Functional Characterization of the C-terminal Domain of the Cytochrome c Maturation Protein CcmE*

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CcmE is a heme chaperone involved in the periplasmatic maturation of c-type cytochromes in many bacteria and plant mitochondria. It binds heme covalently and subsequently transfers it to the apo form of cytochromes c. To examine the role of the C-terminal domain of CcmE in the binding of heme, in vitro heme binding to the apo form of a truncated (immediately before Pro-136) version of the periplasmic domain of the heme chaperone from Escherichia coli was studied. Removal of the C-terminal domain dramatically altered the ligation of non-covalently bound heme in CcmE (the soluble form lacking the membrane anchor) but only slightly affected its affinity for protoporphyrin IX and 8-anilino-1-naphthalenesulfonate. This finding has significant mechanistic implications for in vivo holo-CcmE formation and indicates that the C-terminal region is not required for the recruitment and docking of heme into its binding site but is likely to contain amino acid(s) involved in heme iron axial coordination. Removal of the C-domain significantly impaired in vivo heme binding to CcmE and conversion of apocytochrome to holoprotein by a similar factor, suggesting that the C-terminal domain of the chaperone is primarily involved in heme binding to CcmE rather than in heme transfer to the apo cytochrome.

The post-translational assembly of c-type cytochromes, essential and widespread heme proteins primarily involved in electron transport (1, 2), takes place within eukaryotic cells in the intermembrane space of mitochondria and in the lumen of thylakoids of chloroplasts; it also occurs in the periplasm of Gram-negative bacteria (3). It involves the translocation of the apocytochrome from the cytoplasm to the appropriate compartment, the recognition of the polypeptide by the cytochrome c maturation apparatus, and the transport, delivery, and stereospecific covalent attachment of heme via its vinyl groups to the two cysteines of a CXCH motif in the polypeptide (4).

Among the three c-type cytochrome maturation pathways discovered to date (5), the most complex but best described appears to be System I, also called the Ccm4 (cytochrome c maturation) system, identified in Gram-negative bacteria (6) and plant mitochondria (7). The heme chaperone CcmE plays central roles in this system including the uptake of heme in the periplasm and its storage in a covalently bound form as well as its subsequent stereospecific delivery to the apo-cytochrome c (8). Despite the transient nature of heme binding by the chaperone, CcmE is the first protein to have been reported to bind heme covalently via a histidine residue (9). This covalent bond seems to be formed between the N83 of the histidine and the β-carbon of a heme vinyl group (10), unlike the heme-histidine covalent bond identified in a recombinant cyanobacterial hemoglobin (11), which involves the N82 of the histidine and the α-carbon of the heme 2-vinyl. Evidence has been presented suggesting that it is the 2-vinyl group of heme that is modified in holo-CcmE (12).

CcmE is a membrane protein anchored via an N-terminal hydrophobic region to the cytoplasmic membrane and exposing its main water soluble domain to the periplasm (9). The structures of two apo forms of the periplasmic region of CcmE are available (13, 14), and both reveal a rigid β-barrel core ending with a flexible C-terminal domain. The C-terminal domain is attached to the rigid core in the proximity of the solvent-exposed heme binding histidine (His-130 in Escherichia coli CcmE), itself located close to a hydrophobic platform displayed on the external surface of the β-barrel, which is assumed to be the heme binding region of the protein. Alterations in the functioning of CcmE caused by alanine mutations of amino acids constituting this hydrophobic region (15) support the existence of an exposed binding site, which is consistent with the dynamic nature of the chaperone in that it needs to be able to bind and release heme readily. Because of its apparent flexibility and strategic location close to the predicted heme binding site, the C-terminal domain has been proposed to play major roles in the functioning of the chaperone (13), such as facilitating heme binding and/or transfer to apocytochrome, shielding the bound cofactor from solvent exposure, or being involved in specific interactions with CcmC and/or CcmF, proteins that have been shown to interact directly with CcmE (16, 17). Recent in vivo studies on the effect of various C-terminal deletions on the behavior of CcmE (18) suggested that the C-domain is important for the function of the protein, but its exact role remains enigmatic.

In the present work we used an in vitro approach to investigate the interaction between a truncated variant of CcmE and heme to permit a more refined analysis of the function of the C-domain in heme binding. We produced a version of the periplasmic region of CcmE (lacking its N-terminal membrane anchor up to and including Arg-31, referred to in this paper as CcmE*). We further truncated the C-terminal domain of this construct from and including Pro-136, which is located at the beginning of a short α helix (13), to generate ΔC-CcmE*. It was decided to truncate the protein at this point because the cis-trans isomerization of this highly conserved proline could function as a molecular switch controlling the flexibility of the C-terminal region. This hypothesis was...
tested by replacing Pro-136 with alanine. The truncated construct also retained Tyr-134, which has been shown to participate in iron axial ligation in holo-CcmE (12), the loss of which might have induced overlapping effects on heme binding.

Additionally, we studied the effect of the CcmE C-terminal deletion on cytochrome c maturation and holo-CcmE formation in vivo. Mutagenesis of CcmE was performed on a plasmid containing the whole ccm operon, which was co-expressed in an Δccm genetic background with plasmids encoding c-type cytochromes. The advantage of this system over those used in previous in vivo studies on CcmE (15, 18) is that it preserves the relative physiological stoichiometry of the mutated CcmE and the other Ccm proteins.

MATERIALS AND METHODS

Plasmids and Mutagenesis—All mutations were carried out according to the QuickChange site-directed mutagenesis method (Stratagene). Primers and plasmids generated in this work are listed in Table I. The pE151 plasmid (19) was the expression vector for His$_6$-N-CcmE’ and the Δc mutation. The version of this protein truncated at Pro-136 (His$_6$-N-CcmE) was expressed from pE155, a plasmid derived from pE151 by introducing a stop codon in the place of that encoding for Pro-136 (referred to as Δc mutation). Plasmids pE861, pE863, and pE864 carry the P136A, ΔC, and H130A mutations, respectively, in the ccmE gene of pEC86 (20). A plasmid that contains the entire ccm operon. All plasmids made in this work were sequenced to confirm that only the desired mutation had been incorporated. E. coli XL2-blue and XL10-gold strains (Stratagene) were used as cloning hosts for pE155 and the pEC86 variants, respectively.

Protein Expression and Purification—The E. coli BL21 (DE3) strain (Stratagene) was used for expression of His$_6$-N-CcmE’ and its truncated variant. Transformants carrying pE151 or pE155 were grown at 37 °C with shaking at 200 rpm in LB medium supplemented with ampicillin at a final concentration of 100 µg/ml. At mid-exponential phase, protein expression was induced with 1 mM isopropyl-$\beta$-D-thiogalactopyranoside. Cells were harvested, resuspended in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and lysed by sonication. After sonication, the cell suspension was centrifuged at 15,000 × g for 30 min to remove cell debris, and the supernatant was applied to a Ni$_2$-chelating Sepharose (Amersham Biosciences) column. The hexahistidine-tagged proteins were eluted with 100 mM imidazole in the same buffer. After removal of the imidazole by repeated concentration and dialysis of the eluted fraction in a 5-kDa Vivaspin concentrator (VivaScience), thrombin cleavage of the His$_6$-labeled proteins was performed using a Thrombin Clean Cleave kit (Sigma). Uncleaved protein as well as the hexahistidine tag were removed by re-applying the digested mixture to a Ni$_2$-Sepharose column.

Protein Characterization—Protein concentrations were estimated by absorbance measurements at 280 nm. The extinction coefficients at this wavelength were 7680 M$^{-1}$cm$^{-1}$ for CcmE and 6400 M$^{-1}$cm$^{-1}$ for ΔC-CcmE’, calculated based on amino acid composition. SDS-PAGE analysis was carried out on 10% NuPAGE gels (Invitrogen). Heme staining was performed according to the method of Goodhew (21). Electrospray ionization mass spectrometry was performed on a Micromass Bio-Q II-MS triple quadrupole atmospheric pressure instrument equipped with an electrospray interface. Samples at the concentrations of 10 µM in 1:1 water/acetonitrile, 0.2% formic acid were introduced via a loop injector into the electrospray source at a flow rate of 10 µl/min.

H NMR experiments were performed at 600 MHz using a home-built spectrometer in the Oxford Centre for Molecular Sciences NMR facility. Protein samples were prepared at a concentration of 1 mM in 100 mM NaCl, pH 7.2, in the presence of 5% D$_2$O (Sigma). All experiments were conducted at 25 °C. Two-dimensional total correlation spectroscopy (TOCSY) experiments were recorded with a spectral width of 8000 Hz in both dimensions, 64 acquisitions, and 360 and 1024 complex points in F$_1$ and F$_2$, respectively. An isotropic mixing time of 30 ms was used. The NMR spectra were processed using Felix2.3 (Accelrys Inc., San Diego).

Heme Addition—Hemin (Sigma) was added from a 1 mM stock solution in Me$_2$SO to protein samples prepared in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl. UV/visible absorption spectra of heme-protein mixtures were acquired with a Varian Cary 50 Bio spectrophotometer. Reduced spectra were recorded immediately after the addition of few crystals of disodium dithionite to the mixture.

8-Anilino-1-naphthalenesulphonate (ANS) and Protoporphyrin IX (PPIX) Binding—The affinity of the proteins for hydrophobic molecules, namely ANS (Sigma) and PPIX (Frontier Scientific), was investigated by fluorescence emission spectroscopy. ANS binding was examined both by protein fluorescence quenching (excitation at 280 nm and emission from 285 to 330 nm) and ANS fluorescence enhancement (excitation at 380 nm and emission from 440 to 540 nm) upon the addition of increasing amounts of ANS from 100 µM or 1 mM stock solutions in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl to an 8 µM protein solution in the same buffer. Fluorescence intensities were corrected for the values of free ANS. PPIX binding was studied by protein fluorescence quenching with increasing concentrations of ligand, as described above for ANS. All fluorescence emission spectra were recorded with a PerkinElmer Life Sciences LS 50B fluorimeter (5-nm slit widths and 100-nm/min scan speed), and fluorescence intensities were corrected for primary inner filtering (22). $K_D$ values were determined by standard double-reciprocal plot analysis and are the average of at least three experiments.

In Vivo Cytochrome c Formation—The pEC86 variants were co-transformed into the E. coli ccm deletion strain EC06 (23) either with plasmid pKPD1 (24), containing the gene for Paracoccus denitrificans cytochrome c$_{550}$ or with plasmid pCEB562 (25), encoding the R98C/Y101C variant of E. coli cytochrome b$_{562}$. The EC06 cells as well as the pEC86 plasmid were kindly provided by Prof. L. Thöny-Meyer (Zurich, Switzerland). 500 ml of LB medium supplemented with 0.1% ampicillin, 34 µg/ml chloramphenicol, and 1 mM isopropyl-$\beta$-D-thiogalactopyranoside were inoculated with 0.5 ml of overnight culture and incubated for 24 h at 37 °C with shaking at 200 rpm. After cell harvest-

| Primer | Sequence (5’-3’) | Plasmids made |
|--------|-----------------|---------------|
| ΔC For | CGATGAAAACACTATAGCTGACCAAGATTGGAAAG | pE155, pE863 |
| ΔC Rev | CTITCTCACCCTCTCTGCAGTATGTTTTCATCG | pE155, pE863 |
| P136A For | CGATGAAAACACTACGGCGCAGAATGGAGAAAG | pE861 |
| P136A Rev | CTITCTCACCCTCTCGGCCCGTGATGTTTTCATCG | pE861 |
| H130A For | GTGCGTGGCAGAAAAGCGATGAAACTATAGCCCG | pE864 |
| H130A Rev | CGGCGTATAGTTTCATCGGCTTTCGCCACGC | pE864 |
ing, periplasmic extracts were prepared as described elsewhere (26). Cytochrome c550 production was estimated from the absorbance spectra of the dithionite-reduced periplasmic extracts by measuring their absorbance at 550 and 521.5 nm, wavelengths corresponding to the maxima of ferrous cytochrome c550/H9251 and H9252 bands, respectively. Levels of cytochrome c550 production were normalized by wet cell weight. Pyridine hemochrome spectra were obtained according to the method of Bartsch (27).

Membrane Extraction—120 ml of aerobically grown cultures were harvested and resuspended in 5 ml of 50 mM Tris/HCl, pH 7.4, 100 mM NaCl containing 50 μg/ml DNase I. Cells were lysed by sonication, and debris was removed by centrifugation at 35,000 g for 20 min. The membranes were collected by ultracentrifugation of the supernatant at 150,000 g for 1 h and resuspended in 2 ml of the same buffer. Protein concentration in the membrane fraction was estimated using the Bradford assay (Bio-Rad).

RESULTS

Characterization of CcmE and ΔC-CcmE—Both proteins were produced cytoplastically with a cleavable hexahistidine tag attached at their N termini. After extraction, purification, and thrombin cleavage, the proteins were shown by SDS-PAGE to be highly pure and devoid of the His tags (data not shown). The complete cleavage of the His6 tag was confirmed by electrospray ionization mass spectrometry. CcmE and ΔC-CcmE had masses of 14,762 (+1) Da (theoretical mass, 14,763 Da) and 12,100 (+1) Da (expected mass, 12,101 Da), respectively.

1H two-dimensional NMR experiments were performed to evaluate the effect of the C-terminal deletion on the protein conformation. It is important to ensure that the observed differences in the properties of the truncated variant are due to the physical lack of the C-domain rather than to significant long range protein conformational changes triggered by its removal. Fig. 1 shows a comparison between the fingerprint regions (which contain the Hα-Hα cross-peaks) of the TOCSY spectra of the full-length (panel A) and truncated (panel B) CcmE and indicates that the topology of the β-barrel core is highly preserved in the truncated mutant. More than 25 fingerprint region cross-peaks with Hα chemical shifts downfield of ~4.8 ppm are found in virtually identical positions in the two spectra; these Hα-Hα cross-peaks are characteristic of amino acids located in β-strands (13, 14). On the other hand, significant differences between the two spectra are observed for cross-peaks with Hα chemical shifts upfield of ~4.6 ppm; resonances of residues forming α-helices or less structured parts of the protein are likely to occur in this region of the spectrum (data not shown). This is consistent with the modification undergone by the protein, as the deleted C-terminal domain comprises a short α-helix followed by an unstructured tail of 16 amino acids (13). Methyl resonances shifted upfield of ~0.5 ppm are, in general, characteristic of the specific packing of aromatic and methyl side chains in the hydrophobic core of a protein and provide evidence for the folded state of the protein. The regions of the TOCSY spectra of the two proteins where upfield-shifted resonances of side-chain methyl groups of Leu, Val, and Ile are found are virtually identical (data not shown), suggesting that ΔC-CcmE adopts the same fold as CcmE. Differences in the methyl region of the spectra of the two proteins can be attributed to the deletion of two valine and four alanine residues in ΔC-CcmE.

Heme Ligation Is Dramatically Altered in the ΔC-CcmE Construct—When mixed with heme, apoCcmE spontaneously forms a b-type noncovalent complex (19) in which the heme iron is ligated by residues of the polypeptide. High spin pentacoordinate in its ferric state, the iron in the heme-CcmE complex becomes low spin hexacoordinate upon
C-domain of the Heme Chaperone CcmE

### TABLE TWO

Visible absorption maxima (nm) obtained for heme-CcmE' b-type complexes

| b-Type complex   | Oxidized | Reduced |
|------------------|----------|---------|
|                  | nm       | nm      |
| Heme-WT CcmE'    | 390      | 426     |
| Heme-ΔC CcmE'    | 386      | 416     |
| Free heme        | 386      | 412     |

### FIGURE 2

Absorption spectra of reduced heme-CcmE' (solid line) and heme-ΔC CcmE' (dotted line) mixtures as well as of free heme (dashed line) recorded immediately after the addition of a few grains of sodium dithionite to a heme (4 μM) protein (20 μM) solution in 50 mM Tris HCl, pH 7.4, 100 mM NaCl.

### TABLE THREE

Effect of the C-terminal truncation on the affinity of CcmE’ for ANS and PPIX

| Ligand       | Experiment                | CcmE’ | ΔC-CcmE’ |
|--------------|---------------------------|-------|----------|
| ANS          | Protein fluorescence quenching | 1.72 ± 0.19 | 9.8 ± 1.02 |
| ANS          | ANS fluorescence enhancement | 2.48 ± 0.27 | 9.7 ± 0.77 |
| PPIX         | Protein fluorescence quenching | 0.23 ± 0.02 | 0.5 ± 0.03 |

Reduction (19). To assess the role of the C-terminal domain in heme coordination, the ability of ΔC-CcmE’ to ligate heme was monitored by absorbance spectroscopy. No noticeable change in the visible spectrum of free ferric heme occurred upon the addition of the truncated protein (ratio 1:5, heme to protein), whereas wild-type CcmE’ yielded a high-spin complex, as previously reported (TABLE TWO, spectra not shown). When dithionite was added, the spectrum of the heme-CcmE’ mixture red-shifted to produce the spectrum expected for the low spin ferrous b-type complex (TABLE TWO, Fig. 2). After reduction, the only difference recorded in the spectrum of the heme-ΔC-CcmE’ mixture compared with that of free ferrous heme was a 4-nm red shift of the Soret band, accounting for some weak ligation. However, no α or β bands were detected in this case (inset, Fig. 2).

ΔC-CcmE’ Retained a Significant Affinity for Hydrophobic Molecules—ANS has been widely used as a probe for non-polar binding sites in proteins because its fluorescence depends markedly on the hydrophobicity of its environment (28). It is known that ANS binds at the same site as heme in apomyoglobin and apohemoglobin (29), and it has been shown that this is also the case with apoCcmE’ (19). To determine whether the C-domain is important for the binding of the cofactor, we tested the ability of ΔC-CcmE’ to bind this hydrophobic molecule as well as its physiological cofactor lacking the iron center. As previously observed with apoCcmE’ (19), two ANS binding sites with different affinities were detected for both proteins (data not shown). The dissociation constants of the high affinity binding sites were examined here because the low affinity binding sites could be the result of nonspecific interactions and, therefore, functionally irrelevant (19). The affinity of the truncated mutant for ANS was studied both by protein fluorescence quenching and ANS fluorescence enhancement, and the values for the dissociation constants obtained by the two methods were very similar (TABLE THREE). The affinity of CcmE’ for ANS was diminished after the removal of its C-domain, but the 5-fold increase recorded in its Kd value (TABLE THREE) indicates that the alteration is not dramatic. Because axial coordination contributes to interactions between proteins and their metal cofactors, we used PPIX (heme devoid of iron) instead of heme for affinity studies in an attempt to discern the role of the C-terminal domain in heme attachment to the C-domain of the Heme Chaperone CcmE.

ΔC-CcmE Is Able to Produce Holocytocromes c in Vivo but to a Lower Extent than Wild Type—The importance of the C-terminal domain of CcmE for posttranslational cytochrome c maturation was explored by monitoring the formation of a c-type version of E. coli cytochrome b562 or of P. denitrificans cytochrome c550 in a Δccm genetic background complemented by a ccm operon-containing plasmid carrying the ΔC mutation in the ccmE gene. In the same way, using alanine point mutations, the importance of Pro-136 and the presumed strict requirement of His-130 for this process were investigated.

Because of its artificially generated CXXCH heme binding motif, the R98C/Y101C variant of cytochrome b562 behaves like natural c-type cytochromes, binding heme covalently in the periplasm of E. coli (25). Unusually, this c-type cytochrome is expressed in vivo in its holo form irrespective of the presence of Ccm system, but the stereochemistry of heme attachment is dependent on whether the Ccm proteins are active (30). The product of the Ccm-catalyzed reaction has its porphyrin ring linked to the polypeptide in the same way as in native c-type cytochromes, whereas, in the major product of the uncatalyzed reaction, the heme moiety is rotated by 180° relative to its α,γ axis (30). The two forms can be distinguished by their different absorbance properties, the improperly matured cytochrome displaying a considerably red-shifted visible spectrum. We assessed the ability of our CcmE mutants to process cytochromes c in vivo by monitoring the stereochemistry of heme attachment to the CXXCH version of cytochrome b562, expressed in the presence of pEC86 variants in Δccm cells. The H130A mutation in CcmE completely repressed the Ccm-dependent covalent attachment of heme to the cytochrome, since the periplasmic extracts of cells expressing the CXXCH b562 cytochrome in the presence of H130A CcmE have the same absorbance maxima as the spontaneous form generated in the absence of the Ccm proteins (TABLE FOUR). In contrast, the P136A mutation has no effect on the capacity of the Ccm system to catalyze heme binding to the apocytochrome, as the spectrum of the periplasmic fraction obtained in this case is identical to that observed when wild-type pEC86 was expressed (TABLE FOUR). The removal of the C-terminal domain of CcmE does not seem to have significant consequences on the correct maturation of the c-type cytochrome b562 (TABLE FOUR). However, the 1-nm red shift in the visible spectrum and the 0.5-nm red shift in the α band of the pyridine hemochrome
spectrum of periplasmic proteins from cells containing ΔC-CcmE relative to the wild-type spectra could account for low levels of incorrectly formed holocytochrome.

As for most c-type cytochromes, it was expected that *P. denitrificans* cytochrome c<sub>550</sub> would only be matured in the periplasm of *E. coli* in the presence of the Ccm proteins. We studied the expression levels of this cytochrome in a Δccm genetic background complemented by variants of pEC86. Cytochrome c<sub>550</sub> content in crude periplasmic extracts was estimated by absorbance spectroscopy, and the values were normalized by wet cell weight. Production of endogenous cytochromes in cells transformed only with pEC86 was below the detection limit, and therefore, their contribution to the total absorbance was ignored. The levels of cytochrome c<sub>550</sub> formed in the presence of CcmE mutants were calculated relative to that obtained in the presence of wild-type CcmE, which was assigned a value of 100%. No cytochrome c<sub>550</sub> production was detected in the absence of the Ccm system, and the H130A mutation in CcmE completely abolished holocytochrome formation (TABLE FIVE). Wild-type levels of cytochrome were obtained in the presence of P136A CcmE, indicating that this proline is not important for c-type cytochrome maturation. The deletion of the C-terminal region of CcmE significantly diminished the yield of cytochrome c<sub>550</sub> since ΔC-CcmE was able to generate only one-third of the wild-type cytochrome level (TABLE FIVE).

**FIGURE 3. SDS-PAGE (10%) analysis by heme-staining of membrane proteins (20 μg/lane) from Δccm cells transformed with pEC86 variants carrying WT (lane 1), ΔC (lane 2), or H130A CcmE (lane 3).** The left panel of the figure shows the positions of wild-type and truncated membrane-anchored CcmE. The right panel shows the absorbance spectra of dithionite-reduced periplasmic protein extracts from cells expressing wild-type or mutated CcmE. The spectra were recorded in the presence of dithionite, and the absorbance at 420 nm was set to 1.00. The absorbance at 540 nm is presented as a percentage of that obtained with the wild type (WT) and was determined using the Gel Doc™ software from Bio-Rad (background was subtracted). The intensity values were calculated relative to that obtained with the wild type and were determined using the Gel Doc™ software from Bio-Rad (background was subtracted). The intensities were calculated based on the absorbance at 540 nm.

In *Vivo* Heme Binding to CcmE Is Impaired in the Absence of Its C-domain—The effect of the C-terminal truncation on heme binding to CcmE in *vivo* was investigated by evaluating the amount of holo-CcmE anchored via its N-terminal helix in the membranes of Δccm cells transformed with altered versions of pEC86. Heme-stained SDS-PAGE gels of membrane protein extracts revealed that heme binding is significantly altered in the truncated mutant, since the amount of holo-CcmE is reduced by a factor of 2.5 when its C-domain is missing (Fig. 3, lanes 1 and 2). Interestingly, this factor is close in value to the level of reduction of c-type cytochrome expression in the absence of the C-domain of CcmE (TABLE FIVE), suggesting that a lower amount of available functional chaperone due to impaired heme binding to ΔC-CcmE rather than an altered heme transfer capacity of truncated CcmE is the major cause for the reduced level of cytochrome c<sub>550</sub> observed. As previously reported (15, 19), the H130A CcmE mutant is incapable of binding heme covalently (Fig. 3, lane 3) and, consequently, is completely inactive in cytochrome c maturation (TABLES FOUR and FIVE).

**DISCUSSION**

In this work we used both *in vitro* and *in vivo* approaches to assess the importance of the C-terminal domain of CcmE and to provide insight into its role in holo-CcmE and holocytochrome c formation. As suggested by the two-dimensional NMR analysis, removal of the C-terminal region did not interfere with the general β-barrel fold of CcmE and implicitly should not have affected the integrity of the heme binding site, which has been modeled at the surface of the β-barrel (13). We, therefore, assume that the variations detected in heme binding and ligation between CcmE<sup>+</sup> and ΔC-CcmE<sup>−</sup> are exclusively due to the physical absence of the C-domain in the latter construct. Our *in vitro* studies indicate that the deletion of the C-terminal region decreased the affinity of the protein for PPIX (a variant of the physiological ligand lacking Fe) only slightly, as determined by fluorescence spectroscopy (TABLE THREE), whereas Fe-PPIX ligation was drastically affected, as observed by absorbance spectroscopy (Fig. 2 and TABLE TWO). This suggests that heme can form a non-covalent complex in the absence of C-terminal residues even though the axial coordination of its iron has been altered. This finding has potentially important mechanistic implications for the interaction between heme and CcmE *in vivo*. The fact that non-covalent attachment of heme does not seem to require initial axial ligation suggests that non-covalent binding could represent the first step in *in vivo* holo-CcmE formation, preceding heme-iron chelation by residues of the polypeptide. Therefore, the sequence of events of the *in vivo* process could be envisaged as follows: First, heme binds to CcmE by hydrophobic interactions to form a non-covalent complex. Ligation of the heme iron occurs subsequently to yield the non-covalent complex resembling a β-type cytochrome described above (Fig. 2). Axial coordination of the heme iron in this complex is redox-dependent; the high spin pentacoordinate ferric heme becomes low spin hexacoordinate in the ferrous state. The reduction of the iron center is a requirement for the formation of the covalent bond between His-130 and a heme vinyl group (31), the last phase of holo-CcmE synthesis. Evidence has been presented suggesting that the Ccm proteins could be responsible for the provision of the reductant necessary for this step (32). Components of

**TABLE FOUR**

| pEC86 variant co-transformed with pECB562 | Absorption bands | Pyridine hemochrome α band |
|------------------------------------------|------------------|---------------------------|
|                                          | Maxima           |                           |
|                                          | nm               | nm                        |
| pEC86 (wild type)                        | 420              | 526                       |
|                                         | 556              | 550                       |
| pE861 (P136A)                            | 420              | 526                       |
|                                         | 556              | 550                       |
| pE863 (ΔC)                               | 420              | 526.5                     |
|                                         | 557              | 550.5                     |
| pE864 (H130A)                            | 420              | 529.5                     |
|                                         | 559.5            | 552.5                     |
| pEC86 absent                             | 424              | 529                       |
|                                         | 559              | 552.5                     |

**TABLE FIVE**

| Levels of cytochrome c<sub>550</sub> expression in the presence of pEC86 variants (the CcmE mutation carried by each plasmid is given in parentheses) displayed relative to the amount of cytochrome produced in the presence of pEC86, which was assigned a value of 100%.

| pEC86 variant co-transformed with pKPD1 | % Cytochrome c<sub>550</sub> expression |
|----------------------------------------|----------------------------------------|
| pEC86 (WT)                             | 100 ± 5                                |
| pE861 (P136A)                          | 103 ± 7                                |
| pE863 (ΔC)                             | 34 ± 3                                 |
| pE864 (H130A)                          | 0                                      |
| pEC86 absent                           | 0                                      |
C-domain of the Heme Chaperone CcmE

the Ccm system could play additional roles in the holo-CcmE maturation process, such as facilitating heme delivery to CcmE or catalyzing the formation of the covalent bond between Fe-PPIX and CcmE. CcmC is likely to fulfill at least one of these tasks, as it was shown to be essential for in vivo holo-CcmE formation (33) and to interact directly with the heme chaperone (16).

The observation that the absence of the C-domain affects heme iron ligation rather than overall binding of the porphyrin ring suggests that the C-terminal region is not required for the first step of holo-CcmE formation (the recruitment and accommodation of heme into its binding site) but might contain amino acid(s) involved in heme iron axial coordination. Because heme ligation is redox-dependent and is perturbed in both iron oxidation states (TABLE TWO), more than one iron-ligating residue could be situated in this region of the protein. However, the relatively low sequence homology of the residues in the C-domain in sequence alignments would suggest some plasticity in the selection of ligands.

Two c-type cytochromes were used in this work as probes of the function of the Ccm system, namely a CXXCH-containing form of cytochrome b$_{562}$ and cytochrome c$_{550}$ from P. denitrificans. The latter was selected because its production in the hol form depends entirely on the presence of the Ccm proteins, making it useful for quantitative studies. The holo form of CXXCH b$_{562}$ on the other hand is produced in the absence of the Ccm proteins, but its spectroscopic properties allow us to establish whether correct heme attachment has occurred, allowing a more refined qualitative analysis of cytochrome c maturation. In addition, by performing site-directed mutagenesis on the ccmE gene in the plasmid pEC86, which contains the whole Ccm operon, we sought to maintain the physiological stoichiometry of the Ccm proteins and to avoid possible artifacts of overexpression of one of the component proteins.

Deletion of the C-terminal domain of CcmE at Pro-136 in pEC86 did not prevent the CXXCH version of cytochrome b$_{562}$ from being correctly matured by the Ccm system in vivo, although the major correct product was accompanied by low levels of improperly formed holocytochrome (TABLE FOUR). However, the absence of the CcmE C-terminal region decreased the expression level of cytochrome c$_{550}$ to one-third that of the wild type (TABLE FIVE). Interestingly, the same mutation reduces heme binding to CcmE in vivo by a similar factor (Fig. 3, lanes 1 and 2), suggesting that the reduction observed in cytochrome c$_{550}$ production in the absence of the CcmE C-domain is mainly due to a decrease in the amount of functional chaperone rather than to improper functioning of CcmE in heme transfer to the apocytochrome. This would imply that the C-terminal region of the chaperone has little involvement in heme transfer, being more important for the cooperation between heme and CcmE in the initial formation of the C-domain. This might be achieved by supplying axial ligand(s) to the heme iron, as suggested by the in vitro experiments performed in this work (Fig. 2 and TABLE TWO). Generally, our in vivo data are in agreement with previous studies on the effect of C-terminal truncations of CcmE on its function (18), which show significant alterations in heme binding to CcmE and apocytochrome upon stepwise deletion of C-terminal amino acids.

The in vivo experiments carried out here also confirm the strict requirement of the heme binding His-130 residue of CcmE for cytochrome c maturation (15, 19). The H130A mutation completely abolishes cytochrome production via the Ccm-dependent pathway (TABLES FOUR and FIVE) because of the inability of H130A CcmE to bind heme covalently (Fig. 3, lane 3). This histidine residue is present in the C-terminally truncated variant of CcmE (which terminates at Thr-135) studied in this work.

Many proteins involved in heme transport display a common topology consisting of two domains separated by a flexible linker, generally a proline residue, which allows them to undergo conformational changes that promote binding or release of heme (34). The B-barrel structure of CcmE along with the highly flexible C-terminal domain is consistent with this arrangement. Because of its location at the beginning of the C-terminal domain, the strictly conserved Pro-136 was suspected to be the hinge controlling the flexibility of the C-terminal domain and, thus, modulating the dynamic features of the chaperone. However, our results do not support this hypothesis, as mutation of this residue to alanine has no detectable effect on cytochrome c maturation in vivo (TABLES FOUR and FIVE).

In summary, we have produced the heme chaperone CcmE lacking its flexible C-terminal domain to determine the function of this domain and to examine the mechanism of heme binding. The truncated form was found to be folded and able to bind both ANS and PPIX as ligands but was unable to ligate heme as observed for the wild-type protein. The disrupted ability to ligate heme in a non-covalent complex did not abolish the ability of the protein to function in vivo in cytochrome c biogenesis, as demonstrated with two exogenous cytochromes c. In light of the significant decrease in the in vivo activity of the truncated form, it could be that ligation appropriate for transient heme binding by CcmE could improve the efficiency of the process. The fact that the protein was still biologically active could suggest that ligation of the heme iron is not essential, at least in the initial non-covalent form. The latter is supported by the observation that wild-type CcmE binds PPIX with a similar affinity to heme, indicating that the presence of the iron does not dramatically affect the interaction and, therefore, that the binding may be hydrophobically driven. The significance of this in vivo may be related to the fact that this protein has to interact with other proteins that bind heme (such as CcmC and possibly apocytochromes), and additional studies will be required to address this.

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