c</i> <sub>550</sub> , Amp <sup>R</sup>	ref 46
pEC86	<i>E. coli</i> <i>ccmABCDEFGH</i> , Cam <sup>R</sup>	ref 47
pDD86	<i>D. alaskensis</i> <i>ccmEFABCDI</i> , Cam <sup>R</sup>	ref 1
pCC09	pEC86 carrying the P146A mutation in <i>ccmF</i> , Cam <sup>R</sup>	this work
pCC10	pEC86 carrying the N145A/P146A/F147A mutations in <i>ccmF</i> , Cam <sup>R</sup>	this work
pCC14	pEC86 carrying the H173A mutation in <i>ccmF</i> , Cam <sup>R</sup>	this work
pCC15	pEC86 carrying the H261A mutation in <i>ccmF</i> , Cam <sup>R</sup>	this work
pMC01	pDD86 carrying the H68A mutation in <i>ccmC</i> , Cam <sup>R</sup>	this work
pMC02	pDD86 carrying the K41D mutation in <i>ccmA</i> , Cam <sup>R</sup>	this work
pMC03	pDD86 carrying the H192A mutation in <i>ccmC</i> , Cam <sup>R</sup>	this work
pMC01/3	pDD86 carrying the H68A/H192A mutations in <i>ccmC</i> , Cam <sup>R</sup>	this work
pMC04	pDD86 carrying the Y131A mutation in <i>ccmE</i> , Cam <sup>R</sup>	this work
pMC05	pDD86 carrying an alanine insertion after C127 in <i>ccmE</i> , Cam <sup>R</sup>	this work
pMC06	pDD86 carrying the H173A mutation in <i>ccmF</i> , Cam <sup>R</sup>	this work
pMC07	pDD86 carrying the H263A mutation in <i>ccmF</i> , Cam <sup>R</sup>	this work
pMC08	pDD86 carrying the P147A mutation in <i>ccmF</i> , Cam <sup>R</sup>	this work
pMC12	pDD86 carrying the K41D mutation in <i>ccmA</i> and C127H mutation in <i>ccmE</i> , Cam <sup>R</sup>	this work

pMC12 was generated from pMC02. DNA manipulations were conducted using standard methods. KOD Hot Start DNA polymerase (Novagen) was used for all PCRs, and all constructs were sequenced before use.

**Cell Growth.** Experiments were performed in the wild-type *E. coli* strain JCB387<sup>25</sup> or EC06,<sup>26</sup> which lacks the *ccm* operon and therefore is unable to produce holocytochromes *c*. The *E. coli* *ccm* operon or its variants was constitutively expressed from plasmid pEC86 (or pEC78, pCC12–15) (Table 1). The *D. alaskensis* *ccm* operon or its variants was constitutively expressed from plasmid pDD86 (or pMC01–08, pMC1/3) (Table 1). A *c*-type cytochrome variant of *E. coli* cytochrome *b*<sub>562</sub> bearing a CXXCH motif (*c*-*b*<sub>562</sub>) was coexpressed with the *E. coli* *ccm* operon (or its variants) from plasmid pbS62R98CY101C (Table 1). *Paracoccus denitrificans* cytochrome *c*<sub>550</sub> was coexpressed with the *D. alaskensis* or the *E. coli* *ccm* operons (or their variants) from plasmid pKPD1. Cell growth was conducted in 200 mL of 2× TY medium (16 g L<sup>-1</sup> of peptone, 10 g L<sup>-1</sup> of yeast extract, and 5 g L<sup>-1</sup> of NaCl) in 2.5-liter flasks. Cultures were inoculated from single colonies and incubated at 37 °C for 15–18 h with shaking at 200 rpm. 1 mM isopropyl-1-thio-β-D-galactopyranoside was added to the cultures from inoculation. 100 µg mL<sup>-1</sup> of ampicillin and 34 µg mL<sup>-1</sup> of chloramphenicol were used when appropriate.

**Characterization of the Cytochrome *c*<sub>550</sub> Content of Periplasmic Fractions.** For the extraction of periplasmic fractions, cells were harvested and sphaeroplasted as described.<sup>27</sup> At least six replicates of each experiment were conducted. The production of cytochrome *c*<sub>550</sub> was assessed by SDS-PAGE analysis followed by densitometry of heme-staining bands. SDS-PAGE analysis was carried out on 10% 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol (BisTris) NuPAGE gels (Invitrogen) with MES-SDS running buffer, prepared according to Invitrogen specifications. Prestained protein markers were used (Invitrogen, SeeBlue Plus 2). Samples were denatured at 100 °C for 2 min. Gels were stained for the presence of covalently bound heme according to the method of Goodhew.<sup>28</sup> Gel loadings were normalized according to wet cell-pellet weights. Densitometry was used to quantify cytochrome *c*<sub>550</sub> production (when appropriate) using GeneSnap (Syngene). The linear relationship between the amount of mature cytochrome *c* present on the gel and the amount detected by densitometry was ensured by using subsaturated loading of the gels.<sup>1</sup>

**Analysis of Heme Proteins in Membrane Fractions.** For the isolation of the crude membrane fractions, a French press was used. Disruption of the cells was performed at 16 000 p.s.i. followed by centrifugation at 257 000g for 1 h at 4 °C. The membrane fraction was resuspended in ~25 mL of 50 mM Tris-HCl, 150 mM NaCl (pH 7.5) and was recentrifuged as above. The washed crude membrane fraction was resuspended in 1 to 2 mL of 50 mM Tris-HCl, 150 mM NaCl (pH 7.5). At least two replicates of each experiment were conducted. The production of holo-CcmE, holo-CcmE\*, and cytochrome *c*-*b*<sub>562</sub> was assessed by SDS-PAGE analysis followed by densitometry of heme-staining bands (when appropriate) as described above; in this case, samples were denatured by incubation at 42 °C for 5 min, and gel loadings were normalized according to the total protein content determined using the Pierce BCA Reducing Agent Compatible Protein Assay Kit (ThermoScientific).

We expect, on the basis of the many related amino acid replacements in the proteins of System I, that the variants studied here do not have significantly altered expression levels

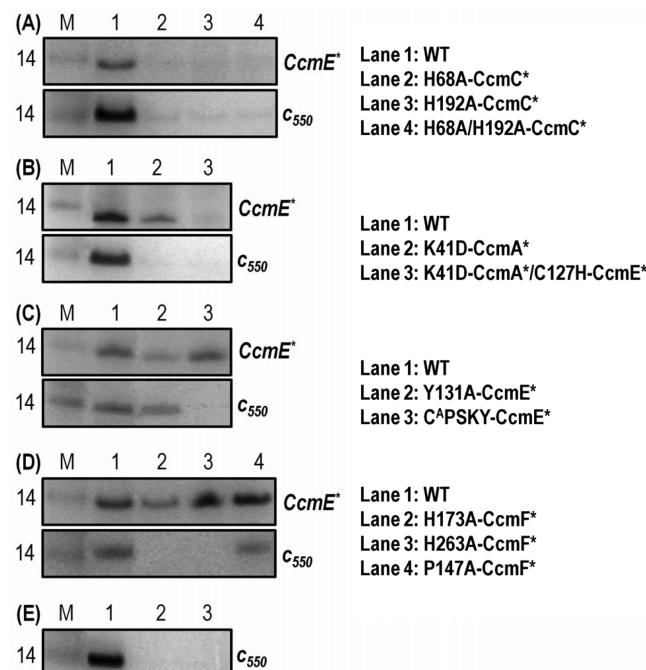
and stability. For example, the Walker A motif replacement in CcmA does not affect the stability of the ATPase subunits.<sup>12,13</sup> Many CcmE variants have also been unaffected by mutation;<sup>29,30</sup> this is not unexpected because the changes were made in the C-terminal domain of the protein, a region demonstrated to be unstructured.<sup>31</sup> In addition, attempts to raise antibodies against the membrane-embedded System I proteins have failed, and the insertion of affinity tags (e.g., Streptavidin II tag) for immunodetection has affected the function of the proteins (unpublished data).

## RESULTS

Mutations were made in a plasmid containing the *ccm* operon of *D. alaskensis* that expresses the proteins constitutively in *E. coli* lacking its *ccm* genes, enabling the resultant strain to produce heterologous holocytochromes *c*.<sup>1</sup> The functions of the variant operons were analyzed for their content of holo-CcmE\* in membrane preparations (shown in the top gel strip of each panel in Figure 2) as well as for their ability to mature a cytochrome *c* in the periplasmic fraction (shown in the lower gel strip in each panel). The variant proteins are described below in the order in which they are thought to be involved in heme handling. Heme first binds to CcmC and is then transferred to CcmE with the aid of CcmAB and CcmD. The heme is finally transferred from CcmE to the apocytochrome by CcmF. Control experiments are shown in Figure 2E: lane 1 shows wild-type System I\* producing holocytochrome *c*<sub>550</sub> that reveals a heme-staining band at the expected mass (~14 kDa) and lanes 2 and 3 show that no such band is detected when the plasmids expressing the cytochrome or the biogenesis operon are absent. Table 2 summarizes the comparative levels of heme attachment to CcmE\* and cytochrome *c* for each System I\* variant examined.

**CcmC\*.** CcmC has been shown to ligate a heme molecule via two conserved histidine residues (H60 and H184 in *E. coli* CcmC),<sup>14,15</sup> which are required for holo-CcmE formation. The two corresponding histidines are H68 and H192 in CcmC\*. We have examined the effect of removal of either (H68A and H192A) or both (H68A/H192A) of these side chains. The effects on holo-CcmE\* production and holocytochrome *c* formation are shown in Figure 2A. Holo-CcmE was not detectable to a significant level in the membranes for the single or the double mutants. As expected, when there is no heme on CcmE\*, no cytochrome *c* was detected in any of the three cases (lanes 2–4). The wild-type levels are shown in lane 1 and they run as expected when compared with the marker proteins indicated on the left of the gels. Both histidine residues appear to be essential for ligating the heme on CcmC\* for it to become covalently bound to CcmE\*.

**CcmA\*.** CcmA has been shown to hydrolyze ATP,<sup>12</sup> and the replacement of a lysine residue (K40 in *E. coli* CcmA) in the Walker A motif abolishes its function in cytochrome *c* maturation.<sup>12,13</sup> The equivalent replacement in CcmA\* is K41D, and its effect was examined here (Figure 2B). The loss of ATP-hydrolysis activity, assumed to be caused by an inability to bind to the nucleotide, abolished the formation of holocytochrome *c* (lane 2 lower strip; wild-type levels are shown in lane 1). Holo-CcmE\* was detected in the membrane at a reproducibly lower level (~35%) with the K41D CcmA variant than the wild-type (lane 2 upper strip). A construct was also produced that expressed both the variant CcmA\* and a replacement of the heme-binding cysteine residue (C127) in CcmE\* with histidine (as found in the classical System I). No



**Figure 2.** Representative SDS-PAGE of cell membranes or periplasmic fractions stained for proteins containing covalently bound heme. The detection of holo-CcmE\* or holo-*c*<sub>550</sub> was performed on washed cell membranes or periplasmic fractions, respectively. In all experiments, wild-type or variants of System I\* were coexpressed with *P. denitrificans* cytochrome *c*<sub>550</sub> in EC06 *E. coli* cells (cells lacking the endogenous *ccm* operon). The lane order is as follows. Panel A: (M) Molecular weight marker (as indicated, in kDa) (1) cells expressing System I\* and *c*<sub>550</sub>, (2) cells expressing (H68A-CcmC\*)-System I\* and *c*<sub>550</sub>, (3) cells expressing (H192A-CcmC\*)-System I\* and *c*<sub>550</sub>, and (4) cells expressing (H68A/H192A-CcmC\*)-System I\* and *c*<sub>550</sub>. Panel B: (M) Molecular weight marker, (1) cells expressing System I\* and *c*<sub>550</sub>, (2) cells expressing (K41D-CcmA\*)-System I\* and *c*<sub>550</sub>, and (3) cells expressing (K41D-CcmA\*/C127H-CcmE\*)-System I\* and *c*<sub>550</sub>. Panel C: (M) Molecular weight marker, (1) cells expressing System I\* and *c*<sub>550</sub>, (2) cells expressing (Y131A-CcmE\*)-System I\* and *c*<sub>550</sub>, and (3) cells expressing (C<sup>A</sup>PSKY-CcmE\*)-System I\* and *c*<sub>550</sub>. Panel D: (M) Molecular weight marker, (1) cells expressing System I\* and *c*<sub>550</sub>, (2) cells expressing (H173A-CcmF\*)-System I\* and *c*<sub>550</sub>, (3) cells expressing (H263A-CcmF\*)-System I\* and *c*<sub>550</sub>, and (4) cells expressing (P147A-CcmF\*)-System I\* and *c*<sub>550</sub>. Panel E: (M) Molecular weight marker, (1) cells expressing System I\* and *c*<sub>550</sub>, (2) cells expressing System I\* with no cytochrome-expressing plasmid, and (3) cells expressing *c*<sub>550</sub> and no cytochrome *c* biogenesis system.

holo-CcmE\* was observed in this case (K41D-CcmA\*/C127H-CcmE\*; comparing lanes 2 and 3 of the top strip of Figure 2B), as expected because C127H-CcmE\* does not acquire heme when the rest of the wild-type System I\* proteins are present.<sup>1</sup>

**CcmE\*.** Some mutations in CcmE from *E. coli* have given insight into the way in which the protein interacts with heme. Two studies have demonstrated that the conserved tyrosine residue four residues away from the heme-binding histidine (i.e., H<sup>130</sup>DENY<sup>134</sup>) is involved in ligating the heme iron in isolated CcmE, although Y134 is not essential for *c*-type cytochrome formation.<sup>30,32,33</sup> We have therefore replaced the equivalent residue in CcmE\* (C<sup>127</sup>PSKY<sup>131</sup>) with alanine (Y131A). The consequence is shown in Figure 2C, lane 2. A decrease in the amount of holo-CcmE\* was observed (~35% compared with the wild-type shown in lane 1), and an

**Table 2. Levels of Cytochrome  $c_{550}$  and Holo-CcmE\* Produced in *E. coli* EC06 Using Wild-Type or Variants of *D. alaskensis* System I\***

variant	level of maturation	
	holo-CcmE*	cytochrome $c_{550}$
wild type (CcmEFABCDI*)	100	100
H68A-CcmC*	— <sup>b</sup>	—
H192A-CcmC*	—	—
H68A/H192A-CcmC*	—	—
K41D-CcmA*	35	—
K41D-CcmA*/C127H-CcmE*	—	—
C <sup>A</sup> PSKY-CcmE*	100	—
Y131A-CcmE*	35	35
H173A-CcmF*	55	—
H263A-CcmF*	125	—
P147A-CcmF*	100	90

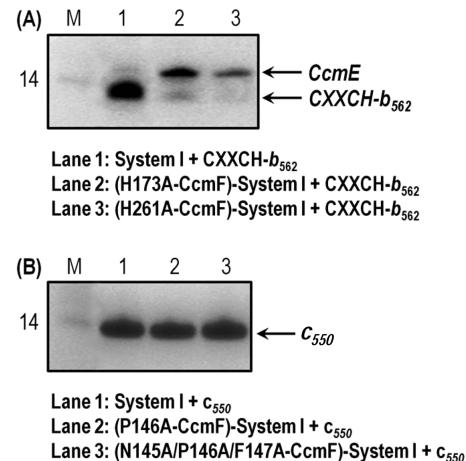
<sup>a</sup>The levels of cytochrome  $c_{550}$  in periplasmic fractions and holo-CcmE\* in membrane fractions were determined by SDS-PAGE analysis followed by densitometry on heme-stained gels. The amount of cytochrome  $c_{550}$  or holo-CcmE\* produced by the wild-type operon is arbitrarily set to 100. The calculation of the errors for the data presented in this table can be found in the Supporting Information (Table S1). <sup>b</sup>Insignificant levels of maturation as detected by densitometry.

equivalent drop in cytochrome  $c_{550}$  maturation was also recorded (Table 2 and Figure 2C). A previous study of *E. coli* CcmE employed the strategy of alanine-insertion mutagenesis to determine the importance of the relative position of the heme-binding histidine to the other residues in the C-terminal domain of CcmE.<sup>30</sup> In a similar experiment in this work, an alanine was inserted into CcmE\* on the C-terminal side of the heme-binding cysteine, yielding a C<sup>A</sup>PSKY motif. Lane 3 of Figure 2C (upper strip) shows that holo-CcmE\* is produced at levels equivalent to the wild-type CcmE\* (lane 1). No holocytochrome, however, was detected (lane 3, lower strip).

**CcmF\*.** CcmF has a WWD motif (see Figure 1) that is proposed to interact with heme, via hydrophobic interactions, on the periplasmic side of the membrane (this heme molecule is transferred from CcmE to the cytochrome). Histidine residues in periplasmic loops in CcmF have also been implicated in ligating this heme.<sup>34</sup> Unexpectedly, purified CcmF has been found to contain heme bound to its transmembrane regions; this heme also has histidines ligating it, forming a second heme-binding site.<sup>35</sup> In this work, we have replaced in CcmF\* one of each of these histidine pairs with alanine and determined the effect on holo-CcmE\* formation and holocytochrome *c* production. In Figure 2D, lane 2 shows the effect of replacement of the histidine (H173), which is thought to interact with heme in the periplasmic loop. A decrease in the amount of holo-CcmE\* was observed compared to the wild-type in lane 1. Removal of the heme-ligating side chain in position 263 (this corresponds to the proposed ligand for the membrane-embedded CcmF heme) did not decrease the amount of holo-CcmE\* in the membranes. Instead, a small increase was observed. No holocytochrome *c* was produced when either of the histidines were absent (lanes 2 and 3 on the lower strip of Figure 2D).

**CcmF.** We performed the corresponding mutations in CcmF in the *E. coli* *ccm* operon for comparison and expressed constitutively the wild-type or variant forms of System I with a

c-type variant of cytochrome  $b_{562}$  ( $c\text{-}b_{562}$ ). The latter has been a useful test substrate for cytochrome *c* maturation and has been shown to be processed readily by System I, expressing abundant bona fide *c*-type cytochrome.<sup>36</sup> The large quantities of  $c\text{-}b_{562}$  produced result in a significant amount of the protein remaining bound to the membrane fraction, even after extensive washing.<sup>37</sup> This procedure allowed us to detect both holo-CcmE and the mature  $c\text{-}b_{562}$  in the membrane fraction by staining for covalently bound heme (Figure 3A) and



**Figure 3.** Panel A: Representative SDS-PAGE of washed cell membranes stained for proteins containing covalently bound heme. In all experiments, wild-type or variants of System I were coexpressed with *E. coli* variant cytochrome  $c\text{-}b_{562}$  in JCB387 *E. coli* cells. It was possible to detect holo-CcmE and holo- $c\text{-}b_{562}$ , although  $c\text{-}b_{562}$  is a soluble periplasmic protein, bands arise from holo- $c\text{-}b_{562}$  that remained bound to the membranes even after washing. The lane order is as follows: (M) Molecular weight marker (as indicated, in kDa), (1) cells expressing System I and  $c\text{-}b_{562}$ , (2) cells expressing (H173A-CcmF)-System I and  $c\text{-}b_{562}$ , and (3) cells expressing (H261A-CcmF)-System I and  $c\text{-}b_{562}$ . Panel B: Representative SDS-PAGE of periplasmic fractions stained for proteins containing covalently bound heme. In all experiments, wild-type or variants of System I were coexpressed with *P. denitrificans* cytochrome  $c_{550}$  in EC06 *E. coli* cells. The lane order is as follows: (M) Molecular weight marker, (1) cells expressing System I and  $c_{550}$ , (2) cells expressing (P146A-CcmF)-System I and  $c_{550}$ , and (3) cells expressing (N145A/P146A/F147A-CcmF)-System I and  $c_{550}$ .

to provide a comparison with the experiments above with CcmF\*. Replacement of H173 and H261 with alanine led to the accumulation of holo-CcmE (at greater amounts than the wild-type system although the holo-CcmE accumulation in the H173A variant is much greater compared to the H261A variant) and loss of cytochrome *c* production.

A quinone-binding site has been proposed in *E. coli* CcmF<sup>34</sup> because of partial sequence similarity to a site known to interact with quinones in other proteins.<sup>38</sup> It is accepted that the heme that becomes attached to apocytochromes must be in the reduced form, whereas a CcmE heme-attachment mechanism that would result in oxidation of the heme iron of CcmE has been proposed.<sup>39</sup> A bound quinol could be a source of reductant for the membrane-bound heme in CcmF, which would in turn reduce the heme bound to CcmE. The putative quinol-binding site is N<sup>145</sup>PF, with the proline being the most highly conserved of the residues. We replaced the proline with alanine, and no effect on cytochrome *c* production was observed, as shown in Figure 3B, lane 2, compared with the

wild-type level seen in lane 1. All three residues of the putative quinol-binding site were then replaced in case the other residues were important for the proposed interaction (N145A/P146A/F147A); these changes also did not alter the level of holocytochrome that matured (lane 3). Replacement of the equivalent conserved CcmF\* proline was also performed in the System I\* plasmid for comparison. Figure 2D (lane 4) shows that holocytochrome levels are slightly reduced (~90% of wild-type levels) by the P147A CcmF\* replacement (compared with the wild-type level in lane 1).

## DISCUSSION

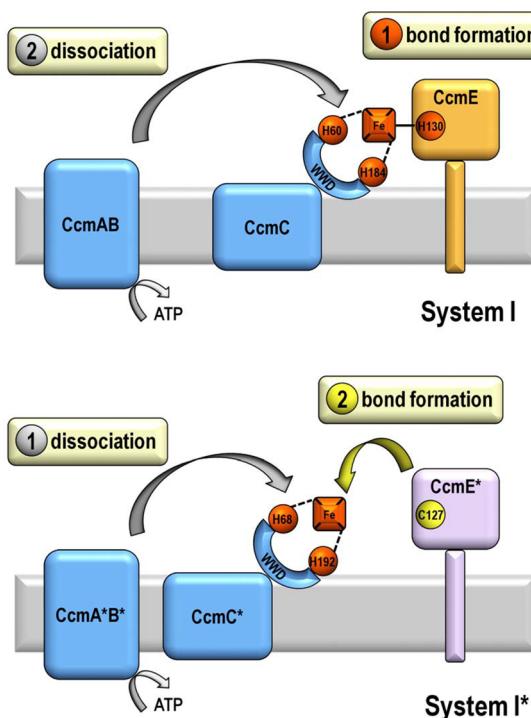
The effects of the mutations described in this work, along with published data on System I, have allowed us to improve our understanding of the process of heme chaperoning from the point of its delivery to the cytochrome *c* assembly site to its attachment to the substrate apocytochrome.

**The Role of CcmABC in Providing Heme to CcmE.** In System I, after its transfer across the membrane, heme is first ligated by two histidine residues (strong iron ligands) on CcmC,<sup>15,40</sup> as illustrated in Figure 4 (step 1, top panel). The heme chaperone CcmE is able to associate with the heme,

which is fixed to CcmC, forming a tightly bound complex. The affinity of CcmE for heme-bound CcmC does not originate from its key histidine residue because it was shown that even when the histidine was replaced with alanine the complex still forms.<sup>15</sup> The formation of the covalent bond between the histidine of CcmE (H130) and a vinyl group of the heme occurs while the heme is still ligated by CcmC; Richard-Fogal et al. observed that a purified CcmC–heme–CcmE complex stained for covalently bound heme.<sup>15</sup> It has been proposed that the mechanism of formation of the covalent heme-histidine bond could be a Michael addition or an imidazole cation radical mechanism.<sup>39</sup> In both cases, the proximity of the attacking group (the imidazole ring of the histidine of CcmE) to the vinyl group of the heme would be crucial. Previous work removing one or both CcmC histidines led to a drastic decrease in holo-CcmE formation and abolished cytochrome *c* maturation, demonstrating the vital role of the histidine ligands for the acquisition and transfer of heme.<sup>15</sup> The occurrence of the CcmC–heme–CcmE complex is integral to the covalent attachment of heme to CcmE. The naturally low reactivity of the bonding histidine toward the heme vinyl would be compensated by fixing the heme near the site of assembly. For the next step of the maturation process to happen (the formation of the intermediate complex between CcmE, heme, and the apocytochrome<sup>37</sup>), CcmA is essential. An ATP-driven conformational change in CcmA, presumably via CcmB, is responsible for removing the heme from CcmC (Figure 4, step 2, top panel). This has been confirmed by the K40D mutation in the Walker A motif of CcmA that abolishes the ATPase activity of the protein; holo-CcmE accumulates in the membrane fraction and no cytochrome *c* is produced.<sup>12,13</sup> The accumulated holo-CcmE is in complex with CcmC because the K40D-CcmA mutation also prevents formation of the CcmE–heme–apocytochrome complex.<sup>37</sup>

In System I\*, the replacement of one or both histidines of CcmC\* had dramatic effects: no holo-CcmE\* or holo-*c*<sub>550</sub> were detected. Therefore, we can conclude that the role of CcmC\* in the variant maturation system is equivalent to System I. CcmC\* ligates heme via H68 and H192. However, when the K41D-CcmA\* variant was examined, a significant decrease in the level of mature holo-CcmE\* in the membrane fraction was observed (~35% compared to wild-type levels, Figure 2B), which contrasts with the significant accumulation of holo-CcmE observed in the K40D-CcmA variant of System I.<sup>30</sup> This implies that in System I\* CcmA\* is required for optimal transfer of the heme from CcmC\* before, or concomitant to, the covalent attachment of CcmE\* to the cysteine (Figure 4, step 1, bottom panel). The cysteine side chain, with an ionizable thiol group close to physiological pH, is reactive and might be expected to act on the vinyl group of the heme via an electrophilic addition (Figure 4, step 2, bottom panel). In the same way as thioether bonds probably form in cytochromes *c*, in System I\* protonation of the heme vinyl, with the reactive cysteine acting as the proton donor, could initiate the attack. The requirement for association of heme-bound CcmC to CcmE, which in System I promotes the covalent bond formation on CcmE, is therefore not necessarily required in the case of System I\*. The decrease in the level of holo-CcmE\* that is matured by the K41D-CcmA\* System I\* could simply reflect the lower accessibility of the heme vinyl by the cysteine thiol of CcmE\* while it is still in complex with CcmC\*.

In general, both histidine and cysteine side chains show nucleophilic behavior in uncatalyzed reactions. Hemoglobin



**Figure 4.** Proposed mechanism of holo-CcmE formation in Systems I (top) and I\* (bottom). Covalent bonds involving the heme moiety are displayed by a solid line, and heme ligation is shown by dotted lines. The numbers indicate the sequence of events. In both systems, heme is transported across the cytoplasmic membrane and fixed at the point of cytochrome *c* assembly by ligation to two essential histidine residues (shown in orange) of CcmC. At this point, the heme chaperone CcmE of System I is able to associate with the heme and covalently bind it via a conserved histidine, forming a stable complex (point 1 of top panel). The ATP-fuelled action of CcmAB is required to dissociate CcmC from the heme (point 2 of top panel) and to allow CcmE to pass the heme to the apocytochrome. In System I\*, the action of CcmA\*B\* dissociates the heme from CcmC\* (point 1 of bottom panel) before the nucleophilic attack of the CcmE\* cysteine (shown in yellow) thiol to the vinyl group of the cofactor occurs.

from *Synechococcus* normally binds heme covalently via a histidine (we note that the histidine–heme bond in this protein occurs between the  $\alpha$ -carbon of the vinyl group and the histidine side chain instead of the  $\beta$ -carbon that binds to the histidine of CcmE). However, it was shown that hemoglobin can also bind heme covalently via a cysteine using a similar reaction mechanism.<sup>41</sup> The different CcmE heme-binding residues in Systems I and I\* lead to very different reaction requirements *in vivo*. The appropriate reaction conditions for heme attachment arise from the environment created by the maturation proteins in a catalyzed process that compensates for the different reactivity of histidine compared to cysteine. The low reactivity of the CcmE histidine is compensated by the high affinity of CcmE for heme-bound CcmC, leading to bond formation before the release of heme from CcmC and only if the CcmC–heme–CcmE complex occurs (accumulation of holo-CcmE in the cases where CcmA is absent or is compromised<sup>13</sup> is a natural consequence). However, the cysteine in CcmE\* is more reactive and initiates the covalent attachment. This is consistent with the decrease in holo-CcmE\* formation with the K41D-CcmA\* variant (the small amount of holo-CcmE\* detected (lane 2, top strip of Figure 2B) might still be in complex with CcmC\* and formed because the cysteine can attack the heme while it is ligated to CcmC\*). A comparable result has been obtained with System I when H130 was replaced by a cysteine.<sup>29</sup> With this mutation, one might expect that the presence of a reactive cysteine on apo-CcmE would promote the accumulation of holo-CcmE. Instead, an insignificant amount of holo-CcmE was detected. Thus, the chemical context for the covalent bond between heme and CcmE must be tailored for histidine in System I and cysteine in System I\*. A double mutation in System I\* supports this hypothesis: when the K41D-CcmA\*/C127H-CcmE\* mutation was examined, heme attachment to holo-CcmE\* was abolished. Although the C127H variant of CcmE\* does not acquire heme,<sup>1</sup> we wondered whether the lack of ATPase activity in CcmA\* might permit heme attachment to C127H-CcmE\* because the putative driving force for disrupting the CcmC–heme–CcmE complex was absent. However, no covalently bound heme was detected on CcmE\*, confirming that the reactivity of the cysteine thiol, and not the proximity to heme-bound CcmC\*, is the driving force for the formation of holo-CcmE\*. In both variants (single and double) of System I\*, no holocytochrome *c* was detected. In the case of the K41D-CcmA\* variant, this would be because any holo-CcmE\* that formed was still trapped in complex with CcmC\*.

**Heme Binding to CcmE.** Mutations in CcmE\* itself support the model in Figure 4. Previously, insertion of an alanine residue into the heme-binding site of CcmE ( $H^A$ DENY) had detrimental effects on heme attachment: no heme-containing CcmE was detected and as a result no cytochrome *c* was matured.<sup>30</sup> In System I\*, an equivalent insertion ( $C^A$ PSKY) did not affect the maturation level of CcmE\*. This could be because attachment of heme to CcmE\* depends on only the propensity for cysteine–heme chemistry. In System I, the chemistry of attachment of heme to the histidine depends not only on the ligation to CcmC but also on the formation of the CcmC–heme–CcmE complex. As explained above, the affinity of CcmE for heme-bound CcmC does not originate from the histidine residue<sup>15</sup> but is more related to the heme-binding pocket of CcmE. Surprisingly, the alanine insertion in CcmE\* abolished cytochrome *c* maturation. This could be because further protein–protein interactions (with the

apocytochrome or the remaining maturation proteins) depend on the integrity of the heme-binding site. Y134 in CcmE has been found to be important but not essential,<sup>30</sup> and a similar result was obtained in this work in System I\* for the equivalent Y131 in CcmE\*. The Y131A-CcmE\* mutation decreased the level of heme attachment to CcmE\* and  $c_{550}$  to ~35% of wild-type levels. The decrease in the level of holo-CcmE\* is mirrored in the level of cytochrome *c* production. As with System I, the tyrosine might ligate the heme in CcmE\*, but because heme ligation in CcmE is known to be dynamic<sup>42</sup> and can accommodate alternative ligands, removal of the tyrosine (Y131A) does not abolish function.

**CcmF Interactions with Heme.** Previous work on the identified heme-ligating histidines of CcmF did not show what effect removal of these histidines would have on the level of holo-CcmE formation.<sup>34</sup> It has been proposed that CcmF-bound heme could provide reductant to the heme of CcmE (which was proposed to become oxidized during the heme attachment process) that is essential for the release of the mature holocytochrome by a reverse Michael addition. Our results show that in both Systems I and I\* the histidine involved in binding the membrane-embedded heme of CcmF (H261 or H263, respectively) is important in the production of holocytochrome *c*. Removal of this residue leads to the slight accumulation of holo-CcmE (or holo-CcmE\*) and the lack of any holocytochrome. Therefore, it is possible that the heme of CcmF plays a role in the release of the mature substrate. The H173A mutation gave different results in the two systems. In System I, it caused significant holo-CcmE accumulation and complete lack of cytochrome *c*, pointing once more to a role of this residue in the release of the mature substrate. However, the H173A-CcmF\* mutation, although equally detrimental to cytochrome *c* maturation, halved the level of holo-CcmE\* production. This implies that H173 of CcmF\* might also be involved in holo-CcmE\* formation. It could be that H173 acts as a ligand for the heme after its release from CcmC\*, securing it for interaction with CcmE\* and putting it into place for the next step. The lack of mature  $c_{550}$  (instead of a ~50% level that would be consistent with the level of holo-CcmE) implies an important role for the ligation of the heme of CcmE by CcmF but also for release of holocytochrome.

Previous work proposed a putative quinone-binding site in CcmF as part of a mechanism for acquiring electrons that would be then transferred to the heme bound to CcmE and allow the release of holocytochrome.<sup>34</sup> In this work, we replaced conserved amino acids in the putative binding sites (NPF) in CcmF proteins in Systems I and I\*. In neither case was cytochrome *c* production abolished, suggesting that interaction with quinols/quinones at this site is not essential for the function of CcmF. If the membrane-embedded heme of CcmF does provide electrons to CcmE, then it would have to have other ways of obtaining reducing power from the cytoplasm. However, we note that although there are suggestions in the literature that the heme cofactor could become oxidized as a result of the heme attachment process,<sup>15,34,35,39</sup> the only experimental demonstration for this is that purified CcmC–heme–CcmE complex or holo-CcmE are 50% oxidized.<sup>15</sup> Heme-containing species, such as holo-CcmE and cytochromes *c*, are readily oxidized. Holo-CcmE with a measured midpoint reduction potential of  $-121\text{ mV}^{30}$  would be rapidly oxidized by atmospheric oxygen during purification.

## CONCLUSIONS

By examining a naturally occurring variant of a well-studied but cryptic cytochrome *c* biogenesis system, we have been able to shed light onto the requirements for heme chaperoning from one protein in the pathway to the next. Heme transfer is dictated by differential affinities during protein–protein interactions as well as by modulating ligation properties and reactivity of residues responsible for heme binding. Elucidating how the heme is driven preferentially between these proteins to the final substrate apocytochrome is central to our understanding of the overall pathway. There are parallel questions in other systems, with one example being how bacterial heme acquisition proteins are able to bind extracellular heme and transfer it, via receptors, to intracellular proteins.<sup>43</sup> Another example is the use of ATP hydrolysis by chaperone proteins (e.g., Hsp90) to drive heme insertion into cytosolic apo hemoproteins.<sup>44</sup> In each of these cases, the binding properties and interactions of the proteins involved need to ensure that the heme reaches its final target, especially given the potential toxicity of heme when free in solution.

## ASSOCIATED CONTENT

### Supporting Information

Calculation of the errors for the data presented in Table 2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS

Ccm, Cytochrome *c* maturation; ABC, ATP-binding cassette; *c*-*b*<sub>562</sub>, cytochrome *b*<sub>562</sub> bearing a CXXCH motif

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