The C-Terminal Flexible Domain of the Heme Chaperone CcmE Is Important but Not Essential for Its Function

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CcmE is a heme chaperone active in the cytochrome c maturation pathway of Escherichia coli. It first binds heme covalently to strictly conserved histidine H130 and subsequently delivers it to apo-cytochrome c. The recently solved structure of soluble CcmE revealed a compact core consisting of a β-barrel and a flexible C-terminal domain with a short α-helical turn. In order to elucidate the function of this poorly conserved domain, CcmE was truncated stepwise from the C terminus. Removal of all 29 amino acids up to crucial histidine 130 did not abolish heme binding completely. For detectable transfer of heme to type c cytochromes, only one additional residue, D131, was required, and for efficient cytochrome c maturation, the seven-residue sequence D130DENYTPP137 was required. When soluble forms of CcmE were expressed in the periplasm, the C-terminal domain had to be slightly longer to allow detection of holo-CcmE. Soluble full-length CcmE had low activity in cytochrome c maturation, indicating the importance of the N-terminal membrane anchor for the in vivo function of CcmE.

CcmE is a heme chaperone involved in the biogenesis of type c cytochromes. In a first step, it binds heme covalently at a strictly conserved histidine (12, 17). The nature of this heme-histidine bond is still unknown and is a subject of great interest. This covalent linkage may be stereospecific and necessary for presentation of the cofactor to type c cytochromes in the correct orientation. Binding of heme to CcmE requires CcmC (14, 16). In addition, CcmD, a small membrane protein, and CcmAB, an ABC transporter with an unknown substrate, are also involved in this process (16). Release of heme from CcmE and formation of the two thioether bonds between the heme vinyl groups and the cysteines of the characteristic CXXCH motif of cytochromes c are catalyzed by CcmF, the putative heme lyase. CcmF was shown to interact directly with CcmE, as well as with CcmH (13). CcmG and CcmH are part of a redox pathway that is required to keep the cytochromes reduced (5, 19). In Escherichia coli, all eight cytochrome c maturation factors (CcmABCDEFGH) are transcribed from an anaerobically expressed operon (7, 20).

Besides the crucial heme-histidine bond, the mechanism by which heme is bound to and released from CcmE remains unknown. Alanine scanning mutagenesis of CcmE did not identify residues that are involved in an acid-base-catalyzed mechanism (3). Incorporation of heme into apo-CcmE and transfer from holo-CcmE to apo-cytochrome c has been shown in vitro, supporting the role of holo-CcmE as an intermediate of the cytochrome c maturation pathway (2). Recently, the structure of soluble CcmE without its natural N-terminal membrane anchor has been solved for E. coli (4) and Shewanella putrefaciens (1) by nuclear magnetic resonance analysis. The compact core of the protein consists of a rigid six-stranded β-barrel. In contrast to many other heme proteins, apo-CcmE contains neither a groove nor a cleft where the heme moiety could bind. Close to heme binding histidine 130 (E. coli numbering), a platform formed by several conserved hydrophobic amino acids and two basic residues that could interact with the propionate groups of the cofactor was postulated to be the heme binding site (4). Solvent-exposed H130 is located at the joint between the rigid core that was resolved with high atomic precision and the less-well-defined, flexible C-terminal domain. This latter part of CcmE is the least conserved in terms of both amino acid sequence and length. Secondary-structure predictions revealed an extended α-helix between P137 and A145 or N146 (http://www.expasy.org/tools/). However, only a short α-helical turn was found for 137PEVE140 by nuclear magnetic resonance analysis (4). It is possible that this helix is extended upon heme binding or interaction with other CcmE proteins (1). The C-terminal domain may shield heme from the solvent. Therefore, we asked whether the dynamic properties of the heme chaperone are functionally important. The role of the flexible C-terminal domain of CcmE was investigated with respect to heme binding and transfer to cytochrome c. Membrane-bound and soluble versions of CcmE and its truncated derivatives were compared.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. Freshly transformed E. coli cells were grown aerobically in Luria-Bertani medium (15) or anaerobically in minimal-salt medium (9) supplemented with 0.4% glycerol, 40 mM fumarate, and 5 mM potassium nitrite as a terminal electron acceptor at 37°C. Induction was performed with 0.05% l-arabinose at an optical density at 600 nm of 0.4 to 0.7, and the cells were harvested 10 to 16 h later. Antibiotics were added at the following final concentrations: ampicillin, 100 μg/ml; chloramphenicol, 10 μg/ml; kanamycin, 50 μg/ml.

Construction of plasmids. E. coli DH5α was used as the host for cloning. Truncated CcmE versions were constructed by using pEC412 as a template for the membrane-bound forms (pEC332, pEC333, pEC324, pEC325, pEC326, pEC312, pEC314, pEC315, pEC316, pEC317, and pEC318) and pEC415 as a template for the soluble forms (pEC311, pEC309, pEC308, pEC307, and pEC306). The NdeI/SalI fragment of a PCR with pINGlaraB and a primer inserting a stop codon after the desired amino acid was cloned into NdeI/SalI-

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digested pEC415. For the construction of pEC301, the plasmid expressing soluble CcmE, pEC458, was amplified with the primers CcmEV and CcmEC. The PCR fragment was digested with SaI and ligated into SaI-digested pEC415; Ap' This work.

Plasmids

pEC101  ccmABCDEFGH (5'-3')-133, and CcmE30-131 were constructed and investigated accordingly. CcmE30-135 and CcmE30-136 produced slightly reduced activity at 200°C compared to CcmE30-137, which is the native anchor of CcmE in C. The substitution of the native membrane anchor of CcmE in C. The substitution of the native membrane anchor of CcmE in C. The substitution of the native membrane anchor of CcmE in C.

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid | Relevant genotype and phenotype | Reference
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**Escherichia coli** strains
DHSa | supE44 lacU169 (Δ880lacZAM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | 8
EC06 | MC1061 Δ(camsABCDEF)Km' | 20
EC65 | MC1061 ΔccmE | 17

**Plasmids**

pEC101 | ccmABCDEFGH (5'-3')-133, and CcmE30-131 were constructed and investigated accordingly. CcmE30-135 and CcmE30-136 produced slightly reduced activity at 200°C compared to CcmE30-137, which is the native anchor of CcmE in C. The substitution of the native membrane anchor of CcmE in C. The substitution of the native membrane anchor of CcmE in C.

**Cell fractionation.** Periplasmic fractions of 400-ml aerobically or anaerobically grown cultures were isolated by treatment with polymyxin B sulfate (Fluka, Buchs, Switzerland). The microsomes were washed with cold 50 mM sodium phosphate (pH 7.2), and resuspended in cold extraction buffer (2 mg of polymerase bovine per ml, 30 mM sodium phosphate, 300 mM NaCl, 5 mM EDTA [pH 7.2], 2 ml of wet cells per g). The suspension was stirred gently for 1 h at 4°C and centrifuged at 40,000 × g for 20 min at 4°C. The supernatant contained the periplasmic fraction.

Membrane fractions of 400-ml aerobically or anaerobically grown cultures were prepared as follows. The cells were harvested by centrifugation at 4,000 × g; washed in cold 50 mM sodium phosphate (pH 7.2); resuspended in 2 ml of wet cells per g in cold 50 mM sodium phosphate (pH 7.2) containing 1 mM phenylmethylsulfonyl fluoride, 20 μg of DNAse I per ml, and 20 μg of RNase A per ml; and passed three times through a French pressure cell at 110 MPa. Cell debris was separated by centrifugation at 40,000 × g for 30 min at 4°C. The supernatant was subjected to ultracentrifugation at 150,000 × g for 1 h. The membrane fraction was resuspended in 100 μl of 50 mM sodium phosphate (pH 7.2).

**Biochemical methods.** Protein concentrations were determined with the Bradford assay (Bio-Rad). Heme staining of proteins separated by sodium dodecyl sulfate (SDS)–15% polyacrylamide gel electrophoresis was carried out with o- diamidocinnolin (Sigma) as the substrate as described previously (18). Protein precipitation with trichloroacetic acid was performed with a 100% solution, which was added to a final concentration of 10%. Samples were incubated on ice for 20 min and then centrifuged at 16,000 × g. For protein precipitation with acetic acid to remove nonspecific signals in the heme stain (6), 4 volumes of 10 M HCl in acetone was added to the sample, which was incubated on ice for 20 min and then centrifuged at 16,000 × g.

Immunohistochemistry was performed with a CcmE-specific antisera directed against the synthetic peptide YD8KHDENYTPPEVEKAM (17). Signals were detected with a goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Bio-Rad) as the secondary antibody and disodium 3-(4-methoxybenzene(1,2-dioxetane-3,2)dioxetane-3,2)-5-(5'-chloro)tricyclo[3.3.1.1(5)]decane-4-yl)phenyl phosphonate (Roche Diagnostics) as the substrate.
shorter constructs, no heme binding was detected by heme
staining (lanes 4 to 6). However, with the more sensitive spec-
trophotometric assay, 1 to 2% of wild-type heme absorption in
the Soret region around 400 nm could be detected in the
periplasmic fraction of soluble CcmE30-134 ending with Y134,
the last 100% conserved amino acid (data not shown). For the
shorter versions, this method did not show heme absorption.
Coomassie staining revealed uniform levels of polypeptide for
all mutants (Fig. 2B).

Soluble CcmE complements cytochrome c maturation in a
ΔccmE background at low levels. Even though structural anal-
yses of CcmE were done with soluble CcmE30-159 (1, 4), the
question of whether or not the soluble form of the heme
chaperone is functional in cytochrome c maturation has not
been addressed previously. To answer this question, pEC412
(CcmE1-159, wild type), pEC301 (CcmE30-159, soluble CcmE),
and pISC-2 (negative control) were transformed into ΔccmE
strain EC65. Cells were grown anaerobically in the presence of
NO₂⁻ to induce expression of the chromosomal ccm genes
(20). The periplasmic diheme type c cytochrome NapB, a sub-
unit of the periplasmic nitrate reductase (11), could only be
found when wild-type CcmE1-159 was present, but not with sol-
uble CcmE30-159, which is the predominant heme-binding pro-
tein in this case (Fig. 3A). Coomassie staining of periplasmic
proteins only showed a prominent band corresponding to sol-
uble CcmE30-159, whereas the NapB protein was not detectable
(Fig. 3B). An immunoblot against CcmE antibodies clearly
showed that the 18-kDa protein seen in panel A did not cross-
react and thus was a different heme protein. By contrast, sol-
uble CcmE was detected (Fig. 3C).

\[ \text{FIG. 1. Alignment of C-terminal CcmE sequences and summary of the results. (A) Alignment of CcmE sequences representing the C-terminal}
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\[ \text{domains from different organisms. Capital letters, conserved residues; lowercase letters, nonconserved residues; bold, heme binding histidine. (B)}
\] 
\[ \text{Analysis of C-terminal truncation of } E. \text{ coli CcmE. nd, not determined.}
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soluble CcmE (Fig. 3D). Even though the band is hardly detectable, this experiment shows that soluble CcmEapo30-159 can complement the biogenesis of the NapC type c cytochrome to some extent. In this case, 18-kDa membrane-bound CcmEapo1-159 was made visible by Coomassie staining, while the 25-kDa NapC protein was not detectable (Fig. 3E).

**Heme binding of membrane-anchored CcmE versions lacking the C-terminal domain.** Because of the low activity of CcmEapo30-159 in cytochrome c maturation, the truncated soluble versions of CcmE were not useful for a complementation assay. Therefore, the membrane-bound truncated CcmE versions ending at amino acids H130, D131, E132, N133, Y134, T135, P136, P137, E138, V139, and E140 were constructed. These constructs were tested first for the ability to bind heme in Δccm strain EC06 cotransformed with pEC101 (ccmABCD). Membrane proteins were analyzed by heme staining for the detection of holo-CcmE (Fig. 4A). In contrast to the soluble forms, even the shortest analyzed construct, CcmEapo1-130, showed residual holo-CcmE formation (lane 10). Heme binding was weak also for the constructs CcmEapo1-134, CcmEapo1-133, CcmEapo1-132, and CcmEapo1-131 (lanes 6 to 9). CcmE versions with longer C-terminal extensions bind heme more efficiently (lanes 1 to 5). A Coomassie stain of membrane fractions showed that the lower levels of holo-CcmE in shorter derivatives did not result from reduced levels of polypeptide (Fig. 4B).

In conclusion, *E. coli* CcmE does not absolutely require the 29 C-terminal amino acids after the crucial histidine to form the covalent linkage to heme. However, in the absence of the amino acid sequence 133DENY134, this first step of heme transfer was drastically diminished. When the C-terminal domain was extended by 135TPPE138, it corresponded in length to the C terminus of the shortest known CcmE homolog of *R. prowazekii*, and holo-CcmE formation was restored to almost wild-type levels. The results are summarized in Fig. 1B.

**Maturation of type c cytochromes does not strictly require the C-terminal domain of CcmE.** The same CcmE versions with truncated C termini were expressed in a ΔccmE background to examine the influence of this domain on heme delivery to cytochrome c. Cells were grown anaerobically in minimal-salt medium with NO2−, allowing expression of periplasmic type c cytochrome NapB. In addition, a second plasmid encoding *Bradyrhizobium japonicum* cytochrome c550 under the control of an arabinose-inducible promoter was co-transformed. The type c cytochromes produced were detected by heme staining of periplasmic extracts (Fig. 5).

Wild-type levels of cytochromes were produced in constructs CcmEapo1-140, CcmEapo1-139, CcmEapo1-138, and CcmEapo1-137 (lanes 2 to 5). Removal of one additional residue in CcmEapo1-136 led to a clear reduction of type c cytochromes (lane 6). When P136 (the last well-conserved residue of CcmE) was also removed in CcmEapo1-135, the level of cytochrome c declined further and only a weak heme staining band was visible (lane 7). Weak bands were also found for CcmEapo1-134, CcmEapo1-133, CcmEapo1-132, and CcmEapo1-131 (lanes 8 to 11). Only CcmEapo1-130, with the heme binding histidine at the C terminus, completely failed to produce detectable amounts of cytochrome c (lane 12). The results are summarized in Fig. 1B.

**Quantification of type c cytochromes by absorption spectroscopy.** To quantify the concentration of soluble type c cytochromes in the different strains, the reduced-minus-oxidized absorption spectra of the periplasmic fractions were recorded. The difference in absorption at 550 and 536 nm is proportional to the amount of soluble type c cytochromes. The value for the wild type was assigned a value of 100%, and the relative levels of cytochromes formed in the truncated CcmE mutants were calculated accordingly. All measurements were performed twice with different samples. The values obtained from the spectra corresponded well to the intensities of the heme-staining bands (Fig. 5). In conclusion, heme delivery from holo-CcmE to cytochrome c can occur at low levels even if the entire C-terminal domain is removed; however, it is only efficient if the well-conserved amino acids of this domain are present (Fig. 1).

**DISCUSSION**

The heme chaperone CcmE is a key player in cytochrome c maturation of α- and γ-proteobacteria, deinococci, and plant mitochondria. It first binds heme at a strictly conserved histidine and subsequently delivers it to the CXCHX motif of apo-cytochrome c. The recently solved structures of apo-CcmE (1, 4) and its biochemical characterization (2, 3, 4) revealed that this protein binds heme initially in a noncovalent complex
FIG. 3. Complementation of cytochrome c maturation by soluble CcmE. ΔccmE strain EC05 was transformed with plasmid pEC412 (CcmE1-159, wild type, lane 1), pEC301 (CcmE30-159, soluble form, lane 2), or pISC-2 (negative control, lane 3). Cells were grown under anaerobic conditions in minimal-salt medium with nitrite as the terminal electron acceptor. Periplasmic extracts and membrane fractions were isolated, separated on an SDS–15% polyacrylamide gel, and analyzed. (A) Periplasmic proteins (40 μg per lane) precipitated with trichloroacetic acid and analyzed by heme staining. (B) Periplasmic proteins (10 μg per lane) analyzed by Coomassie staining. (C) Immunoblot of periplasmic proteins (10 μg per lane) probed with antiserum against CcmE. (D) Membrane proteins (100 μg per lane) extracted with acidic acetone analyzed by heme staining. (E) Membrane proteins (20 μg per lane) analyzed by Coomassie staining. On the left of each panel, the position of the 16-kDa marker is indicated.

FIG. 4. Influence of the C-terminal deletions of membrane-anchored CcmE on heme binding. ΔccmA-H strain EC06 was cotransformed with ccmABCD(pEC101) and a plasmid containing wild-type (wt) or truncated ccmE (lanes: 1, pEC412; 2, pEC316; 3, pEC315; 4, pEC314; 5, pEC312; 6, pEC326; 7, pEC325; 8, pEC324; 9, pEC333; 10, pEC332; 11, pISC-2). Cells were grown aerobically in Luria-Bertani medium. (A) Membrane proteins (50 μg per lane) were extracted with acidic acetone (10 mM HCl), separated on an SDS–15% polyacrylamide gel, and analyzed by heme staining. (B) Coomassie staining of 20 μg of membrane proteins per lane.
at a hydrophobic platform on the surface of the protein. The compact six-stranded β-barrel is separated from a highly flexible C-terminal domain by crucial histidine 130, which is solvent exposed and thus accessible for heme binding. We examined the function of this part of E. coli CcmE by stepwise deletion of the C-terminal end.

CcmE1-130 was the shortest construct with wild-type heme binding (Fig. 4, lane 2) and cytochrome c formation (Fig. 5, lane 4) activities. This shows that the last 21 amino acids of CcmE are functionally not important. Interestingly, this CcmE version corresponds in length to the shortest known CcmE (lanes: 1, pEC412; 2, pEC318; 3, pEC317; 4, pEC316; 5, pEC315; 6, pEC314; 7, pEC312; 8, pEC326; 9, pEC325; 10, pEC324; 11, pEC333; 12, pEC332). Cells were grown under anaerobic conditions in minimal-salt medium with nitrite as the terminal electron acceptor, which leads to the formation of NapB, the soluble diheme type c cytochrome of the periplasmic nitrate reductase. Trichloroacetic acid-precipitated periplasmic proteins (40 μg per lane) were separated on an SDS–15% polyacrylamide gel and analyzed by heme staining. The spectrophotometrically determined concentration of soluble type c cytochromes (cytochrome c550 and NapB; ΔA(436,550) is given as a percentage of the wild-type level below each lane.

FIG. 5. Influence of the C-terminal domain of CcmE on cytochrome c maturation. ΔccmE strain EC65 was cotransformed with plasmid pRJ3291 expressing the B. japonicum cycA gene encoding cytochrome c550 (Cyt c550) and a plasmid expressing wild-type (wt) or truncated ccmE (lanes: 1, pEC412; 2, pEC318; 3, pEC317; 4, pEC316; 5, pEC315; 6, pEC314; 7, pEC312; 8, pEC326; 9, pEC325; 10, pEC324; 11, pEC333; 12, pEC332). Cells were grown under anaerobic conditions in minimal-salt medium with nitrite as the terminal electron acceptor, which leads to the formation of NapB, the soluble diheme type c cytochrome of the periplasmic nitrate reductase. Trichloroacetic acid-precipitated periplasmic proteins (40 μg per lane) were separated on an SDS–15% polyacrylamide gel and analyzed by heme staining. The spectrophotometrically determined concentration of soluble type c cytochromes (cytochrome c550 and NapB; ΔA(436,550) is given as a percentage of the wild-type level below each lane.

indicating that Y134 may be an axial heme ligand, thereby facilitating the initial noncovalent binding of heme to CcmE (2, 3).

The minimal construct leading to normal cytochrome c maturation is CcmE1-137 (Fig. 5, lane 5). CcmE1-136 showed about one-third (lane 6) and CcmE1-135 to CcmE1-131 showed about 10% (lanes 7 to 11) of the wild-type activity. The amount of cytochrome c produced by CcmE1-130 was below the limit of detection (lane 12). As for the binding of heme, the presence of the amino acids immediately after histidine 130 improves the function of CcmE. It is plausible that these residues shield the heme from the solvent (4).

Structure predictions revealed an α-helix in the C-terminal domain of CcmE starting with P137. In the E. coli structure, a short α-helical turn was identified for the sequence PEV140 (4). It was postulated that this helix could be extended in the presence of other subunits of the Ccm complex (1), because similar helices were found in other proteins belonging to the same fold, which all interact with a partner (10). This hypothesis cannot be ruled out by the finding that this part of the protein is not essential, but our results show that the C-terminal domain after residue 138 comprising the short α-helix cannot be the only site of interaction. It is possible that residues 131 to 138 are involved in contacts with other subunits of the cytochrome c maturation apparatus. As CcmE was shown to form a complex with CcmC (14) and CcmF (13) by coimmunoprecipitation, they are candidates for interaction with this domain. Coimmunoprecipitation experiments with the short CcmE versions were not possible because the epitope of our CcmE antibody is also truncated.

Another finding of this work is that soluble CcmE is not efficient at heme transfer to cytochrome c. The N-terminal membrane anchor seems to have an important function and
may be used for the specific interaction with integral membrane proteins CcmC and CcmF. Further investigation of such interactions of the membrane anchor with transmembrane domains of these proteins is required.

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