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Heme transfer to the heme chaperone CcmE during cytochrome *c* maturation requires the CcmC protein, which may function independently of the ABC-transporter CcmAB

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ABSTRACT **Cytochrome *c* maturation in *Escherichia coli* requires the *ccm* operon, which encodes eight membrane proteins (CcmABCDEFGH). CcmE is a periplasmic heme chaperone that binds heme covalently and transfers it onto apocytochrome *c* in the presence of CcmF, CcmG, and CcmH. In this work we addressed the functions of the *ccmABCD* gene products with respect to holo-CcmE formation and the subsequent ligation of heme to apocytochrome *c*. In the absence of the *ccmABCD* genes, heme is not bound to CcmE. We report that CcmC is functionally uncoupled from the ABC transporter subunits CcmA and CcmB, because it is the only Ccm protein that is strictly required for heme transfer and attachment to CcmE. Site-directed mutagenesis of conserved histidines inactivates the CcmC protein, which is in agreement with the hypothesis that this protein interacts directly with heme. We also present evidence that questions the role of CcmAB as a heme exporter; yet, the transported substrate remains unknown. CcmD was found to be involved in stabilizing the heme chaperone CcmE in the membrane. We propose a heme-trafficking pathway as part of a substantially revised model for cytochrome *c* maturation in *E. coli*.**

Heme is the cofactor of proteins involved in a variety of biological processes such as transport of oxygen, oxygenation, electron transfer, and transcriptional regulation. Because of its high hydrophobicity, heme tends to aggregate and bind nonspecifically to biological membranes. Little is known as to how heme is translocated through membranes. Numerous pathogenic bacteria can use heme as a source of iron and/or porphyrin by taking it up via a TonB-dependent heme-acquisition system (1, 2), which argues against the possibility that heme is transported simply by diffusion.

We are interested in how heme is incorporated into *c* type cytochromes during the biogenesis of this class of proteins. Heme attachment generally involves the formation of two thioether bonds between the vinyl groups at positions 2 and 4 of the tetrapyrrole and the cysteine thiols of the apocytochrome *c* sequence motif CXXCH, the heme-binding site. In *Escherichia coli*, this reaction is believed to take place in the periplasm, after both heme and the apocytochrome have been translocated through the cytoplasmic membrane (3–6). We have shown recently that heme is bound transiently to the periplasmic heme chaperone CcmE during cytochrome *c* maturation (7). CcmE is an intermediate of the heme-trafficking pathway because it appears to bind heme covalently by a single histidine and to release it in the presence of apocytochrome *c* and other Ccm proteins. Neither the chemical nature of the heme-histidine linkage nor the mechanisms of how heme binds to, and detaches from, CcmE are understood. In an attempt to characterize the requirements for heme binding to CcmE, we have addressed the question of whether the *ccmABCD* genes are involved in this process. It has been postulated that *ccmABC* encode the subunits of an ABC transporter with a $(CcmA)_2$-CcmB-CcmC composition, perhaps including the small CcmD protein as well (8, 9). Although the idea that such a transporter might be involved in heme export to the periplasm was attractive (3–6), no experimental support for it has been presented. By contrast, the finding that *ccmABC* mutants were capable of producing periplasmic cytochrome *b* (10, 11) and that the failure to produce *c* type cytochromes could not be restored by adding extracellular heme to such mutants rather argued against such a hypothesis (8). Here, we report that CcmAB and CcmCD have separate functions: the CcmAB proteins represent an ABC transporter, and the CcmCD proteins participate in heme transfer to CcmE. The present models for cytochrome *c* maturation thus need to be revised.

MATERIALS AND METHODS

Strains and Plasmids. *E. coli* K-12 strain DH5α (12) was used as host for clonings, and strain MC1061 (13) was used for CcmE and cytochrome *c* expression and analysis. In-frame deletion mutations in *ccmA*, *ccmB*, *ccmC*, *ccmD*, and *ccmF* were constructed by removing 114 internal codons of *ccmA* (coding for S^{35} to W^{148}), 147 internal codons of *ccmB* (Q^{44}–D^{190}), 94 internal codons of *ccmC* (A^{63}–G^{156}), and 76 internal codons of *ccmF* (P^{168}–D^{243}) and by deleting the entire *ccmD* gene. A linker encoding the tripeptide YPG was inserted at the site of the Δ*ccmA* deletion, whereas a decamer linker was inserted in the case of the Δ*ccmC* mutant, which resulted in four new codons for an RRAC tetrapeptide at the site of deletion. Chromosomal in-frame deletion mutants were constructed by marker exchange mutagenesis by using pMAK705 (14). Plasmids encoding different combinations of *ccm* genes were constructed by inserting appropriate DNA fragments into the *Eco*RV site of pACYC184 (15) such that the *ccm* genes were expressed constitutively from the *tet* promoter. Construction of plasmids expressing *ccm* genes from an arabinose-inducible promoter on pISC-derivatives (7) involved amplification of DNA with the PCR by using Vent-Polymerase (New England Biolabs). Site-specific mutations in H_6-*ccmC*^{H60A} and H_6-*ccmC*^{H184A} were introduced by PCR. Detailed information on the construction of plasmids and strains listed in Table 1 is available from the authors on request.

Growth Conditions. Cells were grown aerobically in LB or anaerobically in minimal salts medium with 5 mM nitrite as electron acceptor (16). For the expression of *ccmC*, *ccmD*, and the *Bradyrhizobium japonicum* cytochrome c_{550}, *E. coli* cells

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Table 1. Strains and plasmids used in this work

Strain or Plasmid	Relevant genotype/resistance	Ref.
Strain		
MC1061	*hsdR mcrB araD139* Δ*(araABC-leu)7679* Δ*lacX74 galU galK rpsL thi*	13
EC06	Δ*ccm* derivative of MC1061; Km^R	19
EC21	Δ*ccmA*, (Δ35–148), derivative of MC1061	27
EC75	Δ*ccmB*, (Δ44–190), derivative of MC1061	This work
EC28	Δ*ccmC*, (Δ63–156), derivative of MC1061	27
EC76	Δ*ccmD*, (Δ1–69), derivative of MC1061	This work
EC65	Δ*ccmE*, (Δ3–95), derivative of MC1061	7
EC50	Δ*ccmF*, (Δ168–243), derivative of MC1061	27
EC29	Δ*ccmG*, (Δ23–114), derivative of MC1061	29
Plasmid		
pEC86	*ccmABCDEFGH* cloned into pACYC184; Cm^R	33
pEC94	$ccmA_{\Delta 35-148}BCDEFGH$ cloned into pACYC184; Cm^R	This work
pEC406	*ccmCDE* cloned into pACYC184; Cm^R	This work
pEC408	*ccmDE* cloned into pACYC184; Cm^R	This work
pEC409	*ccmCE* cloned into pACYC184; Cm^R	This work
pEC410	*ccmE* cloned into pACYC184; Cm^R	This work
pEC422	H_6-*ccmC* cloned into pISC-3; Ap^R	This work
pEC433	H_6-$ccmC^{H60A}$ cloned into pISC-3, Ap^R	This work
pEC436	H_6-$ccmC^{H184A}$ cloned into pISC-3; Ap^R	This work
pRJ3268	*B. japonicum cycA* cloned into pISC-2; Ap^R	19
pRJ3291	*B. japonicum cycA* cloned into pISC-2; Km^R	This work

Km^R, Cm^R, and Ap^R, kanamycin, chloramphenicol, and ampicillin resistance, respectively.

were grown to midexponential phase and then induced with 0.4% arabinose.

Cell Fractionation and Analysis. Whole-cell protein analysis, isolation of periplasmic and membrane fractions, determination of protein concentration, and heme staining after SDS/PAGE were performed as described previously (7, 17–19). Western blot detection was performed with CcmE-specific antiserum (7) and with mAbs directed against a tetra-His epitope (Qiagen).

RESULTS

CcmC Is Necessary and Sufficient to Attach Heme Covalently to CcmE. CcmE is an intermediate of the heme-trafficking branch of the cytochrome *c* maturation pathway, which accumulates in its heme-binding form to detectable levels when the *ccm* operon is overexpressed and cytochrome *c* maturation is blocked at a later step. CcmE binds heme covalently at a conserved histidine, H130, and this reaction occurs in the periplasm. Subsequently, CcmE acts as a heme donor for ligation to apocytochrome *c* (7).

Covalent heme attachment to CcmE itself takes place in the absence of the *ccmFGH* genes (7). Overexpression of *ccmE* alone, however, leads to a heme-less CcmE protein, suggesting that at least one of the *ccmABCD* gene products was required for heme trafficking to CcmE (Fig. 1, lane 1). Thus, holo-CcmE formation can be used as an assay to study the requirements for *c* type cytochrome-specific heme transport to the periplasm. The question of whether an intact ABC transporter is required for heme attachment to CcmE was addressed by complementing the Δ*ccm* mutant EC06 (19, 20) with plasmids

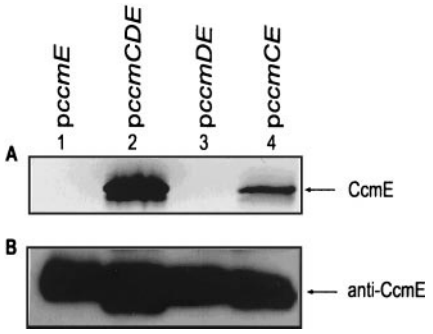

FIG. 1. Requirements of *ccm* gene products for covalent incorporation of heme into CcmE. *E. coli* cells were grown anaerobically in the presence of nitrite. The Δ*ccm* mutant EC06 was complemented with plasmids expressing different combinations of *ccmC*, *ccmD*, and *ccmE*. (*A*) Activity stain for covalently bound heme of membrane protein fractions (50 μg per lane) separated by SDS/15% PAGE. (*B*) Western blot of the same membrane protein fractions (30 μg per lane) as in *A* probed with anti-CcmE serum. Lanes: 1, EC06 Δ*ccm* complemented with pEC410 (*ccmE*); 2, pEC406 (*ccmCDE*); 3, pEC408 (*ccmDE*); 4, pEC409 (*ccmCE*).

expressing *ccmE* constitutively together with *ccmC* and/or *ccmD*, but in the absence of *ccmAB* (Fig. 1, lanes 2–4). An 18-kDa, heme-stainable membrane protein corresponding to holo-CcmE could be detected only when *ccmC* also was coexpressed (Fig. 1*A*, lanes 2 and 4). No heme binding to CcmE was detected in the absence of *ccmC* (Fig. 1*A*, lanes 1 and 3). Conversely, the presence of *ccmD* was not required for the formation of heme-bound CcmE under these steady-state conditions (Fig. 1*A*, lane 4). A Western blot of the same membrane fractions was probed with antiserum directed against CcmE to prove that almost equal amounts of CcmE were present in all lanes (Fig. 1*B*). These results clearly show that in this type of experiment CcmC is necessary and sufficient for covalent incorporation of heme into CcmE.

The Function of CcmC Is Separable from That of CcmAB. The finding that CcmC alone already was sufficient for heme incorporation into CcmE prompted us to test whether holo-CcmE formation also was affected in chromosomal in-frame deletion mutants. To increase the sensitivity of holo-CcmE detection it was necessary to introduce a plasmid-borne copy of *ccmE* (pEC410; Table 1) into the individual deletion strains. Membrane fractions of the wild type and of Δ*ccmE*, Δ*ccmF*, and Δ*ccmG* mutants containing pEC410 produced heme-binding CcmE (Fig. 2*A*, lanes 1 and 6–8). However, the Δ*ccmA*, Δ*ccmB*, and Δ*ccmC* mutants did not produce detectable amounts of holo-CcmE (lanes 2–4), and the Δ*ccmD* mutant produced lower levels of it (lane 5). Western blot analysis of the same proteins revealed similar amounts of CcmE polypeptide in all strains (Fig. 2*B*). These results confirmed that CcmC is essential for heme binding to CcmE. Surprisingly, in this experimental setting *ccmA* and *ccmB* also were required for holo-CcmE formation. This appeared to contradict our findings (cf. Fig. 1) that *ccmA* and *ccmB* are not needed for heme binding to CcmE. We therefore investigated whether an enhanced production of CcmC suppresses the mutant phenotype of the chromosomal Δ*ccmA* and Δ*ccmB* mutants. This was done by coexpressing *ccmC* plus *ccmE* on a plasmid in these strains. Fig. 3*A* shows that high levels of CcmC indeed compensate the otherwise deficient holo-CcmE formation in the Δ*ccmA* and Δ*ccmB* mutants (lanes 2 and 3).

We also assessed the effect of an absence of *ccmA* and *ccmB* on cytochrome *c* production. *E. coli* synthesizes up to five *c* type cytochromes under nonfermentative, anaerobic conditions of growth with nitrate, nitrite, or trimethylamine *N*-oxide (TMAO) as the terminal electron acceptors (16). It was shown previously that a deletion removing the genes *ccmA* to *ccmH*

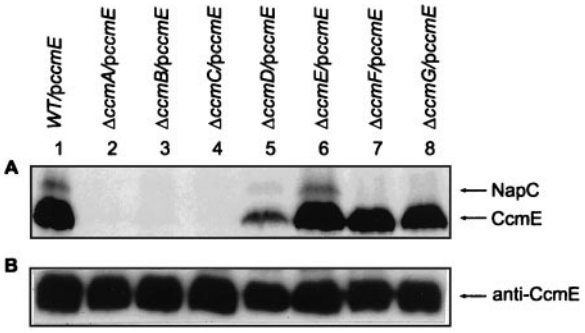

FIG. 2. Functional analysis of chromosomal in-frame *ccm* deletion mutants for formation of holo-CcmE and cytochrome *c*. *E. coli* cells were grown anaerobically in the presence of nitrite. To enhance the level of holo-CcmE detection, pEC410 expressing *ccmE* constitutively was present in all strains. (*A*) Membrane proteins (100 μg per lane) were separated by SDS/15% PAGE and stained for covalently bound heme. The following strains containing pEC410 were analyzed. Lanes: 1, MC1061; 2, Δ*ccmA*; 3, Δ*ccmB*; 4, Δ*ccmC*; 5, Δ*ccmD*; 6, Δ*ccmE*; 7, Δ*ccmF*; 8, Δ*ccmG*. The positions of CcmE and the NapC *c* type cytochrome are indicated on the right. (*B*) Western blot of the same membrane protein fractions (30 μg per lane) as in *A* probed with anti-CcmE serum.

from the *E. coli* chromosome resulted in a complete loss of mature *c* type cytochromes (19, 21). The periplasmic *B. japonicum* cytochrome c_{550} encoded by *cycA* can be expressed in *E. coli* from plasmid pRJ3268 upon induction with arabinose (21). Wild-type and various Δ*ccm* mutant cells were transformed with pRJ3268 and grown under anaerobic conditions with nitrite as terminal electron acceptor to ensure expression of the *ccm* operon and the structural genes *napBC* for the *c* type cytochromes of the periplasmic nitrate reductase. After induction of *cycA* expression, holocytochrome *c* formation was analyzed by heme staining of periplasmic proteins that were separated by SDS/15% PAGE. Cells lacking either of the *ccm* genes were unable to form holocytochrome *c* (not shown). When plasmid pEC409 (Table 1) expressing *ccmCE* was used to complement Δ*ccmC*, Δ*ccmD*, and Δ*ccmE* mutants, cytochrome *c* formation could be restored, as indicated by the presence of periplasmic NapB and cytochrome c_{550} (Fig. 3*C*, lanes 4–6). The Δ*ccmA* and Δ*ccmB* mutants, however, could not synthesize holocytochrome *c* (Fig. 3*C*, lanes 2 and 3), although they did produce heme-bound CcmE (Fig. 3*A*, lanes 2 and 3).

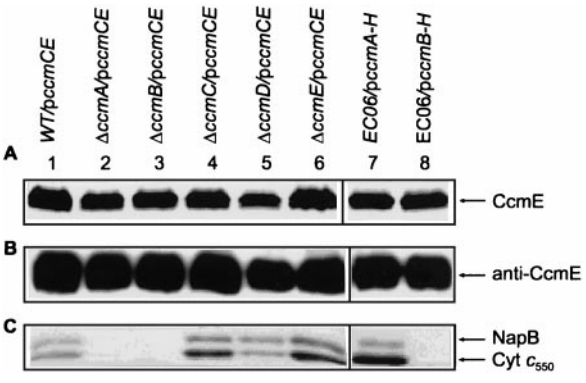

FIG. 3. Influence of CcmC abundance on cytochrome *c* maturation. Membrane fractions were prepared and analyzed as in Fig. 2. The strains in lanes 1–6 contained plasmid pEC409 expressing *ccmC* and *ccmE*. In lanes 7 and 8 the Δ*ccm* strain EC06 was complemented with pEC86 carrying *ccmABCDEFGH* and its Δ*ccmA* mutant derivative pEC94, respectively. In addition, all strains contained pRJ3268 expressing the *B. japonicum cycA* gene encoding cytochrome c_{550} (Cyt c_{550}). (*A*) Heme stain of membrane proteins (50 μg per lane). CcmE and NapC are indicated on the right. (*B*) Western blot of the same membrane protein fractions (30 μg per lane) as in *A* probed with anti-CcmE serum. (*C*) Heme stain of trichloroacetic acid-precipitated periplasmic proteins (20 μg in lanes 1–6 and 8; 3 μg in lane 7). NapB and cytochrome c_{550} are indicated on the right.

When pEC94 containing a *ccmA* in-frame deletion plus wild-type *ccmBCDEFGH* genes (Table 1) was used to complement the Δ*ccm* mutant EC06, heme binding to CcmE occurred, whereas cytochrome *c* maturation was blocked (Fig. 3 *A* and *C*, lane 8). This again reflects that overproduction of CcmC can bypass the need for CcmA with respect to holo-CcmE, but not holocytochrome *c* formation. Our results suggest that the ABC transporter is composed of the ATP-binding CcmA subunit plus the membrane-integral CcmB protein. The ABC transporter seems to be involved in the transport of a molecule that is required (*i*) for attachment of heme to CcmE in the case where CcmC is not abundant and (*ii*) for heme transfer from holo-CcmE and attachment to apocytochrome *c*. Hence, its function in cytochrome *c* maturation clearly is distinct from that of CcmC.

The Conserved Histidines in CcmC Are Required for Heme Transfer to CcmE. CcmC is a homologue of the *Rhodobacter capsulatus* HelC and the *Pseudomonas fluorescens* CcmC proteins, whose membrane topology has been mapped recently (22, 23). Accordingly, the CcmC protein family has six transmembrane helices, a tryptophan-rich motif in the second periplasmic loop, and two strictly conserved histidines in the first and third periplasmic loop. It has been postulated that the tryptophan-rich motif provides a hydrophobic interface for interaction with heme, with the two histidines operating as axial heme ligands (22).

Our finding that CcmC was required for heme transfer onto CcmE prompted us to test the importance of the respective histidines, H60 and H184. We changed them to alanines by site-directed mutagenesis of the *ccmC* variant that codes for the H_6-tagged protein and is expressed under the control of the arabinose-inducible promoter. The Δ*ccmC* mutant containing a plasmid encoding either wild-type or H60A or H184A CcmC variants was cotransformed with pRJ3291 for cytochrome c_{550} expression (Table 1). After induction with arabinose, the formation of holocytochrome *c* was analyzed. Although wild-type CcmC and H_6-CcmC could complement the cytochrome *c* deficiency of the Δ*ccmC* mutant (Fig. 4*A*, lanes 1 and 2), no cytochrome *c* formation was obtained with H_6-CcmC^{H60A} and H_6-CcmC^{H184A} (Fig. 4*A*, lanes 3 and 4). This clearly shows that the two conserved histidines of H_6-CcmC are required for cytochrome *c* maturation.

We also tested the effect of the two point mutations on holo-CcmE formation. The Δ*ccm* mutant EC06 was cotransformed with the plasmid expressing *ccmDE* constitutively and plasmids encoding either of the two different CcmC derivatives. Membrane protein fractions were separated by SDS/15% PAGE and stained for covalently bound heme (Fig. 4*B*). Both H60 and H184 of CcmC were found to be required for the incorporation of heme into CcmE (Fig. 4*B*, lanes 3 and 4). Western blot analysis with CcmE-specific (Fig. 4*C*) and H_6-tag-specific (Fig. 4*D*) antibodies showed that equal amounts of CcmE were present and that the mutant CcmC derivatives were stable. Our data are in agreement with the hypothesis of a direct interaction between CcmC and heme.

CcmD Influences the Level of CcmE in the Membrane. CcmD is a small polypeptide of 69 aa with a predicted molecular mass of 7,745 Da. It was shown that the *Rhodobacter capsulatus* homologue HelD, a putative membrane protein with an N-terminal transmembrane helix, has a COOH-terminus oriented toward the cytoplasm (9).

We observed that an *E. coli ccmD* mutant in a background in which the other *ccm* genes were overexpressed from a tetracycline promoter upstream of *ccmA* (7) allowed the production of small amounts of cytochrome *c* (not shown). This suggested that the lack of CcmD could be compensated

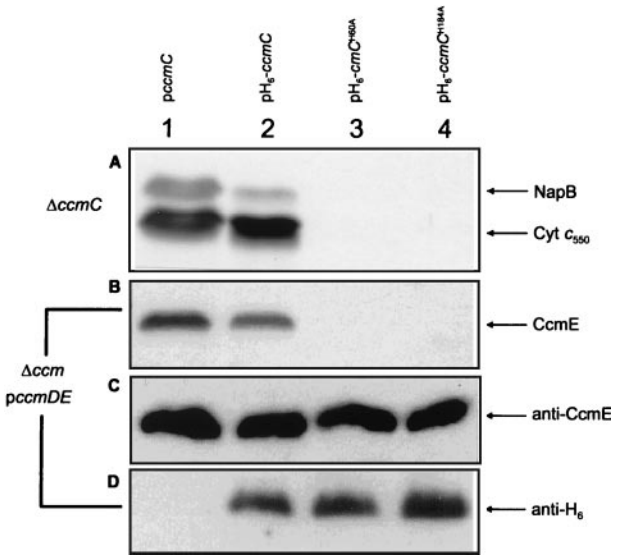

FIG. 4. Phenotypic characterization of mutants with H60A and H184A exchanges of H_6-CcmC. (*A*) The Δ*ccmC* mutant EC28 was cotransformed with plasmids pEC432 (*ccmC*; lane 1), pEC422 (H_6-*ccmC*; lane 2), pEC433 (H_6-*ccmC*^{H60A}; lane 3), pEC436 (H_6-*ccmC*^{H184A}; lane 4), and pRJ3291 (*B. japonicum cycA)*. Cells were grown anaerobically in the presence of nitrite. Periplasmic proteins (50 μg per lane) were separated by SDS/15% PAGE and stained for covalently bound heme. (*B*) The Δ*ccm* mutant EC06 expressing *ccmDE* constitutively from plasmid pEC408 was cotransformed with plasmid pEC432 (*ccmC*; lane 1), pEC422 (H_6-*ccmC*; lane 2), pEC433 (H_6-*ccmC*^{H60A}; lane 3), and pEC436 (H_6-*ccmC*^{H184A}; lane 4). Membrane proteins (50 μg per lane) were separated by SDS/15% PAGE and stained for covalently bound heme. (*C*) Western blot of the same membrane fractions (30 μg per lane) as in *B* probed with antiserum directed against CcmE. (*D*) Identical Western blot probed with a monoclonal antiserum directed against a tetrahistidine epitope.

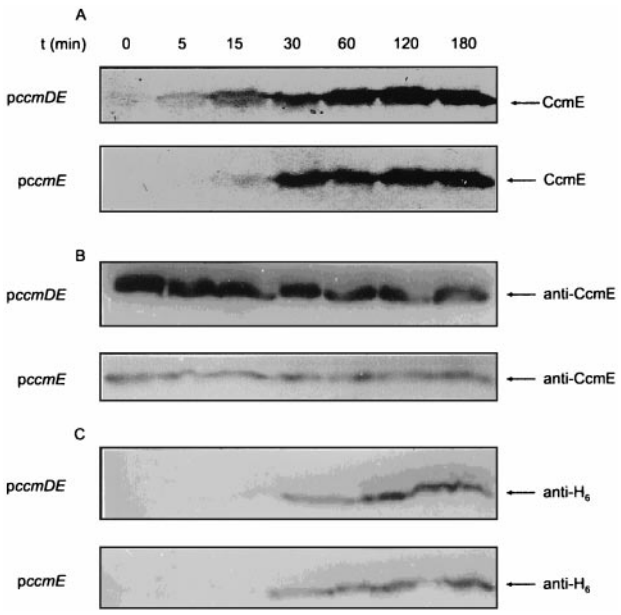

FIG. 5. Kinetics of heme binding to CcmE upon production of H_6-CcmC. The experiment has been repeated at least five times, and similar results have been obtained. The Δ*ccm* mutant EC06 was transformed with plasmids pEC408 or pEC410 to constitutively express either *ccmD* plus *ccmE* or *ccmE* alone, respectively. These strains were cotransformed with plasmid pEC422 encoding H_6-CcmC, whose production was induced with arabinose when cells had reached an OD_{600} of 0.7. At different time points 0.5 ml of cells diluted to OD_{600} = 0.7 were precipitated with trichloroacetic acid and separated by SDS/15% PAGE. (*A*) Activity stain for covalently bound heme. (*B*) Western blot of the same samples as in *A* probed with antiserum directed against CcmE. (*C*) Identical Western blot probed with a monoclonal antiserum directed against a tetrahistidine epitope.

by an enhanced production of other Ccm proteins and that CcmD might have a role in assembly or stability of the latter.

To test specifically whether CcmD is involved in the incorporation of heme into CcmE, the kinetics of this process was investigated *in vivo*. This was done by cotransformation of the Δ*ccm* mutant EC06 with plasmids constitutively expressing either *ccmE* alone, or *ccmD* plus *ccmE* together, and a plasmid expressing H_6-*ccmC* from an arabinose-inducible promoter. H_6-*ccmC* can complement a Δ*ccmC* mutant to wild-type levels (Fig. 4, lanes 1 and 2). Identical amounts of cells were precipitated with trichloroacetic acid at different time points after induction, and proteins were separated by SDS/15% PAGE and stained for covalently bound heme. Holo-CcmE was detected 5 min after induction, when both CcmD and CcmE were present (Fig. 5*A Upper*). In the absence of CcmD, Holo-CcmE was detected several minutes later (Fig. 5*A Lower*). This result suggested that CcmD either facilitates incorporation of heme into CcmE or stabilizes the CcmC and/or CcmE proteins in the membrane. This latter possibility was examined by Western blot analysis of identical samples as in Fig. 5*A*. Parallel blots were probed with anti-CcmE serum (Fig. 5*B*) and with monoclonal tetrahistidine antibodies recognizing H_6-CcmC (Fig. 5*C*). Clearly, CcmE was more abundant in the presence of *ccmD* expression than in its absence (compare Fig. 5*B Upper* and *Lower*). The kinetics of membrane incorporation of H_6-CcmC was not dependent on *ccmD* (Fig. 5*C*). One possible interpretation of this result is that CcmD acts by stabilizing CcmE in the membrane.

DISCUSSION

We have shown recently that CcmE is an obligate intermediate of the heme-trafficking pathway that operates in *E. coli* cytochrome *c* maturation (7). Because heme is bound to CcmE in the periplasm, the formation of holo-CcmE can be used to monitor efficient translocation of heme into the periplasm. This allowed us to determine the minimal requirements for covalent heme attachment to CcmE in the periplasm. CcmC was shown to be necessary and sufficient to incorporate heme into CcmE. According to computer predictions and topological mapping of the homologous *R. capsulatus* HelC and *P. fluorescens* CcmC proteins (22, 23), CcmC is an integral membrane protein with six transmembrane helices and conserved periplasmic elements, i.e., a WGXXWXWD motif in the second periplasmic loop and two invariant histidines in the first and third periplasmic loop. These elements have been postulated to be involved in an interaction of the protein with heme. The H60A and H184A exchanges in the *E. coli* CcmC protein abolished heme attachment to CcmE, which supports the idea that CcmC interacts directly with heme. Presently, we cannot distinguish whether CcmC is required for translocation of heme through the membrane or whether it donates periplasmic heme to CcmE, perhaps by catalyzing the formation of the covalent histidine–heme linkage.

Our data necessitated a change in the current view of both the structure and function of the ABC transporter required for cytochrome *c* maturation. Despite missing experimental support, a model has persisted in the literature that predicted that heme was transported to the periplasm by an ABC transporter composed of the products of the *ccmABC(D)* genes (3–6, 8, 9, 22, 24, 25). The CcmA protein is undoubtedly a member of the ABC transporter family because it contains the ATP-binding site; however, the other proteins, CcmB and/or CcmC, have been only tentatively assigned as subunits of the transporter. Our finding that a strain lacking CcmA and CcmB is able to form heme-binding CcmE (provided that substantial amounts

of CcmC are available), whereas a strain lacking CcmC is not, shows unambiguously that CcmC cannot be a subunit of the CcmAB transporter. The latter, therefore, ought to have a $(CcmAB)_2$ structure (26). The disparate functions of CcmAB and CcmC also might be a reflection of the physical separation of their respective genes, *ccmAB* and *ccmC*, by a gap of 41 bp, whereas all other *ccm* genes are translationally coupled by overlapping start and stop codons. CcmC also has been reported to be essential for pyoverdine production in *P. fluorescens*, and some amino acids identified as being required for this ability do not overlap with those required for holocytochrome *c* formation (23). CcmC may be able to recognize and transfer different hydrophobic, organic iron complexes.

Despite the inability of Δ*ccmA* and Δ*ccmB* mutants to produce holo-CcmE, several lines of evidence indicate that CcmAB is not a heme exporter. (*i*) The transfer of periplasmic heme onto CcmE seems to require either CcmAB or enhanced levels of CcmC. One possible explanation is that CcmC can be activated for heme transfer by a transported substance that promotes oligomerization or specific protein–protein or protein–heme interactions, which, alternatively, can be facilitated when CcmC is overproduced. It is unlikely that this substance is heme, because substantial amounts of heme are bound to CcmE in this situation. We cannot exclude the formal possibility that heme translocation through the membrane occurs normally via CcmAB, but high abundance of CcmC allows heme to cross the membrane in the absence of CcmAB. Yet, such an explanation would question the need for CcmAB at all. (*ii*) It was demonstrated previously that several Δ*ccm* mutants, including a Δ*ccmA* mutant, can form periplasmic holocytochrome *b* (27), supporting the view that heme translocation to the periplasm is *ccm*-independent. (*iii*) The parallel occurrence of holo-CcmE and lack of holocytochrome *c* in the Δ*ccmA* mutant complemented with plasmid-borne *ccmC* indicates that the substance translocated by the ABC transporter also is involved in a step after heme transfer onto CcmE, i.e., the attachment of heme to apocytochrome *c*. Two obvious questions remain to be answered in this context. (*i*) How is heme translocated to the periplasm? It appears as if heme is translocated either by CcmC in an ABC transporter-independent way or by an entirely Ccm-independent system. (*ii*) What then is the substrate of the ABC transporter encoded by *ccmAB*? It emerges from our studies that besides the compounds to be transported during cytochrome *c* maturation (heme, apocytochrome), there seems to be a requirement for an additional component that has not yet been discovered. One possibility is a reductant that keeps heme reduced before and after binding to CcmE. It is known that cytochrome *c* maturation requires the presence of reduction equivalents in the oxidative environment of the periplasm to keep the cysteines of the heme-binding site in the apocytochrome reduced (28–31). A Δ*ccmA* in-frame deletion mutant was not found to complement cytochrome *c* maturation after the addition of various reductants such as cysteine, glutathione, or 2-mercapto-ethanesulfonic acid (R.A.F., unpublished data). Thus, the question of what kind of a molecule the transported substrate is remains open.

Another requirement for obtaining functional CcmE appears to be CcmD. This small protein is thought to be anchored in the membrane by a hydrophobic N terminus, facing the cytoplasm with a positively charged, C-terminal domain (9). We have investigated its role in the kinetics of heme incorporation into CcmE. Rather than influencing the heme transfer, CcmD appears to influence the level of the CcmE polypeptide in the membrane. Interestingly, in the *P. fluorescens* strain 09906, the CcmD and CcmE sequences are fused as a single polypeptide (32), which suggests a close physical interaction of the two individual proteins also in *E. coli*.

Our findings can be compiled in a revised model for cytochrome *c* maturation, which takes into account that the CcmAB ABC transporter may not be involved in heme export, but in another step of cytochrome *c* maturation, and that CcmC is essential for heme transfer to the CcmE heme chaperone. The linear pathway in which cytochrome *c* maturation is organized consists of two main branches, a polypeptide-trafficking branch and a heme-trafficking branch. The former includes the *sec*-dependent translocation of apocytochrome *c* and a periplasmic redox system consisting of CcmG and CcmH, which guarantees that the cysteines of the apocytochrome heme-binding site are kept reduced to allow the formation of the thioether bonds. The heme-trafficking branch involves CcmC that transfers heme onto CcmE and, to some extent, the ABC transporter CcmAB. This transporter appears to translocate a factor that also is needed for cytochrome *c* maturation after the apocytochrome has been released into the periplasm and holo-CcmE has been formed. This factor may participate in heme trafficking to and from CcmE, perhaps by keeping the heme iron reduced. It is possible that CcmABCD function in a supramolecular complex, with CcmAB and CcmD playing an accessory and CcmC playing an obligatory role in making heme available to CcmE. The two branches are joined in the last step of the cytochrome *c* biogenesis, the heme ligation that may be catalyzed by CcmF. Heme ligation remains as the least-understood reaction of the entire pathway.

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