Physical Interaction of CcmC with Heme and the Heme Chaperone CcmE during Cytochrome c Maturation

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Running title: CcmC interacts with heme and CcmE

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SUMMARY

Biogenesis of c-type cytochromes requires the covalent attachment of heme to the apoprotein. In Escherichia coli, this process involves eight membrane proteins encoded by the ccmABCDEFGH operon. CcmE binds heme covalently and transfers it to apocytochromes c in the presence of other Ccm proteins. CcmC is necessary and sufficient to incorporate heme into CcmE. Here, we report that the CcmC protein directly interacts with heme. We further show that CcmC co-immunoprecipitates with CcmE. CcmC contains two conserved histidines and a signature sequence, the so-called tryptophan-rich motif, which is the only element common to cytochrome c maturation proteins of bacteria, archae, plant mitochondria and chloroplasts. We report that mutational changes of these motifs affecting the function of CcmC in cytochrome c maturation do not influence heme binding of CcmC. However, the mutants are defective in the CcmC-CcmE interaction, suggesting that these motifs are involved in the formation of a CcmC-CcmE complex. We propose that CcmC, CcmE and heme interact directly with each other, establishing a periplasmic heme delivery pathway for cytochrome c maturation.

Keywords: co-immunoprecipitation / cytochrome c maturation / heme binding protein / heme chaperone / heme transport
INTRODUCTION

C-type cytochromes are electron transfer proteins that carry covalently-bound heme as a prosthetic group. During the last decade there has been an increasing interest in their biogenesis, which involves the transport of both the apoprotein and heme across membranes and their covalent ligation (1-4). Three different systems for the maturation of c-type cytochromes have been identified in living cells (eubacteria, plant and protist mitochondria chloroplasts) (2,4,5). In system I and II, cytochrome c maturation proteins contain at least one membrane protein with several membrane-spanning segments that possesses the well-conserved, tryptophan-rich sequence motif WGXXWXWD in an extracytoplasmically oriented domain, which represents the only signature common to cytochrome c maturation factors of all three kingdoms. By contrast, system III operating in mitochondria of animals and fungi does not involve such a protein.

In gram-negative bacteria, the synthesis of heme and apocytochrome c takes place in the cytoplasm, whereas the covalent attachment of heme to the conserved cysteines in the CXXCH heme binding site of the polypeptide is a periplasmic process. This key step of cytochrome c maturation referred to as heme ligation occurs after both heme and the apocytochrome have been translocated across the cytoplasmic membrane (6,7). In Escherichia coli apocytochrome c is translocated via the general type II secretion (sec) pathway of protein translocation to the periplasm (7). However, no system for directed translocation of heme between the cytoplasmic and periplasmic compartments has yet been described. Recently, it was shown that heme can be transported into everted membrane vesicles of E. coli by an energy-independent mechanism, but no evidence for a specific
heme export system was obtained (8).

In *E. coli*, eight genes essential for cytochrome c maturation (*ccmA-H*)\(^1\) have been identified (9,10). Analysis of mutants with deletions in individual *ccm* genes showed that each of the gene products is essential for c-type cytochrome biogenesis (11-15). A key player in the maturation pathway has been identified in the heme chaperone CcmE that binds heme covalently at a single histidine residue and subsequently transfers it to apocytochrome c (11). The attachment of heme to CcmE requires the function of another Ccm protein, CcmC, and can be improved by the small, integral membrane protein CcmD, which apparently stabilizes CcmE in the membrane (12). It was also reported that CcmC of *Pseudomonas fluorescens* and *Paracoccus denitrificans* had an additional function in the biogenesis and/or secretion of the iron chelator pyoverdine (16,17).

The membrane topology of the *Rhodobacter capsulatus* CcmC homologue HelC (18) and the *P. fluorescens* CcmC (19) has been established. CcmC contains six transmembrane helices, separated by two cytoplasmic and three periplasmic loops. A conserved tryptophan-rich motif is present in the second periplasmic loop, which is flanked by two conserved histidines located in the first and third periplasmic loops (3,4,7,20). In *E. coli*, site-directed mutagenesis of each of these histidines (12) and the tryptophan-rich motif (21) affected the function of CcmC. It has been speculated that the tryptophan-rich motif of CcmC provides a hydrophobic platform for the interaction of CcmC with heme, with the two histidines operating as axial heme ligands (18,21). However, no biochemical approach has been used to support this hypothesis. Here, we tested whether CcmC is a heme binding protein and

\(^1\) Ccm, cytochrome c maturation; δ-ALA, δ-aminolevulinic acid; TCA, trichloroacetic acid; CSPD, 3-\(\|\)4-methoxyspiro[1.2-dioxethan-3,2’,(5’chloro)tricyclo(3.3.1.1\(3,7\))decan]-4-yl\(\|\) phenyl-phosphate; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; Cyt c\(_{550}\), cytochrome c\(_{550}\).
investigated the prediction that CcmC interacts directly with CcmE in the course of the heme transfer.
EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* C600ΔhemA::kan and pFR2 were gifts from C. Wandersman. pFR2 contains the hasR gene of *Serratia marcescens*, which can be expressed in *E. coli* upon induction with 0.02% arabinose (22). The EC28ΔhemA mutant was constructed by P1 transduction (23) of the *E. coli* strain C600ΔhemA::kan by selection for kanamycin resistance, followed by testing the requirement of δ-ALA on minimal medium containing glycerol as non-fermentable C-source (24).

Bacteria were grown aerobically in LB medium or anaerobically in minimal salts medium (25) supplemented with 0.4% glycerol, 40 mM fumarate and 5 mM potassium nitrite as the terminal electron acceptor. For maximum holo-CcmE formation, cells were grown in minimal salts medium. Hemin was dissolved in 20 mM NaOH to a final concentration of 10 mM. Antibiotics were added at the following final concentrations: ampicillin, 200 µg/ml; chloramphenicol, 20 µg/ml; kanamycin, 50 µg/ml. For the expression of *ccmC*, cells were induced with 0.05% arabinose at mid-exponential growth phase.

Construction of plasmids

*E. coli* strain DH5α (26) was used as host for cloning. For construction of pEC705, which contains a double mutant *ccmC* allele (H60A, H184A), the 363-bp NsiI-NdeI fragment of pEC433 (12) was ligated to the 5.8 kb NsiI-NdeI fragment of pEC436 (12). To express *ccmC* W119A/(W122-D126)A from the inducible *Para* promoter, the 1.2-kb EcoRI-NsiI fragment of plasmid pEC477 (21) was cloned into the 5.7-kb EcoRI-NsiI fragment of
pEC422 (12), resulting in pEC706. pEC707, which contains the combination of H60A and W119A/(W122-D126)A of ccmC, was obtained by inserting 363-bp NsiI-NdeI fragment of pEC433 into 6.6-kb NsiI-NdeI fragment of pEC706. pEC708 expressing ccmC with W119A/(W122-D126)A and H184A and pEC709 expressing the ccmC H60A-W119A/(W122-D126)A-H184A referred to as triple mutant were constructed by QuickChange™ site-directed mutagenesis (Stratagene Europe-Switzerland, Amsterdam, NL) using pEC706 and pEC707, respectively, as templates. Primers 5’-ttggtgaacacctggctcaggggtcgacgc-3’ and 5’-gcgtcgacccctgagccagggtttccacca-3’ were purchased from Microsynth (Balgach, Switzerland). The mutant constructs were confirmed by DNA sequencing using an ABI Prism 310 Genetic Analyzer (Perkin Elmer).

Analysis of holo-CcmE from whole cells

500 µl (OD_{600} = 0.5) cells were precipitated with 250 µl TCA (30%) on ice for 15 min. and then centrifuged at 14,000 rpm, 4°C for 15 min. The pellets were resuspended in 13 µl 2 x SDS loading dye and neutralized with 5 µl 1 M Tris-HCl (pH 9.5). Holo-CcmE formation was analyzed after SDS-PAGE by heme stain (11).

Cell fractionation

Periplasmic fractions of 500 ml anaerobically grown cultures were isolated by treatment with polymyxin B sulfate (Sigma, Buchs, Switzerland). The cells were harvested by centrifugation at 4 000 g, washed with cold 50 mM Tris-HCl pH 8.0, and resuspended (2 ml/g wet cells) in cold extraction buffer (1 mg/ml polymyxin B sulphate, 20 mM Tris-HCl, 500 mM NaCl, 10 mM EDTA, pH 8.0). The suspension was stirred for 60 min at 4 °C and
centrifuged at 40 000 g for 20 min at 4 °C. The supernatant contained the periplasmic fraction.

Membrane fractions of 500 ml anaerobically grown cultures were prepared as follows. The cells were harvested by centrifugation at 4 000 g, washed with cold 50 mM Tris-HCl, pH 8.0, resuspended in 2 ml cold 50 mM Tris-HCl, pH 8.0 containing 10 µg/ml DNase I and passed three times through a French pressure cell at 110 MPa. Cell debris was separated by centrifugation at 40 000 g for 20 min at 4 °C. The supernatant was subjected to ultracentrifugation at 150 000 g for 60 min at 4 °C. The membrane fraction (pellet) was washed once with 1 ml cold Tris-HCl buffer (50 mM, pH 8.0) and resuspended in 200 µl of the same buffer.

**Biochemical methods**

Protein concentrations were determined using the Bradford assay (Bio-Rad).

Heme staining of proteins separated by SDS-PAGE was carried out using o-dianiside (Sigma) as substrate. The gel was incubated for 10 min in 10% TCA. After extensive washing with water, the gel was stained using 20 mg of o-dianisidine freshly dissolved in 20 ml of 50 mM tri-Na-citrate, pH 4.4, 0.7% H₂O₂ (11).

Immunoblot analysis of the histidine-tagged versions of CcmC (H₆-CcmC) was performed using monoclonal tetra-His antibodies (Qiagen) at a dilution of 1:2000. Antibodies directed against CcmE (11) were used at a dilution of 1:5000. Signals were detected using goat anti-mouse IgG or goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad), respectively, as secondary antibody and CSPD (Roche Diagnostics) as substrate.
Affinity chromatography of CcmC with hemin-agarose

150 µg total membrane proteins were solubilized at 4°C for 1 h in 1 ml of solubilization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5 mM PMSF, and 0.5% lauroylsarcosine). After centrifugation (15 000 g, 20 min., 4°C), the supernatant was incubated with 10 µl of hemin-agarose (Sigma, 7.7 µmol heme per ml of packed gel) on a rotating wheel at 30°C for 1 h. Aminododecyl-agarose beads (Sigma) were used as control to rule out non-specific interactions between membrane proteins and agarose. Agarose beads were washed three times with solubilization buffer and twice with PBS to remove unbound material. Agarose beads were then treated with 2 x SDS loading dye (containing 50 mM DTT) and incubated at room temperature for 20 min. The mixture was subjected to 12% SDS-PAGE. Proteins in the gel were visualized by immunoblot analysis.

Competition of free heme with hemin-agarose for CcmC

Membrane proteins (150 µg) were solubilized at 4°C for 1 h in 1 ml of solubilization buffer (see above). After centrifugation (15 000 g, 20 min., 4°C), the supernatant was incubated with different concentrations of heme: 0, 5 µM, 10 µM, 20 µM and 50 µM at 30°C for 30 min on a rotating wheel. Heme was dissolved in 20 mM NaOH (final concentration, 10 mM). The mixture was then centrifuged at room temperature, 15 000 g for 10 min. The supernatant was further incubated with 10 µl hemin-agarose at 30 °C on a rotating wheel for 1 h. The agarose beads were collected and washed. 30 µl SDS loading dye (containing 50 mM DTT) were added to resuspend the agarose beads. Samples were then incubated at room temperature for 20 min. The proteins (20 µl) were subjected to a 15% SDS-gel, transferred to a PVDF membrane and probed with antisera directed against the tetra-His-tag.
Co-immunoprecipitation of CcmC and CcmE

500 µg total membrane proteins were solubilized in solubilization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5 mM PMSF, and 1% n-dodecyl-β-D-maltoside) at 4°C for one hour. After centrifugation at 15,000 g for 20 min, 4°C, the supernatant was incubated with 5 µl CcmE antibodies or 10 µl tetra-His antibodies (0.2 mg/ml) at 4°C for 1.5 hours on a rotating wheel. 5 mg protein A sepharose (Sigma) was pretreated by washing twice with 1 ml HEPES (20 mM, pH 7.5), twice with 1 ml HNMG (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% n-dodecyl-β-D-maltoside, 10% glycerol). Protein A sepharose was then added to the membrane/antibody mixture, and the mixture was incubated at 4°C for 1.5 hours on a rotating wheel. The sepharose was precipitated by centrifugation at 15,000 g for 2 min., washed three times with 1 ml solubilization buffer, and once with 1 ml PBS. The proteins associated with the antibody/proteinA sepharose complex were treated with 2 x SDS-loading dye (containing 50 mM DTT) and incubated at room temperature for 20 min. The proteins were subjected to 12% SDS-gel, transferred to a PVDF membrane and probed with antisera against the tetra-His-tag or CcmE.
RESULTS

The conserved histidines in CcmC are not absolutely required for heme transfer to CcmE

Previously, it was shown that CcmC is sufficient to trigger heme binding to CcmE, and the conserved histidines as well as the tryptophan-rich motif of CcmC are required for heme transfer to CcmE (12). In an attempt to look for minor phenotypic variations between the different mutants, experimental conditions were improved such that detection of holo-CcmE became more sensitive (see Experimental Procedures). With the optimized conditions, we found that the individual histidines (H60 and H184) of CcmC are not absolutely required for heme transfer to CcmE. The Δccm mutant EC06, in which the ccmA-H genes are deleted (9), was co-transformed with plasmid pEC410 (pccmE) and with plasmids expressing different ccmC mutant alleles. Fig. 1A (left panel) shows that in the presence of wild-type CcmC heme was incorporated into CcmE (lane 2), whereas in the negative control (vector only) and in all tested ccmC mutants no heme attachment to CcmE was detected (lanes 1, 3-6). A different picture emerged when the Δccm mutant was transformed with pEC408 (pccmDE) and the plasmids expressing different ccmC mutant alleles. Fig. 1A (right panel) shows that in the presence of wild-type CcmC heme binding to CcmE was enhanced (lane 8), whereas in the negative control (vector only) no heme attachment to CcmE occurred (lane 7). In contrast to former results (12), upon enhanced expression of ccmD, the single CcmC histidine point mutants (H60A and H184A) showed some activity of heme attachment to CcmE (lanes 9 and 10). However, substitution of both histidines by alanines (H60A/H184A) abolished detection of heme-binding to CcmE (lane 11). As reported previously, mutations within the tryptophan-rich motif (W119A/(W122-D126)A) resulted in loss of heme transfer to CcmE at our level of detection (lane 12). When the 5-fold amount of membrane proteins

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was analyzed from these samples, the same results were obtained (not shown). These results demonstrate that the conserved histidines and the tryptophan-rich motif are necessary for holo-CcmE formation in the absence of CcmD. Expression of ccmD partially suppressed the phenotype of the single histidine mutants. The differing abilities of the ccmC mutants to form holo-CcmE observed in Fig. 1A were not due to different levels of CcmE or CcmC (Fig. 1B and 1C), but rather to different activities of CcmC mutant proteins.

The ability of the CcmC point mutants to support cytochrome c maturation was also tested. The periplasmic B. japonicum cytochrome c$_{550}$ (Cyt c$_{550}$) encoded by cycA can be expressed in E. coli from plasmid pRJ3291 upon induction with arabinose (12). E. coli strain EC28 (13), containing a ccmC in-frame deletion, was transformed with pRJ3291 and with plasmids expressing different ccmC alleles. Upon induction of cycA expression, holocytochrome c formation was analyzed by heme staining of periplasmic proteins. As shown in Fig. 1D, wild-type ccmC (lane 2) and the ccmC mutants containing the H60A or the H184A (lanes 3 and 4) were able to complement the ΔccmC mutant phenotype, although the mutants H60A and H184A showed a significant reduction in cytochrome c formation. As expected, the mutants H60A/H184A and W119A/(W122-D126)A did not synthesize detectable amounts of holocytochrome c (Fig. 1D, lanes 5 and 6). These results substantiate our finding that both histidines together and the tryptophan-rich motif per se are important for CcmC-mediated heme transfer during cytochrome c maturation (12).

*Extracellular heme cannot complement holo-CcmE formation in a ΔccmC mutant*

Is CcmC a heme transporter? If so, it might be possible to restore holo-CcmE formation in a ΔccmC mutant by adding heme to the medium, provided heme can pass the outer membrane
and becomes available in the periplasm for interaction with the CcmE biosynthetic apparatus. An *E. coli* hemA mutant deficient in the synthesis of the heme precursor δ-ALA cannot grow under respiratory conditions unless δ-ALA is added to the medium. Heme can only be used to restore this auxotrophy when an outer membrane heme receptor is co-expressed in *E. coli*. To create a *ccmC* deficient strain that can be used to test for extracellular heme complementation, we transduced the hemA::kan allele into the ΔccmC mutant EC28. The resulting heme auxotroph transductant was co-transformed with the plasmids pFR2 (*hasR* expressed from an arabinose-inducible promoter) and pEC406 (*pccmCDE*) or pEC408 (*pccmDE*). HasR, an outer membrane heme receptor of *Serratia marcescens*, is sufficient for heme uptake in *E. coli* (24). The recombinants were grown in MS minimal medium in the presence and absence of heme (5 μM) and induced with 0.02% arabinose. Without heme supplied, no growth was observed. Cells grown in the presence of heme were collected, and holo-CcmE formation was analyzed by heme stain. Fig. 2 shows that in the presence of CcmC, heme was incorporated into CcmE from cells grown with external heme (lane 1), whereas in the absence of CcmC, holo-CcmE was not formed (lane 2). Cell growth indicated that the externally added heme had been taken up. Nevertheless, exogenous heme did not complement CcmC function. Similar findings have been reported for *P. denitrificans ccmA* and *ccmB* mutants (27).

**CcmC is a heme binding protein**

Does CcmC directly interact with heme or does it indirectly assist heme delivery? To tackle this question, we tested the ability of CcmC to bind heme by affinity chromatography on hemin-agarose. The Δccm mutant EC06 containing plasmid pEC422 expresses a histidine-
tagged (H$_6$-) CcmC protein (Fig. 3A, lane 3). Membranes of anaerobically grown cells were solubilized with various detergents and tested for specific binding to hemin-agarose. It was found that lauroylsarcosine could solubilize CcmC without causing non-specific interactions between CcmC and hemin-agarose. Solubilized proteins before (Fig. 3A) and after (Fig. 3B) incubation with hemin-agarose were separated by 12% SDS-PAGE and probed with anti-His antibodies. Membrane proteins of a control strain containing vector only and membrane proteins containing H$_6$-CcmC incubating with agarose rather than hemin-agarose were used as negative controls. The wild-type CcmC was able to bind to hemin-agarose (panel B, lane 3), whereas the negative controls did not give any detectable CcmC signal on the immunoblot (panel B, lanes 1 and 2), ruling out the possibility of non-specific interactions. We conclude that CcmC is a heme binding protein.

The conserved histidines and the tryptophan-rich motif in CcmC are not essential for heme binding

If the tryptophan-rich motif and the two histidines of CcmC constitute a heme binding site, we should expect reduction or loss of heme binding in mutants with changes in these motifs. Therefore, we performed hemin-agarose affinity chromatography with the different ccmC mutant proteins. When membrane preparations containing similar amounts of CcmC proteins (Fig. 3A) were applied under identical conditions as for the wild type, the H60A, H184A, H60A/H184A and W119A/(W122-D126)A mutant proteins bound hemin-agarose at wild-type levels (Fig. 3B, lanes 3-7). Hence, the two conserved histidines together or the tryptophan-rich motif alone might be sufficient for heme binding of CcmC. By consequence, three mutants were constructed, in which the double mutant pairs H60A-
W119A/(W122-D126)A and W119A/(W122-D126)A-H184A, and the triple mutant H60A-W119A/(W122-D126)A- H184A were combined. Further affinity chromatography analysis of these three mutants with hemin-agarose revealed that heme binding was not affected (Fig. 3B, lanes 3, 8-10).

To confirm the specificity of heme binding to CcmC, pre-treatment of the membrane proteins with heme before applying hemin-agarose was done in a titration experiment. Membrane preparations containing CcmC or the triple mutant H60A-W119A/(W122-D126)A-H184A were first incubated with different concentrations of free heme, followed by addition of hemin-agarose. Proteins bound to hemin-agarose were subsequently analyzed by SDS-PAGE and immunoblotting. The more CcmC protein can be saturated with free heme, the less will bind to hemin-agarose. Fig. 4 reveals that with the increase of free heme, the amount of both wild-type and mutant CcmC decreased on the immunoblot (Fig. 4). Saturation appeared to be reached at a concentration of 50 µM free heme, because neither of the CcmC proteins could be detected on the immunoblot (Fig. 4). These results demonstrate that heme binding of CcmC is independent on residues H60, H184 and the tryptophan-rich motif.

*CcmC interacts with CcmE for heme delivery*

It has been speculated that the heme chaperone CcmE forms a protein complex with CcmC during heme transfer to CcmE (21). To determine if CcmE interacts with CcmC, we performed co-immunoprecipitation experiments. Treatment of membranes containing CcmE and/or CcmC proteins with various detergents revealed that n-dodecyl-β-D-maltoside could solubilize the majority of both proteins. Immunoprecipitation of detergent-solubilized proteins was performed with the anti-His antibodies or with the anti-CcmE serum proteins.
The precipitated complexes were subjected to SDS-PAGE and immunoblot analysis. Anti-His-tag precipitates were probed with anti-CcmE immunoglobulines to detect co-immunoprecipitated CcmE protein (Fig. 5A). Anti-CcmE precipitates were analyzed for the presence of CcmC using anti-His antibodies (Fig. 5B). In membrane preparations containing both CcmE and CcmC, the CcmC immunoprecipitates contained CcmE antigen (panel A, lanes 2 and 3), and the CcmE precipitates contained CcmC (panel B, lanes 2 and 3). By contrast, the control membrane preparations with either CcmC or CcmE alone did not contain the corresponding co-precipitates (lane 1 in panel A and B, respectively). When CcmD was present, more CcmE could be detected in the CcmC immunoprecipitates compared to when CcmD was absent (panel A, lane 3 versus 2), whereas the amount of CcmC in the CcmE immunoprecipitates was not influenced by the absence of CcmD (panel B, lanes 2 and 3). These results indicate that CcmC interacts with CcmE, resulting in a macromolecular CcmCE complex.

Both histidines and the tryptophan-rich motif are required for the interaction of CcmC and CcmE

The finding that the conserved histidines and the tryptophan-rich motif of CcmC are not essential for heme binding prompted us to test other possible functions of these motifs, such as interaction with CcmE. Fig. 6A shows that CcmE did not co-immunoprecipitate with the CcmC mutants even in the presence of CcmD (lanes 3-6), albeit the abundance of CcmE and CcmC polypeptides in the membrane fractions (Fig. 6B and C, respectively) was not affected drastically by the point mutations. Thus, both conserved histidines and the tryptophan-rich motif are required for the formation of a complex between CcmC and CcmE.
Discussion

Our understanding of the mechanism of Ccm protein action is very limited. The present paper extends previous knowledge on the function of CcmC by providing evidence that (i) CcmC interacts directly with heme; (ii) CcmC interacts directly with CcmE; and (iii) contrary to previous suggestions (see Introduction), the conserved residues in CcmC are not essential for binding heme, but are involved in forming a CcmC-CcmE complex.

In gram-negative as well as gram-positive bacteria, plant and protist mitochondria and chloroplasts at least one cytochrome c maturation protein contains a strictly conserved tryptophan-rich motif, which is the only common element shared between system I and II of cytochrome c maturation (5). In this work we addressed for the first time the question of whether these proteins physically interact with heme using CcmC as a model. We could show that CcmC binds heme. Histidines are common axial ligands of heme-iron in proteins (28,29). However, the functionally important, conserved histidine residues of CcmC are not essential for heme binding. Similar results have been reported for the heme receptor protein HemR of Yersinia enterocolitica, where mutations of two conserved histidines (H128 and H461) did not affect heme binding to HemR (30). The hydrophobic nature of the tryptophan-rich motif gave rise to the speculation that it functions as a heme binding site. However, according to our analysis of mutants with amino acid substitutions in this motif, the heme binding characteristics were not changed. Even the construction of a triple mutant with both histidines and the tryptophan-rich motif changed did not decrease the binding of CcmC to hemin agarose, suggesting that heme binding occurs elsewhere in the polypeptide. It is important to state that this result does not preclude any kind of transient interaction of the conserved sequence motifs with heme.
It is possible that CcmC is also involved in translocating heme across the cytoplasmic membrane. In this case, one would expect a heme binding site to lay on the cytoplasmic side of the membrane. However, the cytoplasmically oriented domains of CcmC are not particularly well conserved. Alternatively, heme may insert spontaneously into the lipid bilayer and just involve periplasmic domains of the protein for passing it onto CcmE. They would constitute a heme delivery site that is not designed to hold heme, but rather provides a path or physical connection to the heme recipient, CcmE.

Our attempts to bypass the lack of CcmC by providing heme in the medium and transport it across the outer membrane with the help of the heme receptor HasR were not successful. Since we controlled for heme uptake of the cells by using a hemA mutant that can only grow on glycerol-minimal medium when heme is available, the failure of the experiment can be explained in different ways: (i) heme transport is directly linked to heme delivery, which is in agreement with our finding that CcmC interacts with CcmE, (ii) heme uptake by HasR and translocation to the cytoplasm are coupled such that heme cannot be released into the periplasm. Certainly, CcmC cannot be a general heme exporter, because a ccmC mutant still incorporates heme into periplasmic cytochrome b562 (13). At present, we cannot exclude a role of CcmC in heme transport across the membrane.

In vivo function of CcmC as the only factor strictly required for heme insertion into CcmE suggested a protein-protein interaction between CcmC and CcmE. Here, we showed by co-immunoprecipitation that a complex between the two polypeptides is formed in the membranes. Clearly, the conserved sequence motifs of CcmC contribute to this interaction. The formation of a CcmC-CcmE complex is in agreement with a function of CcmC in heme transfer that involves a directed, perhaps even stereospecific positioning of heme onto apo-
CcmE to catalyze the formation of the covalent heme-histidine bond.

Our findings support a new model for holo-CcmE synthesis, which takes into account that the conserved histidines and tryptophan-rich motif of CcmC may not be involved in binding heme, but rather in the delivery of heme to CcmE. Our data give experimental evidence for the first time that CcmC functions as a CcmE heme lyase.

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References

Figure legends

FIG. 1. **Phenotypic characterization of mutants with amino acid changes in H60, H184 and/or the tryptophan-rich motif of CcmC.** The ΔccmA-H mutant EC06 expressing ccmE (lanes 1-6) or ccmDE (lanes 7-12) constitutively from plasmids pEC410 or pEC408, respectively, was co-transformed with plasmids pISC-3 (vector; lanes 1 and 7), pEC422 (H₆-ccmC; lanes 2 and 8), pEC433 (H₆-ccmCH60A; lanes 3 and 9), pEC436 (H₆-ccmCH184A; lanes 4 and 10), pEC705 (H₆-ccmCH60A, H184A; lanes 5 and 11), pEC706 (H₆-ccmCW119A(W122-D126)A; lanes 6 and 12). Cells were grown anaerobically in the presence of nitrite. Membrane proteins (100 µg per lane) were separated by 12% SDS-PAGE and stained for covalently bound heme (A). B. Western blot of the same membrane fractions as in A probed with antiserum directed against CcmE. C. Identical immunoblot probed with a monoclonal antiserum directed against a tetra-histidine epitope. The positions of holo-CcmE, CcmE and CcmC are indicated on the right. D. Ability of CcmC mutants to form holocytochrome c. The ΔccmC mutant EC28 was co-transformed with plasmids pRJ3291 (B. japonicum cycA) and pISC (vector, lane 1), pEC422 (H₆-ccmC; lane 2), pEC433 (H₆-ccmCH60A; lane 3), pEC436 (H₆-ccmCH184A; lane 4), pEC705 (H₆-ccmCH60A, H184A; lane 5), or pEC706 (H₆-ccmCW119A(W122-D126)A; lane 6). Cells were grown anaerobically in the presence of nitrite. Periplasmic proteins (100 µg per lane except 20 µg in lane 2) were separated by 15% SDS-PAGE and stained for covalently-bound heme. The position of holo-cytochrome c₅₅₀ is indicated on the right.
FIG. 2. **Extracellular heme cannot complement holo-CcmE formation in the ΔccmC mutant.**

EC28ΔhemA::kan was co-transformed with pFR2 (phasR) and pEC406 (pccmCDE, lane 1) or pEC408 (pccmDE, lane 2). The recombinants were grown in MS glycerol-minimal medium in the presence or absence of heme (5 µM) and induced with 0.02% arabinose for hasR expression. Without heme supplied, no growth was observed. Cells grown in media supplemented with heme were collected, and holo-CcmE formation was analyzed by heme stain. The position of holo-CcmE is indicated on the right.

FIG. 3. **Binding of heme to the wild-type and mutant CcmC proteins.** EC06 was transformed with plasmid pISC-3 (vector, lane 2), pEC422 (H6-ccmC; lane 3), pEC433 (H6-ccmCH60A; lane 4), pEC436 (H6-ccmCH184A; lane 5), pEC705 (H6-ccmCH60A, H184A; lane 6), pEC706 (H6-ccmCW119A/(W122-D126)A; lane 7), pEC707 (H6-ccmCH60A, W119A/(W122-D126)A; lane 8), pEC708 (H6-ccmCW119A/(W122-D126)A, H184A; lane 9), pEC709 (H6-ccmCH60A, W119A/(W122-D126)A, H184A; lane 10). Cells were grown anaerobically in the presence of nitrite. A. Western blot of membrane proteins (150 µg per lane) from the indicated strains. Immunodetection of CcmC was done with tetra-His antibodies. B. Membrane proteins (150 µg per lane) from the indicated strains were incubated with hemin-agarose. EC06 (pEC422 H6-ccmC) (panel A, lane 3) incubating with agarose was used as a control to rule out non-specific interactions between CcmC and agarose (lane 1). The agarose-bound proteins were separated by 12% SDS-PAGE and probed with tetra-His antibodies. The position of CcmC is indicated on the right.
FIG. 4. **Heme titration.** EC06 (pEC422, \(H_6-ccmC\)) and EC06 (pEC709 (\(H_6-ccmCH60A, W119A/(W122-D126)A, H184A\)) were grown anaerobically in the presence of nitrite. Membrane preparations (150 µg per lane) containing wild-type CcmC (A) and the H60A/W119A/(W122-D126)A/H184A mutant CcmC (B) were pre-incubated with different concentrations of hemin: 0 µM, 5 µM, 10 µM, 30 µM, 50 µM. Samples were then further incubated with 10 µl hemin-agarose. Proteins bound to hemin-agarose were separated by 12% SDS-PAGE and probed with tetra-His antibodies. The position of CcmC is indicated on the right.

FIG. 5. **Co-immunoprecipitation of CcmC and CcmE.** *E. coli* cells were grown anaerobically in the presence of nitrite. The \(\Delta ccm\) mutant EC06 was transformed with plasmids expressing different combinations of ccmC, ccmD and ccmE. A. Membrane proteins (500 µg per lane) from the indicated strains were immunoprecipitated with tetra-His antibodies, separated on a 15% SDS-gel, and stained with anti-CcmE serum. Lanes: EC06 carrying pEC410 (ccmE); 1; pEC422 (\(H_6-ccmC\)) and pEC410, 2; pEC422 and pEC408 (ccmDE), 3. B. Membrane proteins (500 µg per lane) from the indicated strains were immunoprecipitated with anti-CcmE serum, separated on a 12% SDS-gel, and stained with tetra-His antibodies. Lanes: EC06 carrying pEC422 (\(H_6-ccmC\)), 1; pEC422 and pEC410 (ccmE), 2; pEC422 and pEC408 (ccmDE), 3. The positions of CcmE and CcmC are indicated on the right. The signals above CcmC in Panel B are from the light-chain of CcmE antibodies.

FIG. 6. **Two histidines and the tryptophan-rich motif are necessary for the interaction of**
**CcmC with CcmE.** The Δccm mutant EC06 was transformed with plasmids expressing different combinations of **ccmC** allele, **ccmD** and **ccmE**. Cells were grown anaerobically in the presence of nitrite. A. Membrane proteins (500 µg per lane) from the indicated strains were immunoprecipitated with tetra-His antibodies, separated on a 15% SDS-gel, and stained with anti-CcmE serum. Lanes: EC06 carrying pEC410 (**ccmE**) and pEC422 (**ccmC**), 1; pEC408 (**ccmDE**) and pEC422 (**ccmC**), 2; pEC410 and pEC705 (**H6-ccmCH60A, H184A**), 3; pEC408 and pEC705, 4; pEC410 and pEC706 (**H6-ccmCW119A/W122-D126**), 5; pEC408 and pEC706, 6. The positions of CcmE and CcmC are indicated on the right. B. Western blot of the same membrane fractions (150 µg per lane) as in A probed with antiserum directed against CcmE. C. Identical Western blot probed with a monoclonal antiserum directed against tetra-His antibodies. The positions of CcmE and CcmC are indicated on the right.
Table 1. Bacterial strain and plasmids used in this study

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
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<tr>
<td>DH5α</td>
<td><em>SupE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</em></td>
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<tr>
<td>C600ΔhemA</td>
<td>Δ<em>hemA::kan</em>, derivative of C600, Km^R^</td>
<td>C. Wandersman</td>
</tr>
<tr>
<td>EC06</td>
<td>Δ<em>ccmA-H</em>, derivative of MC1061, Km^R^</td>
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<tr>
<td>EC28</td>
<td>Δ<em>ccmC</em>, derivative of MC1061</td>
<td>(13)</td>
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<tr>
<td>EC28ΔhemA</td>
<td>Δ<em>ccmC</em>, Δ<em>hemA::kan</em>, derivative of EC28, Km^R^</td>
<td>this study</td>
</tr>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pRJ3291</td>
<td>B. japonicum cycA cloned into pISC-2, Km^R^</td>
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<td><em>ccmE</em> cloned into pACYC184, Cm^R^</td>
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<tr>
<td>pEC408</td>
<td><em>ccmDE</em> cloned into pACYC184, Cm^R^</td>
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</tr>
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<tr>
<td>pEC433</td>
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<td>pEC477</td>
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<tr>
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<td>this study</td>
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<td>pEC708</td>
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<td>this study</td>
</tr>
</tbody>
</table>
pEC709

\( H_6-ccmC^{H60A, \ W119A, \ (W122-\ D126)A, \ H184A} \) this study

cloned into pISC-3, Ap^R

pFR2

\( hasR \) cloned into pBAD24, Ap^R

C. Wandersman
FIG. 4

A

Wild-type ccmC

0 5 10 20 50

B

ccmC^{H80A, W119A, (W122-D126)A, H184A}

0 5 10 20 50

Free heme (μM)

CcmC
FIG. 5

A

B

ccmE  ccmCE  ccmCDE

1  2  3

CcmE

ccmC  ccmCE  ccmCDE

1  2  3

CcmC
Physical interaction of CcmC with heme and the heme chaperone CcmE during cytochrome c maturation
Qun Ren and Linda Thöny-Meyer

J. Biol. Chem. published online May 30, 2001

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