Probing the Heme-Binding Site of the Cytochrome *c* Maturation Protein CcmE†

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ABSTRACT: Maturation of *c*-type cytochromes in many bacterial species and plant mitochondria requires the participation of the heme chaperone CcmE that binds heme covalently via a His residue (H130 in *Escherichia coli*) before transferring it stereospecifically to the apo form of cytochromes *c*. Only the structure of the apo form of CcmE is known; the heme-binding site has been modeled on the surface of the protein in the vicinity of H130. We have determined the reduction potential of CcmE, which suggests that heme bound to CcmE is not as exposed to solvent as was initially thought. Alanine insertions in the vicinity of the heme-binding histidine (which we showed by NMR do not perturb the protein fold) strikingly abolish formation of both holo-CcmE and cytochrome *c*, whereas previously reported point mutations of residues adjacent to H130 gave only a partial attenuation. The heme iron coordinating residue Y134 proved to be strictly required for axial ligation of both ferrous and ferric heme. These results indicate the existence of a conformationally well-defined heme pocket that involves amino acids located in the proximity of H130. However, mutation of Y134 affected neither heme attachment to CcmE nor cytochrome *c* maturation, suggesting that heme binding and release from CcmE are hydrophobically driven and relatively indifferent to axial ligation.

Covalent attachment of heme to the CXXCH motif of *c*-type cytochromes is catalyzed in vivo by numerous proteins. Their function and number vary greatly depending on the organismal type (*1*−*3*). Three different *c*-type cytochrome biogenesis systems have, for several years, been thought to exist (*4*), but recent work suggests that this number is likely to be higher (*5, 6*). In plant mitochondria and many Gram-negative bacteria, holocytochrome *c* assembly employs a heme chaperone, CcmE,[1] together with at least 10 other gene products, involved in either heme handling or redox control of the cysteines of the heme-binding motif of the cytochrome polypeptide (*7*). In the *Escherichia coli* periplasm, CcmE binds heme transiently, but covalently, with the participation of other proteins expressed from the *ccm* operon, the membrane protein CcmC (*8*), and the ATP-binding cassette protein CcmAB (*9, 10*), and transfers it in a stereospecific manner to the reduced apocytochrome (*11*). CcmF, another integral membrane protein, is believed to facilitate the latter step (*12*).

CcmE binds its cofactor via an unusual His−heme bond, in which $N^{\delta 1}$ of the histidine (H130 in *E. coli*) becomes covalently linked to the β-carbon of a vinyl group of heme, as established by NMR studies of a peptide derived from CcmE (*13*). This bond is significantly different from the only other heme−His covalent bond discovered to date, which occurs in recombinant cyanobacterial hemoglobin (*14*). The structure of the apo form of the periplasmic domain of CcmE (lacking its N-terminal membrane anchor and termed CcmE′) was determined by NMR (*15, 16*) and reveals a rigid β-barrel core together with a flexible C-terminal domain, which follows H130, as illustrated in Figure 1. An accurate description of the location of the heme-binding site has not been possible in the absence of a three-dimensional (3D) structure of the holoprotein, but the available structure of the apo form allowed prediction of the location of the heme-binding site, which was modeled on the surface of the β-barrel core, in the immediate vicinity of the heme-binding His and at the beginning of the C-terminal domain (*16*). This model implies a solvent-exposed heme-binding site. The level

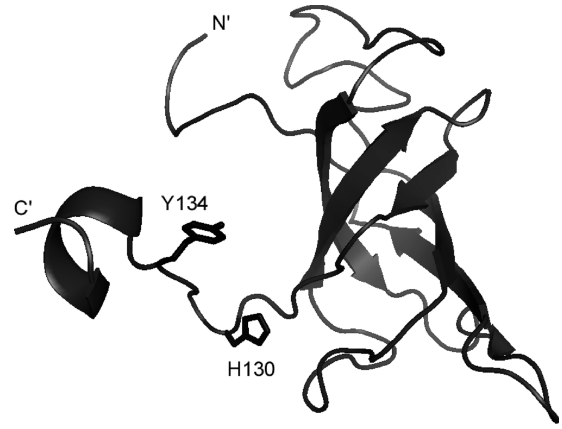

FIGURE 1: Structure of the apo form of *E. coli* CcmE determined by NMR (*16*). The side chain of heme-binding histidine H130 is shown, as well as heme-ligating tyrosine Y134, indicating the location of the proposed heme-binding site. The structure does not include the N-terminal membrane anchor of the protein. This image was prepared using PyMOL (http://www.pymol.org).

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[1] Abbreviations: Ccm, cytochrome *c* maturation; CcmE′, soluble periplasmic domain of CcmE (S32 at the N-terminus); TOCSY, two-dimensional (2D) total correlation spectroscopy.

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Table 1: Oligonucleotides Used for Mutagenesis

primer	sequence (5′−3′)	plasmid made
Y134F-For	GCGAAACACGATGAAAACTTTACGCCGCCAGAAGTTGAG	pE862
Y134F-Rev	CTCAACTTCTGGCGGCGTAAAGTTTTCATCGTGTTTCGC	pE862
KAH-For	GAAAGAAGTGCTGGCGAAAGCTCACGATGAAAACTATACGC	pE866, pE1515
KAH-Rev	GCGTATAGTTTTCATCGTGAGCTTTCGCCAGCACTTCTTTC	pE866, pE1515
HAD-For	GAAGTGCTGGCGAAACACGCTGATGAAAACTATACGCCG	pE867, pE1516
HAD-Rev	CGGCGTATAGTTTTCATCAGCGTGTTTCGCCAGCACTTC	pE867, pE1516
PAP-For	GATGAAAACTATACGCCGGCTCCAGAAGTTGAGAAAGCG	pE868
PAP-Rev	CGCTTTCTCAACTTCTGGAGCCGGCGTATAGTTTTCATC	pE868
DAE-For	GTGCTGGCGAAACACGATGCTGAAAACTATACGCCGCCA	pE870
DAE-Rev	TGGCGGCGTATAGTTTTCAGCATCGTGTTTCGCCAGCAC	pE870
LAA-For	CTCGCGAAAGAAGTGCTGGCTGCGAAACACGATGAAAAC	pE871
LAA-Rev	GTTTTCATCGTGTTTCGCAGCCAGCACTTCTTTCGCGAG	pE871
AAK-For	GCGAAAGAAGTGCTGGCGGCTAAACACGATGAAAACTAT	pE872
AAK-Rev	ATAGTTTTCATCGTGTTTAGCCGCCAGCACTTCTTTCGC	pE872
EAN-For	GCTGGCGAAACACGATGAAGCTAACTATACGCCGCCAGAAG	pE873
EAN-Rev	CTTCTGGCGGCGTATAGTTAGCTTCATCGTGTTTCGCCAGC	pE873
NAY-For	GGCGAAACACGATGAAAACGCTTATACGCCGCCAGAAGTTG	pE874
NAY-Rev	CAACTTCTGGCGGCGTATAAGCGTTTTCATCGTGTTTCGCC	pE874
YAT-For	GAAACACGATGAAAACTATGCTACGCCGCCAGAAGTTGAG	pE875
YAT-Rev	CTCAACTTCTGGCGGCGTAGCATAGTTTTCATCGTGTTTC	pE875
TAP-For	CACGATGAAAACTATACGGCTCCGCCAGAAGTTGAGAAAG	pE876
TAP-Rev	CTTTCTCAACTTCTGGCGGAGCCGTATAGTTTTCATCGTG	pE876
Y134M-For	GCGAAACACGATGAAAACATGACGCCGCCAGAAGTTGAG	pE3011, pE869
Y134M-Rev	CTCAACTTCTGGCGGCGTCATGTTTTCATCGTGTTTCGC	pE3011, pE869

of solvent exposure has been indicated as one of the most important factors in modulating the reduction potential of heme centers in *c*-type cytochromes (*17*). Low redox potentials have been correlated with surface exposure of the heme moiety, whereas solvent exclusion by burial of the cofactor inside the protein has been shown to increase its midpoint redox potential (*18*−*21*). We have investigated the reduction potential of heme in holo-CcmE′ to gain insight into the chemical (dielectric) environment of the bound heme and into possible in vivo requirements for CcmE function related to redox control.

A site-directed mutagenesis approach has been used previously to identify the heme-binding site in CcmE. Extensive alanine substitution screening showed that, with the exception of H130, whose loss proved to be completely deleterious for the protein activity, no amino acid tested appeared to be specifically required for heme binding and/or transfer to or from CcmE (*22*). Double or triple point mutations in the predicted heme-binding site were needed to generate a drastic reduction in the function of CcmE, and even then complete abolition of CcmE function was surprisingly difficult to achieve (*22*). To study the importance and location of the heme-binding site relative to essential histidine H130, which is in one of the most conserved regions of the protein, we inserted Ala residues in the proximity of this amino acid and investigated the effect of these sequence perturbations on holo-CcmE formation and holocytochrome *c* production in vivo. If a heme-binding site located on the surface of the protein is strictly required, then changing the position of H130 relative to other residues in the putative binding site might be expected to impair the attachment of heme to CcmE. The heme iron in holo-CcmE has been found to be ligated by a tyrosine residue [Y134 in *E. coli* (Figure 1)] in both ferrous and ferric forms (*23, 24*). We reported properties of a Y134F variant which were consistent with Y134 binding to heme iron in wild-type holo-CcmE (*23*). Ala insertions also allowed us to examine the effect of changing the relative positions of the heme-binding histidine and a heme iron ligating residue. In addition, we assessed the contribution of the tyrosine ligation to heme handling by CcmE and so substituted Y134 with another potential iron ligating residue, Met, as well as Phe, which cannot ligate heme.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis. Mutations were introduced according to the QuikChange site-directed mutagenesis method (Stratagene). Primers and plasmids generated in this work are listed in Table 1. Plasmids pE862 and pE869 carry the Y134F and Y134M mutations, respectively, in the *ccmE* gene of pEC86 (*25*), a plasmid that contains the entire *E. coli ccm* operon. Similarly, pEC866−868 and pE870−876 contain codons for alanine insertions after each residue of the L^{127}AKHDENYTP^{136} sequence of CcmE. Plasmid pE3011 was the expression vector for periplasmically targeted Y134M CcmE′ (CcmE′ refers to CcmE lacking its membrane anchor; S32 is the N-terminal residue) and was derived from pEC301 (*22*). Plasmids expressing the two Ala insertion variants for expression and purification were produced using pE151 (*26*) as the template. All plasmids produced were sequenced to confirm that only the desired mutation had been incorporated. The *E. coli* XL10-gold strain (Stratagene) was used as the cloning host for the pEC86 variants, and strain XL2-blue (Stratagene) was used for the other plasmids.

Protein Expression and Purification. Extraction of periplasmic and membrane proteins from cells transformed with the pEC86 variants was carried out as described previously (*27*). The cytoplasmically expressed variants used for the NMR studies were purified and prepared as described previously (*26, 27*). The untagged holo forms of CcmE′ and the Y134M variant were produced as follows. JM109(DE3) transformants containing pEC301 and pEC86 were grown aerobically in Luria-Bertani medium supplemented with ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL). At midexponential phase, protein expression was induced with 0.1% L-arabinose and growth was continued for 14 h. After cells had been harvested, periplasmic extracts were

prepared as described previously (*28*) and loaded on a DEAE-Sepharose anion-exchange column (XK 26/20 Pharmacia column) with a resin bed volume of 70 mL. The chromatography buffer was 50 mM Tris-HCl (pH 8.0), and protein was eluted with a 0 to 0.5 M NaCl gradient (volume of 500 mL in the same buffer) at a flow rate of 7 mL/min. Fractions (7 mL) were collected, and those containing holo-CcmE′ devoid of the apo form, identified by heme-stained SDS−PAGE gels and absorbance spectroscopy, were pooled, concentrated to 1 mL, and loaded on a prepacked HiLoad 16/60 Superdex 75 gel filtration column. The column was equilibrated and run with 50 mM Tris-HCl (pH 7.5) and 300 mM NaCl at a flow rate of 0.6 mL/min, and the eluant was collected in 1 mL fractions. After this step, holo-CcmE′ in the relevant fractions was pure and monomeric, as shown by SDS−PAGE and electrospray mass spectrometry. A yield of 2 mg of protein per liter of culture was obtained. All the purification steps were carried out at 4 °C.

Protein Characterization. The concentration of holo-CcmE′ was determined from its pyridine-hemochrome spectrum, as previously described (*29*). The membrane protein concentration was estimated using the BCA protein assay kit (Pierce), following the manufacturer's instructions. SDS−PAGE analysis was carried out on 10% NuPAGE gels (Invitrogen). Heme staining was performed according to the method of Goodhew et al. (*30*). Western blotting was performed with rabbit antiserum raised against the K^{129}HDENYTPPEVEKAME^{144} sequence of *E. coli* CcmE (a gift from Prof. Thöny-Meyer, ETH, Zurich, Switzerland) and goat anti-rabbit alkaline phosphatase-conjugated antibody (Sigma), as primary and secondary antibodies, respectively. Electrospray ionization mass spectrometry (ES-MS) was performed on a Micromass Bio-Q II-ZS triple-quadrupole atmospheric-pressure instrument with an electrospray interface. Samples, at 10 μM in a 1:1 water/acetonitrile mixture with 0.2% formic acid, were introduced via a loop injector into the electrospray source. UV−visible absorption spectra were recorded with a Varian Cary 50 Bio spectrophotometer. Reduced spectra were recorded immediately after the addition of a few crystals of disodium dithionite to the protein samples. ^{1}H NMR experiments were performed at 600 MHz using a home-built spectrometer in the Department of Biochemistry NMR facility. Protein samples were prepared at a concentration greater than 2 mM in 100 mM NaCl (pH 7.2) in a 95% H_2O/5% D_2O mixture (Sigma). All experiments were conducted at 25 °C. Two-dimensional (2D) total correlation spectroscopy (TOCSY) experiments were conducted with a spectral width of 8000 Hz in both dimensions, 64 acquisitions, and 360 and 1K complex points in F_1 and F_2, respectively. An isotropic mixing time of 30 ms was used.

Determination of the Reduction Potential of Holo-CcmE′. Potentiometric titrations were performed using the method of Dutton (*31*) with a Varian Cary 50 Bio spectrophotometer and with the protein sample under argon. Sodium dithionite was used as a reductant and potassium ferricyanide as an oxidant. The electrochemical titrations were carried out in the dark, with constant stirring, at 25 °C, in 4 mL of 50 mM Tris-HCl (pH 7.3) and 150 mM NaCl at a protein concentration of 18 μM. The presence of salt is required for the stability of the protein in solution and does not appear to influence the midpoint redox potential of the protein. The following mediators (purchased from Sigma-Aldrich) were added to the protein solution at a concentration of 20 μM: anthraquinone-2-sulfonic acid sodium salt ($E^\circ = -225$ mV), 2-hydroxy-1,4-naphthoquinone ($E^{\circ\prime} = -145$ mV), phenazine ethosulfate ($E^{\circ\prime} = 55$ mV), phenazine methosulfate ($E^{\circ\prime} = 80$ mV), and 2,3,5,6-tetramethyl-1,4-phenylenediamine ($E^{\circ\prime} = 260$ mV) (*31*). The electrochemical potential of the solution was monitored with a silver/silver chloride electrode connected to a Hanna Instruments meter. The electrode was calibrated using a $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ standard (*32*). The protein was initially titrated in the reductive direction and then back in the oxidative direction, and 10−15 min of equilibration time was allowed after each addition of reductant or oxidant. The titration was repeated three times. All potential values are reported versus the standard hydrogen electrode (*31*).

The data were analyzed using Origin (Microcal) and fitted to eq 1, which was derived by extension of the Nernst equation and the Beer−Lambert law.

$$A = A_{ox} + \frac{A_{red} - A_{ox}}{1 + e^{(E_0 - E)nF/RT}} \quad (1)$$

In eq 1, A is the measured absorbance at a given wavelength, A_{ox} and A_{red} are the absorbances of the fully oxidized and fully reduced protein, respectively, n is the number of electrons transferred, E_0 is the oxidation or reduction potential, and E is the measured potential. RT/F was considered to be equal to 59 mV at 25 °C.

RESULTS

Reduction Potential of CcmE. A soluble form of CcmE with heme covalently attached (holo-CcmE′) was produced in the periplasm of *E. coli* in the absence of an affinity tag, using plasmid pEC301. After purification, the protein was found to be pure by SDS−PAGE (not shown) and of the correct mass by ES-MS [mass observed for the holoprotein, 15120 ± 2 Da; expected, 15118 Da = 14502 Da (apoprotein) + 616 Da (heme)].

Holo-CcmE′ was found to be oxidized after purification, as suggested by its UV−visible spectrum. A 612 nm band was observed in the spectrum. Upon reduction, the intensity of this band decreases and α and β bands typical for a low-spin hexacoordinate heme iron appear at 556 and 525 nm, respectively (Figure 2A). Both reductive and oxidative titrations were reversible and yielded midpoint potentials of −118 ± 5 and −123 ± 3 mV, respectively; in each case, the best fit to the Nernst equation was to $n = 1$. Since no significant hysteresis was observed, the data from both oxidative and reductive titrations were averaged and fitted to eq 1, with an average midpoint potential of −121 mV and an n value of 1 (Figure 2B). Rapid reoxidation by atmospheric oxygen of reduced holo-CcmE′ was noted to occur during the mixing time in a spectrophotometer cuvette, which is consistent with the relatively low potential we have measured.

Effect of Mutations in the Heme-Binding Site of CcmE on Cytochrome c Maturation. One of the strictly conserved residues in CcmE is Y134. Changing this residue to Ala considerably attenuated (~80%) the attachment of heme to CcmE and transfer of heme to cytochrome *c* but did not abolish it, suggesting that this amino acid is important, but not essential, for CcmE activity (*22*). However, since Y134

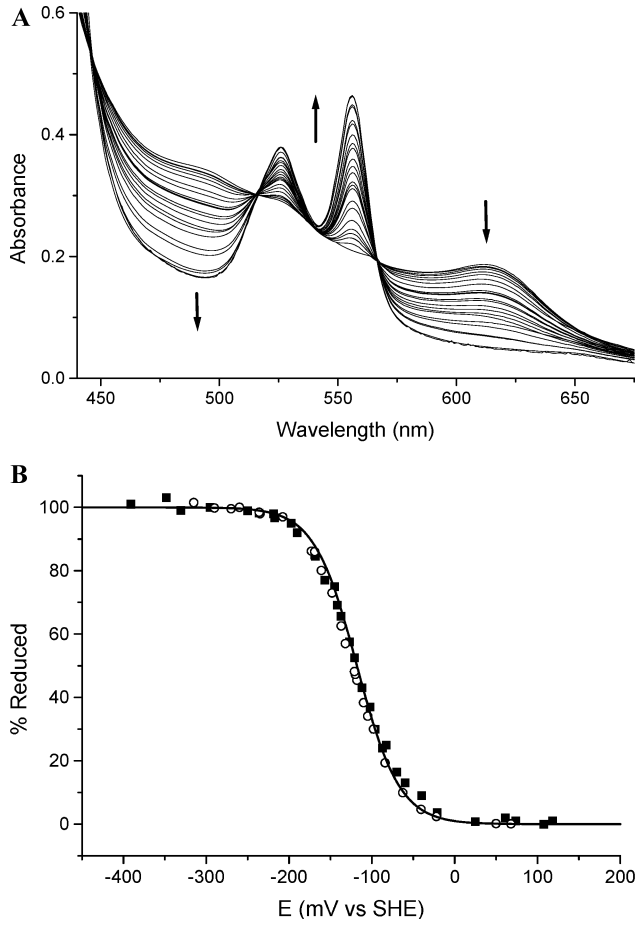

FIGURE 2: (A) Reductive potentiometric titration of holo-CcmE′. The arrows indicate the direction of the absorbance changes during the titration. Protein (18 μM), in the presence of the mediators listed in the experimental procedures, in 50 mM Tris-HCl (pH 7.3) and 150 mM NaCl was titrated using dithionite as the reductant. (B) Reductive (■) and oxidative (○) titration of holo-CcmE′ monitored at 556 nm. Data are fitted to eq 1 with an n value of 1 and a midpoint potential of −121 mV.

has been demonstrated to be involved in heme ligation in CcmE (*23, 24*), its mutation to Ala did not allow determination of whether the impaired activity of CcmE was due to the removal of a heme iron ligand or caused by the loss of its aromatic ring, which might be participating in hydrophobic interactions with the heme. To distinguish between these two possibilities, phenylalanine and methionine substitutions of Y134 were performed and their effect on the activity of CcmE was examined. The Y134F variant preserves the hydrophobic character of the native protein but loses its iron coordinating function, whereas Y134M CcmE lacks the phenyl ring but contains a potential heme coordinating group. In addition, mutants of CcmE carrying Ala insertions in the highly, but not completely, conserved $L^{127}AKHDENYTP^{136}$ region of CcmE were constructed to test for the existence of a well-defined heme-binding site within the protein.

The mutations were introduced in pEC86, a plasmid that encodes the entire *ccm* operon, so that native copies of all the other *ccm* genes are transcribed at the same level as the variant CcmE proteins. The effect of these mutations on cytochrome *c* maturation was studied by monitoring expression levels of *Paracoccus denitrificans* cytochrome c_{550} in a Δ*ccm* genetic background complemented by variants of pEC86. Maturation of *P. denitrificans* cytochrome c_{550} is strictly dependent on the presence of the Ccm apparatus (*33*). The level of expression of cytochrome c_{550} was estimated from normalized periplasmic extracts by absorbance spectroscopy. The absorbance of the dithionite-reduced periplasmic fractions was monitored at 550 and 521.5 nm, wavelengths corresponding to the maxima of ferrous cytochrome c_{550} α and β bands, respectively. The levels of cytochrome c_{550} formed in the presence of CcmE mutants were calculated relative to those obtained in the presence of wild-type CcmE, which was assigned a value of 100%. The growth conditions were aerobic, which represses transcription of the *E. coli* endogenous cytochromes. Thus, in the absence of the pEC86 plasmid, no exogenous *c*-type cytochrome is produced (Table 2). Y134F CcmE was capable of producing almost wild-type levels of the cytochrome *c*, whereas the Y134M mutant retained three-quarters of the wild-type activity (Table 2).

Table 2: Levels of Cytochrome c_{550} Production for CcmE Variants Expressed from pEC86[a]

pEC86 variant cotransformed with pKPD1	% cytochrome c_{550} production
pEC86 (WT)	100 ± 5
pEC862 (Y134F)	92 ± 3
pEC869 (Y134M)	73 ± 6
pEC871 (L^{127}*A^{128})	9 ± 3
pEC872 (A^{128}*K^{129})	11 ± 4
pEC866 (K^{129}*H^{130})	0
pEC867 (H^{130}*D^{131})	0
pEC870 (D^{131}*E^{132})	0
pEC873 (E^{132}*N^{133})	13 ± 2
pEC874 (N^{133}*Y^{134})	17 ± 7
pEC875 (Y^{134}*T^{135})	41 ± 3
pEC876 (T^{135}*P^{136})	33 ± 6
pEC868 (P^{136}*P^{137})	52 ± 5
pEC86 absent	0

[a] The CcmE mutation carried by each plasmid is given in parentheses (an asterisk indicates an alanine insertion). The values are relative to the amount of cytochrome produced in the presence of wild-type pEC86, which was assigned a value of 100%. The values are the average of at least three experiments.

We then sought to examine the effect of a series of alanine insertions in the heme-binding region of CcmE on its function. In contrast with other CcmE variants, the Ala insertions in the immediate vicinity of the heme-binding histidine of CcmE [after K129, H130 (shown in Figure 1), or D131] proved to be highly deleterious for cytochrome *c* formation, as no holocytochrome *c* was detected with these variants (Table 2). The Ala insertions after L127, A128, E132, and N133 also markedly reduced the activity of the chaperone, to approximately 10% of the wild-type level, whereas Ala insertions further downstream in the sequence [after Y134 (see Figure 1)] had a less dramatic effect (Table 2).

Effect of Mutations in the Heme-Binding Site of CcmE on Holo-CcmE Formation. The effect of the CcmE mutations described above was further examined by evaluating the amount of full-length heme-bound CcmE anchored in the membranes of Δ*ccm* cells transformed with the modified versions of pEC86. The holo-CcmE cannot donate its heme to an apo *c*-type cytochrome under these conditions, since no exogenous cytochrome was coexpressed in these experiments and the expression of endogenous cytochromes is aerobically repressed. Holo-CcmE is the only protein revealed on the SDS−PAGE gel of membrane protein extracts stained for covalently bound heme (the heme staining band

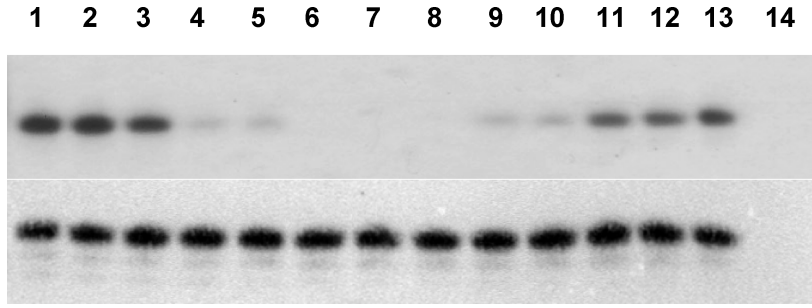

FIGURE 3: SDS−PAGE gel stained for heme covalently bound to CcmE (top panel; 50 μg of membrane protein/lane) and Western blot with an antibody against CcmE (bottom panel; 10 μg of membrane protein/lane) of membrane extracts from Δ*ccm* cells transformed with pEC86 variants carrying WT (lane 1), Y134F (lane 2), Y134M (lane 3), L^{127}*A^{128} (lane 4), A^{128}*K^{129} (lane 5), K^{129}*H^{130} (lane 6), H^{130}*D^{131} (lane 7), D^{131}*E^{132} (lane 8), E^{132}*N^{133} (lane 9), N^{133}*Y^{134} (lane 10), Y^{134}*T^{135} (lane 11), T^{135}*P^{136} (lane 12), or P^{136}*P^{137} CcmE (lane 13). Membrane extracts from untransformed Δ*ccm* cells were used as a negative control (lane 14). An asterisk indicates an alanine insertion.

at a mass corresponding to that of CcmE in lane 1 of the top panel of Figure 3; lane 14 shows a negative control). Interestingly, the levels of heme-bound CcmE detected follow a trend similar to that of the holocytochrome *c* expression pattern in the presence of these mutants (comparison of the top panel of Figure 3 with Table 2). Equivalent levels of holo-CcmE were detected in the membranes from cells expressing WT and Y134F CcmE (Figure 3, lanes 1 and 2, respectively), whereas slightly less heme was bound to the Y134M mutant (Figure 3, lane 3). Ala insertions after K129, H130, and D131 completely prevented CcmE from binding heme covalently (Figure 3, lanes 6−8, respectively), whereas Ala insertions after L127, A128, E132, and N133 reduced the level of holo-CcmE by ~90% (Figure 3, lanes 4, 5, 9, and 10, respectively). Finally, the intensities of the bands corresponding to holo-CcmE with Ala insertions after Y134 (Figure 3, lanes 11−13) are approximately half of that of the wild type (as determined by densitometry with a Gel Doc apparatus from Bio-Rad). A Western blot performed on the membrane extracts with an antibody against CcmE (*29*) showed that similar amounts of CcmE polypeptide were inserted in the membranes in all cases (Figure 3, bottom panel).

To ensure that the alanine insertions in the heme-binding region of CcmE′ did not disrupt the structure of the protein, we examined by NMR spectroscopy two of the variants that had the most dramatic effects on function in vivo. Point mutations were made in a plasmid expressing soluble CcmE [pE151 (*26*)] with a cleavable His_6 tag. The two proteins, K^{129}*H^{130} and H^{130}*D^{131}, were overexpressed, purified, cleaved of their affinity tags, and shown to be pure by SDS−PAGE analysis (not shown). Assignments of NMR spectra of CcmE are available (*15, 16*). ^{1}H 2D NMR experiments were performed on samples, at concentrations greater than 2 mM, of the two proteins; the 2D TOCSY spectra of the fingerprint regions, containing the H^N−H^α cross-peaks of the β-barrel core, are shown in Figure 4. Panel A shows the spectrum of wild-type CcmE′ for comparison with those of the variant proteins, which are shown in panels B (K^{129}*H^{130}) and C (H^{130}*D^{131}). This region of the spectrum shows more than 20 fingerprint region cross-peaks and demonstrates that the conformation of the β-barrel core of CcmE (shown in Figure 1) is very similar in the variant proteins and the wild-type protein. Methyl resonances which are shifted upfield of ~0.5 ppm are considered characteristic of the specific packing of aromatic and methyl side chains in the hydrophobic protein core and are indicative of the

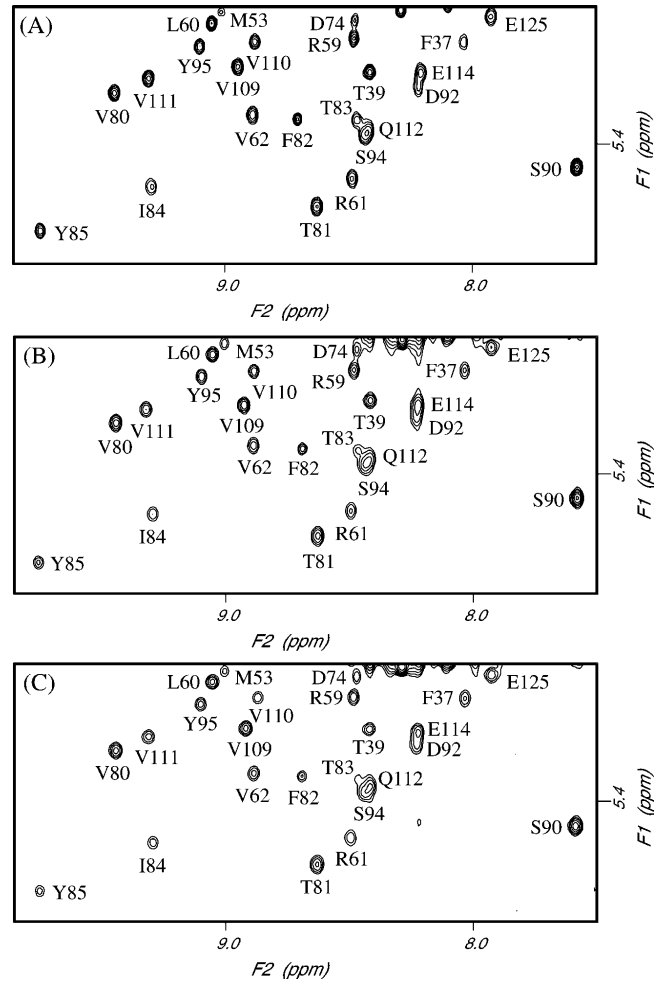

FIGURE 4: Portion of the fingerprint region of the two-dimensional TOCSY spectrum of wild-type (A), K^{129}*H^{130} (B), and H^{130}*D^{131} (C) CcmE′ showing H^N−H^α cross-peaks arising from β-sheet regions of the structure of the protein.

folded state of the protein. The regions of the TOCSY spectra of the three proteins where upfield-shifted resonances of Leu, Val, and Ile side chain methyl groups are located are virtually identical (data not shown), indicating that the alanine insertion variants possess the same fold as wild-type CcmE′. Although the C-terminal region is relatively unstructured in the apoprotein and thus difficult to study by NMR, it can be concluded that the alanine insertions did not induce any significant ordering of structure in this region.

Met Cannot Substitute for Y134 as a Heme Ligand in Holo-CcmE. To study the ligation of the heme in the Y134M

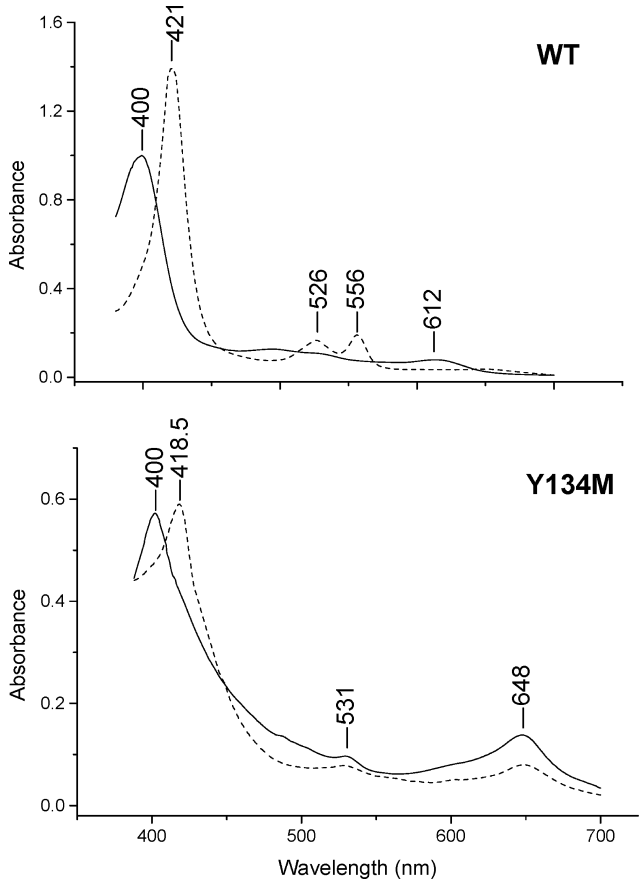

FIGURE 5: Visible spectra of wild-type and Y134M holo-CcmE′. The top panel shows data for the purified wild-type protein and the bottom panel data for the Y134M variant. Samples contained either form of the holoprotein at 12 μM in 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl. The spectra of ferrous proteins (---) were collected after the addition of disodium dithionite to the ferric samples (—).

mutant, an untagged version of holo-CcmE′ carrying this mutation was expressed from plasmid pE3011 in the periplasm of *E. coli* and purified. After the protein was confirmed to be pure by SDS−PAGE (not shown) and of correct mass by ES-MS [mass observed for the holoprotein, 15088 ± 1 Da; mass expected, 15088 Da = 14472 Da (apoprotein) + 616 Da (heme)], its spectroscopic properties were investigated. Interestingly, the absorbance spectra of the Y134M variant in both reduced and oxidized states (Figure 5) were identical to those of the Y134F mutant used for resonance Raman analysis (*23*), indicating that the heme iron in the reduced Y134M CcmE is high-spin pentacoordinate, as in the Y134F variant, as opposed to the wild-type protein, which contains a low-spin hexacoordinate ferrous iron (Figure 5). This shows that Met cannot substitute for Y134 as a heme ligand in holo-CcmE. Nevertheless, substantial cytochrome *c* formation is seen in the Y134M variant (Table 2). It would be interesting to determine the redox potential of the Y134M and Y134F variants, but we have not been able to obtain satisfactory titrations for these forms.

DISCUSSION

The existence of a solvent-exposed heme-binding site on the surface of CcmE has been inferred from the structure of the apoprotein (*16*) and mutagenesis analysis (*22*); however, its exact location has not been determined, and the resistance to many nonconservative sequence variations adjacent to H130, which covalently attaches heme, is puzzling. We have sought to learn more about the heme-binding site through measurement of redox potential and the unorthodox approach of inserting alanines both on the N-terminal side of H130 and into the relatively unstructured C-terminal part of the protein following this heme-binding histidine.

Reduction Potential of CcmE. A good correlation between the exclusion of water from the heme surface and heme reduction potential values has been observed in heme-containing proteins. Exposure of heme moieties to aqueous solvents has been shown to lower reduction potentials (*17, 18, 34*). However, other factors are involved; axial ligation is another important factor regulating midpoint potential values of heme cofactors (*35, 36*). Ligation of ferrous heme in purified holo-CcmE has been shown by resonance Raman spectroscopy to involve Y134 and a histidine residue, whereas covalently attached ferric heme in CcmE is pentacoordinate with a tyrosine ligand (*23*); tyrosine coordination was confirmed by EPR (*24*). For *E. coli* CcmE, His147 can be an axial ligand (see later). HasA is a bacterial hemophore that exhibits His/Tyr axial heme coordination (that is not redox-dependent) and is involved in binding extracellular heme in an iron acquisition pathway (*37*). A reduction potential of −550 mV has been reported for this protein (*38*), and this unusually low value has been attributed to a high level of solvent exposure, which was subsequently confirmed by the X-ray structure of the protein (*39*). The dramatic difference between the redox potential of CcmE determined here (−121 mV) and that of HasA, in which similar ligation of the heme iron occurs, suggests that the level of heme contact with the solvent in CcmE is probably not as high as initially proposed. Amino acids located in the C-terminal domain of the heme chaperone might provide a protective function of the heme center of CcmE from the aqueous environment, which has been proposed previously in our work examining the function of the C-terminal region of CcmE (*27*). We have found evidence for this in our studies of the ligation of noncovalently bound heme in CcmE, which differs significantly from holo-CcmE in that H130 ligates the heme in the ferric form, to be replaced by other ligands in the reduced form. H147 was identified in the latter case as a ligand, demonstrating that a residue in the flexible C-terminal domain can interact with the heme. However, one of the current puzzles concerning CcmE is the failure to identify a conserved axial ligand to the heme other than Y134, although H147 can be the second ligand in the *E. coli* protein. Note that, unlike CcmE, HasA does not covalently attach its heme cofactor, but covalent modification of the vinyl groups of the porphyrin ring has been found to have little effect on the redox properties of the heme iron within a protein (*41*). Therefore, it is reasonable to expect that the main contributor to the difference between the reduction potential of the two heme carriers, CcmE and HasA, is the heme environment, including exposure to water. This is supported by previous resonance Raman studies on CcmE (*23*), which indicate that the heme in holo-CcmE is not highly solvent exposed. When CO is bound to heme in a hemoprotein, line broadening of the C−O stretching mode in resonance Raman spectra is caused by the presence of water molecules; this band was not broadened in the case of CcmE (*23*), again suggesting

the exclusion of solvent from the heme-binding region. There are other examples of hemoglobin and myoglobin variants (naturally occurring and produced by mutation) that have His/Tyr coordination of the heme iron (*42*−*45*). One study showed that the tyrosine-ligated variant had a 250 mV lower reduction potential that the wild-type protein (*45*), indicating that tyrosine ligation contributes to significant lowering of the redox potential of a heme iron. Overall, although the redox potential of a protein-bound heme reflects many local environmental effects, a tyrosine axial ligand and high solvent exposure seem always each to contribute to a low potential, relative to histidine ligation and a buried environment. Thus, a value for CcmE, where tyrosine is a ligand, of only −120 mV is certainly suggestive of a relatively buried heme.

Alanine Insertions around the Heme-Binding Histidine of CcmE. Alanine insertion scanning mutagenesis within transmembrane α-helices has been used to probe membrane protein structure and function (*46*−*48*). We have applied this approach to a soluble domain to confirm further the existence of a solvent-protected heme-binding pocket in CcmE. Single Ala insertions were carried out in the highly conserved L^{127}AKHDENYTP^{136} sequence of CcmE [consensus motif, $\underline{Hy}^{126}$LAKHDE$\underline{X}$Y$\underline{X}$P^{136}, as derived from the sequence analysis of 151 nonredundant homologues from bacterial and plant species (*49*)]. Insertion of Ala after K129, H130, and D131 of CcmE abolishes cytochrome *c* formation and heme binding to CcmE (Table 2 and Figure 3, lanes 6−8, respectively) while not affecting insertion of the protein into the cytoplasmic membrane or disrupting the structure of CcmE. The only point mutation in CcmE that generated a similar phenotype is replacement of H130 with Ala. H130A CcmE was incapable of maturing *c*-type cytochromes because of its inability to bind heme covalently (*26*). It is notable that K129A, D131A, E132A, and D131A/E132A variants of CcmE resulted in continued covalent attachment of heme to H130 and either an only marginal (the two single mutants) or 30% decrease in cytochrome *c* production in previous work (*22*). In contrast, when positions 129, 131, and 132 become alanine as a result of mutational insertion in our experiments, cytochrome *c* formation is abolished. Thus, whereas alanine at positions 129, 131, and 132 per se is compatible with physiological function, transfer of heme to apocytochrome *c*, the consequence of insertion of an extra alanine at these positions clearly shows that there is some required structural feature in holo-CcmE around H130. It cannot just be a question of the relative position of Y134 with respect to H130 as Y134 can be replaced by others (e.g., F or M in this work) without a loss of function. We would emphasize that the unorthodox use of alanine insertions rules out the possibility, as implied by the results of point mutations, that the polypeptide chain adjacent to H130 is of little significance for the function of CcmE.

The activity-disrupting Ala insertions before H130 could be explained by a shift of this residue away from a spatially well defined binding site that would place the reacting vinyl group of the docked heme out of the reach of the H130 imidazole. The insertions after the heme-binding histidine could prevent the C-terminal domain from adopting a conformationally rigid heme pocket between the heme docking platform, modeled on the surface of the β-barrel core, and the C-terminal region of the protein. Enggist et al. have proposed a model for heme binding to CcmE (*16*) in which K129 was suggested to interact with a propionate of heme. This interaction is not essential as K can be replaced with alanine with only moderate attenuation of function and heme binding (*22*). The fact that an insertion of alanine at position 129, as opposed to a substitution (*22*), cannot be tolerated suggests that the binding site for heme relies upon a set of noncovalent interactions and that while there is limited sequence conservation, there is a cooperative effect of many residues in the vicinity of H130 that is lost with the insertion of one alanine.

A plausible explanation for our results is that a heme pocket would involve the amino acids in the vicinity of H130, because Ala insertions further downstream in the sequence, after Y134, proved to have less dramatic effects on the binding of heme to CcmE and the activity of the chaperone (Figure 3, lanes 11−13, and Table 2). In fact, the C-terminal domain of the shortest CcmE homologues from *Rickettsia* (*50*) and *Wolbachia* (*49*) species terminates four amino acids after this tyrosine residue (Y134 in *E. coli*), suggesting that the amino acids downstream of this position are not essential for the function of the protein. NMR spectroscopy showed that the region on the C-terminal side of H130 is relatively unstructured in apo-CcmE (*16*) containing a single helical turn, which is followed by an unstructured tail. In light of the alanine insertion mutagenesis study presented here, it seems that the presence of the cofactor, and/or in vivo interacting partner proteins, orders the C-terminal region of the CcmE, presumably to render it structurally suitable for accommodating heme, and for receiving it from other components (e.g., CcmC) of the Ccm system.

Heme Ligation in CcmE. The formation of a heme pocket could be required to shield the cofactor from solvent exposure and/or nonspecific interactions with other proteins. The internal 3D configuration of the heme pocket in CcmE might be more important than the nature of the contributing amino acids, which would explain why amino acid substitutions in the binding site are only partially detrimental to heme binding, but more significant spatial perturbations, such as those induced by Ala insertions, completely abolish activity. The conformational rigidity of the heme pocket would account for the observation that Y134 is irreplaceable in heme ligation. Substitution of this tyrosine with Met (Figure 5) did not provide axial coordination to the bound heme, presumably because the side chain of this residue is not sufficiently long to reach the iron center. Archaea and some bacteria have recently been shown to possess homologues of CcmE with the heme-binding histidine replaced with a cysteine residue (*49*). With the exception of the tyrosine residue involved in heme iron coordination (Y134 in *E. coli*), a different conservation pattern was observed in the heme-binding motif of the archaeal-type CcmEs. These additional sequence variations may compensate for the structural perturbation induced by the His for Cys substitution in these proteins and preserve the overall conformation of the heme-binding site.

Investigation of a Y134F CcmE variant by resonance Raman showed that the heme iron in this mutant is pentacoordinate high-spin in the ferrous form, as opposed to the wild type, which displays a hexacoordinate low-spin iron under reducing conditions, implying that one of the heme axial ligands has been lost in the variant protein (*23*). The

fact that Y134F CcmE is capable of binding heme (*23*) and producing cytochrome *c* at almost wild-type levels (Figure 3, lane 2, and Table 2) indicates that the heme iron axial ligation in CcmE is not essential for binding of the cofactor to the chaperone. Interestingly, site-directed mutagenesis in the predicted heme-binding site of CcmE showed that loss of hydrophobic residues was more detrimental to heme binding than mutations affecting polar amino acids, suggesting that the mechanism of heme binding and release relies mainly on hydrophobic interactions and the charged residues are not essential for this process (*22*). The mutagenesis results presented here suggest the same, supporting a hydrophobically driven heme interaction rather than ligation-induced binding. This is also supported by the finding that CcmE binds heme and protoporphyrin IX (heme devoid of Fe) in vitro with similar affinities (*27*), which indicates that the presence of Fe does not significantly affect the heme−protein interaction. In vivo, the heme cofactor could be transferred to the chaperone following the interaction between a putative heme-loaded CcmC and the high-affinity binding site of CcmE. The docking of the heme moiety could be followed by axial ligation of the heme iron and, ultimately, formation of the covalent bond with H130, which might stabilize the interaction and fix heme into a correct spatial orientation that facilitates its subsequent stereospecific transfer to the apocytochrome. This is supported by the observation that in vitro, heme forms a noncovalent complex with CcmE, before the covalent reaction occurs (*26, 51*).

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