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Biochemical and Mutational Characterization of the Heme Chaperone CcmE Reveals a Heme Binding Site

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CcmE is a heme chaperone that binds heme transiently in the periplasm of *Escherichia coli* and delivers it to newly synthesized and exported *c*-type cytochromes. The chemical nature of the covalent bond between heme and H130 is not known. We have purified soluble histidine-tagged CcmE and present its spectroscopic characteristics in the visible range. Alanine scanning mutagenesis of conserved amino acids revealed that H130 is the only residue found to be strictly required for heme binding and delivery. Mutation of the hydrophobic amino acids F37, F103, L127, and Y134 to alanine affected CcmE more than mutation of charged and polar residues. Our data are in agreement with the recently solved nuclear magnetic resonance structure of apo-CcmE (PDB code 1LIZ) and suggest that heme is bound to a hydrophobic platform at the surface of the protein and then attached to H130 by a covalent bond. Replacement of H130 with cysteine led to the formation of a covalent bond between heme and C130 at a low level. However, the H130C mutant CcmE was not active in cytochrome *c* maturation. Isolation and characterization of the heme-binding peptides obtained after a tryptic digest of wild-type and H130C CcmE support the hypothesis that heme is bound covalently at a vinyl group.

The biogenesis of *c*-type cytochromes is a complex process by which the heme cofactor is bound to the apocytochrome polypeptide via two covalent thioether bonds. In *Escherichia coli*, eight gene products are necessary for this posttranslational maturation. A key function has been attributed to CcmE. This membrane-anchored protein is oriented with its active domain towards the periplasm and binds heme covalently prior to delivering it to apocytochromes (18). Heme is bound transiently, and thus, holo-CcmE is a true intermediate in the pathway leading to the formation of *c*-type cytochromes. Extending the concept of metallochaperones (4, 9, 15), which represent proteins that bind redox-reactive metals and transport them to their biological partners while preventing premature redox reactions, CcmE was called a heme chaperone (18).

Remarkably, CcmE binds heme covalently at the histidine H130, as demonstrated by Edman degradation, ion spray, and tandem mass spectrometry of the CcmE-derived tryptic heme peptide (18). However, the nature of the heme-histidine bond is not known yet. It has also been shown previously that H130 is required for heme binding of CcmE and also for cytochrome *c* maturation (18). Therefore, the covalent attachment of heme to H130 is not an artifact but represents a true intermediate of the heme delivery pathway (20). Heme binding to CcmE has a strict requirement only for CcmC, but it is most efficient in the presence of CcmABCD (17). CcmC was shown to interact directly with both heme and CcmE (14).

It was the aim of this work to characterize CcmE biochemically and genetically. The soluble domain of CcmE was purified in a His-tagged form and analyzed spectroscopically. Different methods were applied to achieve information about the heme binding site, the nature of the covalent heme-histidine bond, and the mechanism of the transfer of heme to *c*-type cytochromes. A sequence alignment of 27 bacterial and plant CcmE homologues revealed 26 residues with >90% sequence identity. From these, 11 mostly invariant charged, polar, or hydrophobic amino acids were selected and changed to alanine by site-directed mutagenesis. In addition to these 11 mutants, another three less-conserved residues were investigated. In a first step, single amino acids were exchanged to seek for CcmE point mutants expected to be deficient in either heme binding or heme release. With the exception of H130, no other residue was found to be required strictly for CcmE function. H130 was also mutated to a cysteine to mimic the situation in *c*-type cytochromes, where a covalent heme-cysteine bond is formed. Wild-type and H130C mutant CcmE were overproduced and purified, and their heme binding characteristics were compared. The results are discussed in the context of the structure of apo-CcmE that has been solved recently by nuclear magnetic resonance (6), and a model of heme binding is presented.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* cells were grown aerobically at 37°C in Luria-Bertani (LB) medium (16) or anaerobically at 30°C in minimal salts medium (10) supplemented with 0.4% glycerol, 40 mM fumarate, and 5 mM potassium nitrite as the terminal electron acceptor. If necessary, induction was performed with 0.1% arabinose at the mid-exponential growth phase. 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. All used primers were purchased from Microsynth (Balgach, Switzerland). They are listed in Table 2.

Plasmid pEC302 was constructed by QuikChange site-directed mutagenesis (Stratagene) with pEC415 as the template. For the likewise construction of pEC469, pEC472, pEC479, pEC480, pEC481, pEC482, pEC485, pEC491, pEC492, pEC493, and pEC496, the template pEC458 was used. pEC472 served as the template for constructing pEC488 and pEC495. pEC489 resulted from a one-step PCR mutagenesis with pEC458 as the template. The 947-bp *Bgl*II/*Sal*I fragment was ligated into *Bgl*II/*Sal*I-digested pEC482. The product of a similar PCR with different primers ligated into pEC481 yielded pEC494. For pEC498,

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant genotype and/or phenotype	Reference
E. coli strains		
DH5α	*supE*44 Δ*lacU169*(ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	8
EC06	MC1061 Δ(*ccmABCDEFGH*) Km^r	21
EC65	MC1061 Δ*ccmE*	18
Plasmids		
pEC86	*ccmABCDEFGH* cloned into pACYC184; Cm^r	1
pEC101	*ccmABCD* 2.38-kb *Nsi*I/*Ssp*I fragment in *Pst*I/*Fsp*I-digested pACYC184; Cm^r	This work
pEC415	*ompA′-′ccmE*$_{sol}$-H_6 cloned into *Nde*I/*Xba*I-digested pISC-2; Ap^r	18
pEC419	*ccmE* H130A 1.5-kb *Eco*RV/*Bam*HI fragment cloned into *Eco*RV/*Bam*HI-digested pACYC184; Cm^r	18
pEC439	*ccmC* 0.98-kb *Afl*II/*Bam*HI fragment cloned into *Eco*RV/*Bcl*I-digested pACYC184; Cm^r	19
pEC458	*ccmE* 1.0-kb *Fsp*I/*Bam*HI fragment cloned into *Eco*RV/*Bam*HI-digested pBR322; Ap^r	19
pEC467	*ccmE* H130A derived from pEC419 and pEC458; Ap^r	This work
pEC469	*ccmE* D131A derived from pEC458; Ap^r	This work
pEC472	*ccmE* K129A derived from pEC458; Ap^r	This work
pEC476	*ccmE* E132A derived from pEC458; Ap^r	This work
pEC479	*ccmE* D86A derived from pEC458; Ap^r	This work
pEC480	*ccmE* D101A derived from pEC458; Ap^r	This work
pEC481	*ccmE* R104A derived from pEC458; Ap^r	This work
pEC482	*ccmE* E105A derived from pEC458; Ap^r	This work
pEC485	*ccmE* Y134A derived from pEC458; Ap^r	This work
pEC488	*ccmE* K129A/D131A/E132A derived from pEC472; Ap^r	This work
pEC489	*ccmE* F103A/R104A/E105A derived from pEC458 and pEC482; Ap^r	This work
pEC491	*ccmE* F37A derived from pEC458; Ap^r	This work
pEC492	*ccmE* S70A derived from pEC458; Ap^r	This work
pEC493	*ccmE* Y95A derived from pEC458; Ap^r	This work
pEC494	*ccmE* F103A derived from pEC458 and pEC481; Ap^r	This work
pEC495	*ccmE* L127A derived from pEC472; Ap^r	This work
pEC496	*ccmE* H130C derived from pEC458; Ap^r	This work
pEC497	*ccmE* R104A/K129A derived from pEC472 and pEC481; Ap^r	This work
pEC498	*ccmE* K129A/D131A derived from pEC472; Ap^r	This work
pEC499	*ccmE* D131A/E132A derived from pEC476; Ap^r	This work
pEC302	*ompA′-′ccmE*$_{sol}$-H_6 H130C derived from pEC415; Ap^r	This work
pRJ3291	*B. japonicum cycA* cloned into *Nde*I/*Sal*I-digested pISC-2 *bla*::*kan*; Km^r	17

pEC472 served as the template. The 869-bp *Nhe*I/*Sal*I fragment was ligated into *Nhe*I/*Sal*I-digested pEC472. For the construction of pEC499, the 377-bp *Nhe*I/*Sal*I fragment from the PCR with pEC476 as the template was cloned into *Nhe*I/*Sal*I-digested pEC476, which was the result of a two-step PCR. A 105-bp fragment was amplified with primers *ccmE*C and *ccmE*E132Af with pEC458 as the template. This fragment was used as a primer together with *ccmE*N2, again with pEC458 as the template. The resulting 535-bp fragment was digested with *Bam*HI/*Eco*RV and ligated into *Bam*HI/*Eco*RV-digested pBR322. Cloning of the 707-bp *Nru*I fragment from pEC419 into *Nru*I-digested pEC458 yielded pEC467, and the ligation of the 585-bp *Nru*I fragment of pEC472 into *Nru*I-digested pEC481 resulted in pEC497.

For the construction of pEC101 expressing *ccmABCD*, a 1,189-bp *Afl*II/*Ssp*I fragment containing *ccm′BCD* was ligated into *Afl*II/*Fsp*I-digested pEC86 with *ccmAB′*. All mutations of PCR-derived plasmids were confirmed by DNA sequencing with an ABI Prism 310 genetic analyzer (Perkin Elmer).

Cell fractionation. Periplasmic fractions of 500 ml of anaerobically grown cultures were isolated by treatment with polymyxin B sulfate (Sigma). The cells were harvested by centrifugation at 4,000 × *g*, washed in cold 50 mM Tris-HCl, pH 8.0, and resuspended in cold extraction buffer (1 mg of polymyxin B sulfate/ml, 20 mM Tris-HCl, 500 mM NaCl, 10 mM EDTA [pH 8.0]) (1.5 ml/g of wet cells). The suspension was stirred 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 250 ml of aerobically grown cultures were prepared as follows. The cells were harvested by centrifugation at 4,000 × *g*, washed in cold 50 mM Tris-HCl (pH 8.0), resuspended in 3 ml of cold 50 mM Tris-HCl (pH 8.0) containing 5 μg of DNase I/ml, and passed twice through a French pressure cell at 110 MPa. Cell debris were separated by centrifugation at 40,000 × *g* for 20 min at 4°C. The supernatant was subjected to ultracentrifugation at 150,000 × *g* for 1 h. The membrane fraction was washed once with 1 ml of cold 50 mM Tris-HCl, pH 8.0, and resuspended in 200 μl of the same buffer.

Purification of His-tagged soluble CcmE. The *E. coli* strain EC06 carrying pEC415 overproduced soluble apo-CcmE-H_6 (hexahistidine tagged). One liter of LB was inoculated with 10 ml of culture grown overnight. The pellet obtained 12 h after induction with 0.1% arabinose was washed once with 50 mM sodium phosphate, pH 7.2, and resuspended in 6 ml of polymyxin buffer (150 mM NaCl, 1 mM EDTA, 50 mM sodium phosphate [pH 7.2], and 2 mg of polymyxin B [Fluka]/ml). The solution was stirred gently for 1 h at 4°C. After centrifugation, the pellet was resuspended in the same amount of polymyxin B buffer for a repeated extraction of periplasmic proteins. The two supernatants containing the periplasmic proteins were precipitated with ammonium sulfate that was added slowly to 60% saturation. After centrifugation, the pellet was dialyzed twice against 2 liters of 300 mM NaCl and 50 mM sodium phosphate, pH 7.2. Purification of CcmE-H_6 was performed by affinity chromatography on nickel-nitrilotriacetic acid (NTA)-agarose (Qiagen) with 3 column volumes of a linear gradient from 0 to 250 mM imidazole in 300 mM NaCl and 50 mM sodium phosphate, pH 7.2. Apo-CcmE-H_6 was present in fractions eluted with 100 to 200 mM imidazole. After dialyzing twice against 2 liters of 300 mM NaCl and 50 mM sodium phosphate, pH 7.2, soluble CcmE-H_6 was concentrated with Centricon-10 (Millipore) up to 60 mg/ml.

When plasmid pEC101 containing *ccmABCD* was overexpressed in cells containing pEC415, a mixture of soluble holo- and apo-CcmE-H_6 was obtained and purified by the same procedure. To isolate holo-CcmE-H_6, the affinity-purified proteins were subjected to hydrophobic interaction chromatography (phenyl Sepharose 6 fast flow, high sub; Amersham Biosciences). The sample was brought to 1 M ammonium sulfate, 300 mM sodium chloride, and 50 mM sodium phosphate (pH 7.2) and eluted with a linear gradient of 3 column volumes from 1 to 0 M ammonium sulfate. Both components eluted shortly after reaching 0 M ammonium sulfate in two peaks, and apo-CcmE-H_6 eluted first. The purity of the samples was checked by reverse-phase chromatography on a LiChrospher 100 RP-18 high-performance liquid chromatography (HPLC) cartridge (Hewlett-Packard) with a linear acetonitrile gradient in 0.1% trifluoroacetic acid. The protein was monitored at 215 and 398 nm. Samples with an A_{400}/A_{280} ratio of >2.7 were found to contain only holo-CcmE. To remove degradation products, which had lost some C-terminal amino acids, including the His-tag, a second

TABLE 2. Oligonucleotides used for plasmid construction and sequencing

Primer	Sequence (5′–3′)[a]	Restriction site	Use[b]	Plasmid(s)
*ccmE*F37Af	CGAATATCGATCTC**GCT**TATAC**C**CCGGG**G**GGAAATTCTCTACG	*Sma*I	QC	pEC491
*ccmE*F37Ar	CGTAGAGAATTTCC**C**CCGGG**G**GTATAA**GC**GAGATCGATATTCG	*Sma*I	QC	pEC491
*ccmE*S70Af	GGCGGGATGGTGATG**C**CCGGG**G**GCTGTGCAGCGCGATCCC	*Sma*I	QC	pEC492
*ccmE*S70Ar	GGGATCGCGCTGCACA**GC**CCC**G**GGCATCACCATCCCGCC	*Sma*I	QC	pEC492
*ccmE*D86Af	CCTTCACCATTTACG**C**TGCTGAAGGCTCAGTGG		QC	pEC479
*ccmE*D86Ar	CCACTGAGCCTTCAGCA**G**CGTAAATGGTGAAGG		QC	pEC479
*ccmE*Y95Af	GGCTCAGTGGATGTCT**CTGCAG**AAGGCATTTTGCCGG	*Pst*I	QC	pEC493
*ccmE*Y95Ar	CCGGCAAAATGCCTT**CTGCAG**AGACATCCACTGAGCC	*Pst*I	QC	pEC493
*ccmE*D101Af	CGAAGGCATTTT**G**CCGG**C**TCTGTTCCGTGAAGGGC	*Nae*I	QC	pEC480
*ccmE*D101Ar	GCCCTTCACGGAACAGA**G**CCGG**C**AAAATGCCTTCG	*Nae*I	QC	pEC480
*ccmE*F103A	GGCATTTTGCC**A**GATCT**G**G**C**CCGTGAAGGGCAGGGCG	*Bgl*II	PCR	pEC494
*ccmE*R104Af	CGAAGGCATTTTGCC**A**GATCTGTTCGCTGAAGGGCAGGGCG	*Bgl*II	QC	pEC481
*ccmE*R104Ar	CGCCCTGCCCTTCA**GC**GAAC**A**GATCTGGCAAAATGCCTTCG	*Bgl*II	QC	pEC481
*ccmE*E105Af	CGAAGGCATTTTGCC**A**GATCTGTTCCGTG**C**AGGGCAGGGCGTTG	*Bgl*II	QC	pEC482
*ccmE*E105Ar	CAACGCCCTGCCCT**G**CACGGAAC**A**GATC**T**GGCAAAATGCCTTCG	*Bgl*II	QC	pEC482
*ccmE*L127Af	CCTCGCGAAAGAAGTG**GCA**GCGAAACACGATGAAAACTATACG		QC	pEC495
*ccmE*L127Ar	CGTATAGTTTTCATCGTGTTTCGC**TGC**CACTTCTTTCGCGAGG		QC	pEC495
*ccmE*K129Af	CGCGAAAGAAGT**GCTAGC**GGCACACGATGAAAACTATACG	*Nhe*I	QC	pEC472
*ccmE*K129Ar	CGTATAGTTTTCATCGTGT**GC**C**GCTAGC**ACTTCTTTCGCG	*Nhe*I	QC	pEC472
*ccmE*H130Cf	CGAAAGAAGT**GCTAGC**AAA**TG**CGATGAAAACTATACG	*Nhe*I	QC	pEC302, pEC496
*ccmE*H130Cr	CGTATAGTTTTCATCG**CA**TTTC**GCTAGC**ACTTCTTTCG	*Nhe*I	QC	pEC302, pEC496
*ccmE*D131Af	GTGCTGGCGAAACACG**CA**GAAAACTATACGCCGCC		QC	pEC469
*ccmE*D131Ar	GGCGGCGTATAGTTTTC**TG**CGTGTTTCGCCAGCAC		QC	pEC469
*ccmE*E132Af	GTGCTGGCGAAACACGATG**C**AAACTATACGCCGCC		PCR	pEC476
*ccmE*Y134Af	GAAGTGC**TGGCCA**AACACGATGAAAAC**GC**TACGCCGCCAG	*Msc*I	QC	pEC485
*ccmE*Y134Ar	CTGGCGGCGTA**GC**GTTTTCATCGTGTTT**TGGCCA**GCACTTC	*Msc*I	QC	pEC485
*ccmE*DE-Af	AAGAAGTGCT**A**GCGAAACACG**C**TG**C**AAACTATACG		PCR	pEC499
*ccmE*KD-Af	AAGAAGTGCT**A**GCG**GC**ACACG**C**TGAAAACTATACG		PCR	pEC498
*ccmE*KDE-Af	GCGAAAGAAGTGCTGGCG**GC**ACACG**C**TG**C**AAACTATACGCCG		QC	pEC488
*ccmE*KDE-Ar	CGGCGTATAGTTT**G**CA**G**CGTGT**GC**CGCCAGCACTTCTTTCGC		QC	pEC488
*ccmE*FRE-A	GGCATTTTGCC**A**GATCTGGCC**GC**TGCAGGGCAGGGCG	*Bgl*II	PCR	pEC489
*ccmE*C	CG**GGATCC**TCATGATGCTGGGTCC	*Bam*HI	PCR	pEC476
*ccmE*N2	GC**GATATC**GGGCGCGTGAGGCGCGTTTACG	*Eco*RV	PCR	pEC476
pBR322 770–751	CACCTGTCCTACGAGTTGC		PCR	pEC489, pEC494, pEC498, pEC499
*ccmF*17283–264	TACACGGACAGCAGCAGCGC		SEQ	
pACYC184 1613–1629	CGGCACCGTCACCCTGG		SEQ	
pACYC184 1959–1943	CCCCATCGGTGATGTCG		SEQ	
pING1*araB*	GGCCGACGAAATCACTCG		SEQ	
pBR322 4269–87N	CGAAAAGTGCCACCTGACG		SEQ	

[a] Underlined nucleotides indicate the restriction site, and boldface characters are point mutations.
[b] QC, QuikChange mutagenesis; SEQ, sequencing.

purification by nickel-NTA-agarose was performed, in which only full-length protein was present.

Biochemical methods. Protein concentrations were determined by using the Bradford assay (Bio-Rad). Heme staining of proteins separated by sodium dodecyl sulfate (SDS)–15% polyacrylamide gel electrophoresis was carried out with *o*-dianisidine (Sigma) as the substrate as described previously (19). Immunoblot analysis was performed with a CcmE-specific antiserum directed against the synthetic peptide ^{129}KHDENYTPPEVEKAME^{144} (18). Signals were detected by using goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate (Bio-Rad) as a secondary antibody and disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate (Roche Diagnostics) as the substrate.

Purified soluble CcmE-H_6 was digested with 1% (wt/wt) trypsin (sequencing grade; Roche Diagnostics) overnight at 37°C. After centrifugation, the sample was prepared for HPLC by filtering it through a 0.22-μm-pore size filter. The peptides were separated on a Zorbax 300SB-C18 reverse-phase column (Hewlett-Packard) with a linear acetonitrile gradient (20 to 30%) in 0.1% (vol/vol) trifluoroacetic acid. Peptides were monitored at 215 nm, and heme was monitored at 398 nm. The peak fractions containing the heme-binding peptides were collected and lyophilized.

Protein precipitation with trichloroacetic acid was performed with a 100% (wt/vol) solution, which was added to a final concentration of 10%. Samples were kept on ice for 15 min and then centrifuged at maximal speed. The pellet was washed quickly with 2 M Tris-HCl, pH 8.0, and resuspended in 3× SDS loading dye.

Spectroscopic methods. Optical spectra were recorded on a Hitachi model U-3300 spectrophotometer. Samples were reduced by adding a few grains of sodium dithionite and oxidized by adding $K_3Fe(CN)_6$ to a final concentration of 0.05 mM. For the CO difference spectrum, the reduced sample was bubbled for 2 min with CO and measured immediately. Pyridine hemochrome spectra were used for quantification of heme with an extinction coefficient at 551 nm of 29.1 mM^{-1} cm^{-1} (7).

For mass spectroscopy, the peptides were resuspended in a saturated solution of sinapinic acid in H_2O-acetonitrile (2:1) and 0.1% trifluoroacetic acid. One microliter was deposited directly onto the mass spectrometer target. After drying, the sample was washed again with 1 μl of H_2O-acetonitrile (2:1) and 0.1% trifluoroacetic acid. Mass spectroscopy was performed on a Perseptive Biosystem Voyager Elite matrix-assisted laser desorption ionization–time of flight (mass spectrometry) with reflector and delayed extraction.

RESULTS

Point mutations of charged amino acids in CcmE. The periplasmic membrane-anchored protein CcmE functions as a heme chaperone during cytochrome *c* maturation by binding heme covalently and subsequently delivering it to *c*-type cytochromes. The aim of this work was to investigate the role of specific classes of amino acids in heme transfer by alanine scanning mutagenesis. To test whether heme binding and release were dependent on an acid- or base-catalyzed mechanism, we first changed some of the best conserved charged

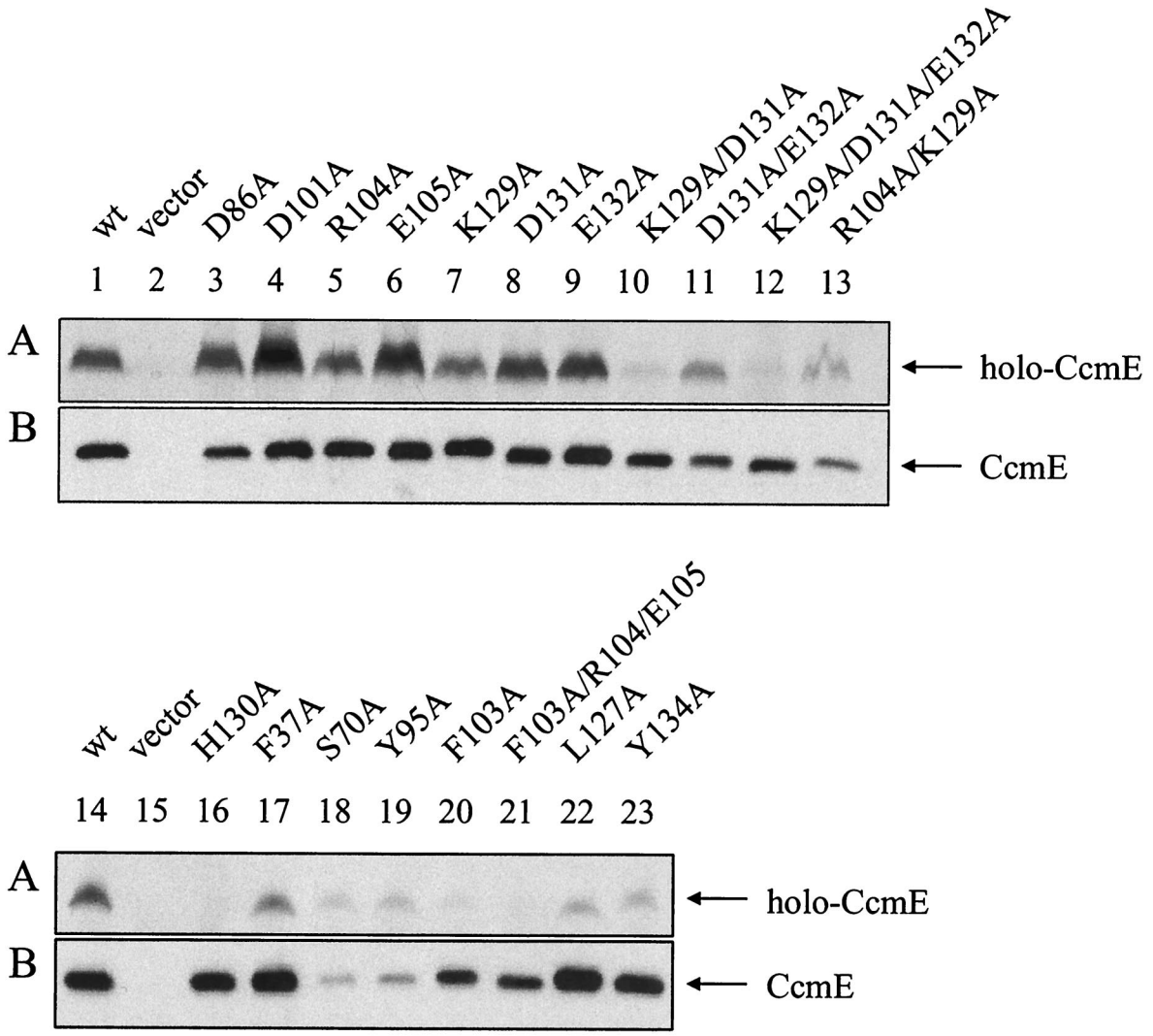

FIG. 1. Phenotypic characterization of *ccmE* mutants. The Δ*ccmA-H* strain EC06 was cotransformed with *ccmC* (pEC439) and a plasmid containing the mutated *ccmE* alleles. Cells were grown aerobically in LB medium. (A) Membrane proteins (100 μg per lane) were separated on an SDS–15% polyacrylamide gel and analyzed by heme staining. (B) Western blot of the same membrane fractions (20 μg per lane) probed with antiserum directed against CcmE. Upper panel, point mutants of charged amino acids; Lower panel, point mutants of aromatic, hydrophobic, and uncharged polar amino acids. wt, wild type.

amino acids of CcmE. The two best conserved motifs of CcmE, ^{99}LPDLFREG^{106} and ^{127}LAKHDE^{132}, contain several charged amino acids. Residues D101, R104, E105, K129, D131, and E132 were mutated. In addition, the invariant D86 was also changed to alanine. All constructed plasmids were pBR322 derivatives with *ccmE* expressed under the control of the tetracycline promoter of the vector.

The constructed plasmids were cotransformed with a plasmid carrying *ccmC* into strain EC06 (Δ*ccmA-H*). In this minimal system, wild-type CcmE can bind heme, but no *c*-type cytochromes are formed due to the lack of the other *ccm* genes (17). The mutants were tested for their ability to form holo-CcmE by heme staining of membrane proteins (Fig. 1A). Different quantities of holo-CcmE were detected in the mutants (Fig. 1A, lanes 3 to 9). No single mutant showed a complete deficiency of heme binding as it is found for mutant H130A (18) (Fig. 1A, lane 16). The presence of the CcmE polypeptide in the membrane was assessed by Western blot analysis. All mutants produced wild-type or only slightly reduced levels of CcmE (Fig. 1B, lanes 3 to 9).

To check if these mutants were affected in heme delivery to cytochrome *c*, a Δ*ccmE* in-frame deletion strain was complemented with a plasmid carrying either wild-type or mutant *ccmE* alleles. Coexpression of the heterologous *Bradyrhizobium japonicum* gene *cycA* under anaerobic growth conditions with nitrite as the terminal electron acceptor allowed us to analyze the formation of two soluble periplasmic *c*-type cytochromes, the foreign *B. japonicum* cytochrome c_{550} (2), and the endogenous NapB diheme cytochrome *c* (12). Heme staining of the periplasmic fractions showed that all CcmE versions with point mutations of charged residues were able to complement the Δ*ccmE* strain for cytochrome *c* biogenesis (Fig. 2, lanes 1 and 3 to 9).

We next attempted to introduce more drastic changes by

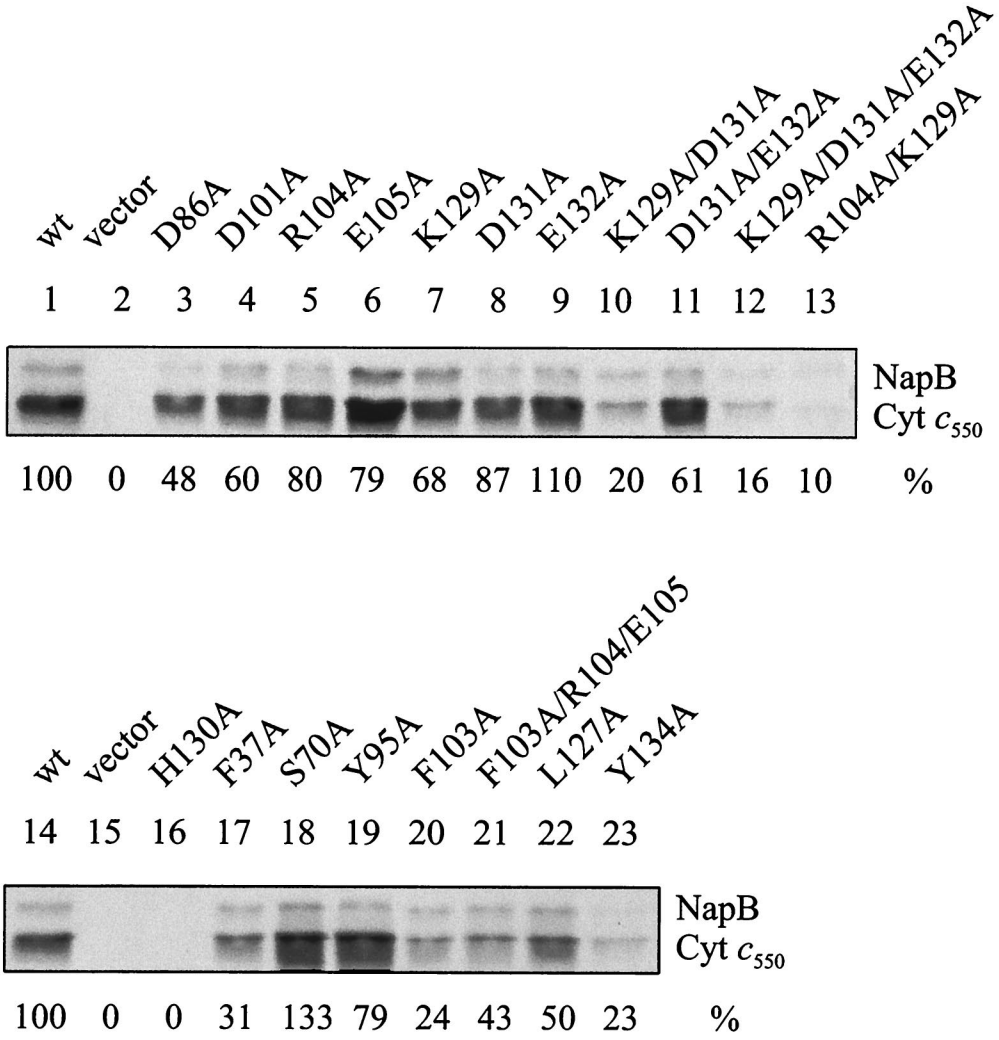

FIG. 2. Influence of *ccmE* mutants on cytochrome *c* maturation. The Δ*ccmE* strain EC65 was cotransformed with plasmid pRJ3291 expressing the *B. japonicum cycA* gene encoding cytochrome c_{550} (Cyt c_{550}) and a plasmid expressing the different *ccmE* alleles. Cells were grown under anaerobic conditions in minimal salt medium with nitrite as the terminal electron acceptor. Trichloroacetic acid-precipitated periplasmic proteins (50 μg per lane) were separated on an SDS–15% polyacrylamide gel and analyzed by heme staining. The spectrophotometrically determined concentration of soluble *c*-type cytochromes ($\Delta A_{550\text{-}536}$) is given as a percentage of the wild-type (wt) level below each lane.

constructing the double and triple mutants K129A/D131A, D131A/E132A, K129A/D131A/E132A, and R104A/K129A. All tested combinations yielded CcmE versions that retained some ability to bind heme (Fig. 1A, lanes 10 to 13) and to produce *c*-type cytochromes (Fig. 2, lanes 10 to 13). However, multiple changes also led to a decrease of the CcmE signal (Fig. 1B, lanes 10 to 13), perhaps affecting the stability of the protein. In conclusion, we have not found evidence for acid- or base-catalyzed heme binding or release by removing specific charged residues.

Point mutations of conserved, noncharged amino acids in CcmE. Binding of the hydrophobic heme molecule to CcmE is expected to involve hydrophobic interactions between cofactor and polypeptide. Thus, some of the best conserved noncharged and hydrophobic amino acids were changed to alanine: the aromatic residues F37, Y95, F103, and Y134, the hydrophobic L127, and the polar S70. In addition, the triple mutant F103A/R104A/E105A was constructed in order to introduce a drastic change in the conserved motif ^{99}LPDLFREG^{106}. Expression of these CcmE versions together with CcmC resulted in the heme staining bands presented in Fig. 1A (lanes 17 to 23). Mutants F37A and L127A were only slightly impaired in heme binding, whereas the other mutants produced low levels of holo-CcmE. The polypeptide levels of mutants S70A and Y95A were also strongly affected as detected by Western blot analysis (Fig. 1B, lanes 18 and 19). However, the corresponding mutant proteins were able to transfer heme as well as the wild type was (Fig. 2, lanes 18 and 19). Most likely these mutations affected the stability, but not the function, of CcmE. The decreased amount of mutated CcmE is thus not limiting with respect to cytochrome *c* maturation.

Mutant F103A contained the lowest levels of heme of all of the single mutants, but it also showed reduced protein levels and was affected in cytochrome *c* maturation. The additional mutation of R104 and E105 did not alter this phenotype, which excludes a cooperative behavior of these residues. L127A and

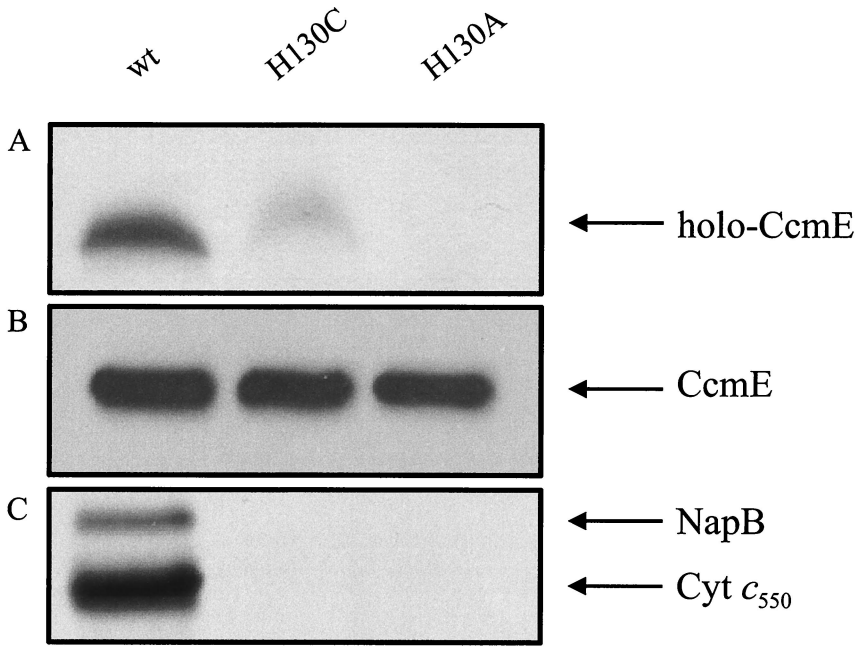

FIG. 3. Characterization of the CcmE point mutant H130C. (A) Membrane proteins (100 μg per lane) of the Δ*ccm* strain EC06 cotransformed with plasmid pEC439 expressing *ccmC* and a plasmid encoding wild-type (wt) CcmE, CcmE H130C, or CcmE H130A were separated by SDS–15% polyacrylamide gel electrophoresis and analyzed by heme staining. (B) Western blot of the same membrane fractions (20 μg per lane) as in panel A probed with antiserum directed against CcmE. (C) Trichloroacetic acid-precipitated periplasmic proteins (50 μg per lane) of the Δ*ccmE* strain EC65 cotransformed with plasmid pRJ3291 expressing the *B. japonicum cycA* gene encoding cytochrome c_{550} (Cyt c_{550}) and a plasmid encoding wild-type or mutant CcmE were separated on an SDS–15% polyacrylamide gel and analyzed by heme staining.

Y134A showed a decreased heme binding capacity but wild-type levels of CcmE polypeptide. Both mutants were impaired in heme transfer to cytochrome *c*. In summary, hydrophobic residues play a major role in the binding of heme to CcmE. Likewise, changes of hydrophobic residues F37, F103, L127, and Y134 led to the most significant loss of cytochrome *c* formation.

Quantification of *c*-type cytochromes by absorption spectroscopy. To quantify the concentration of soluble *c*-type cytochromes in the different strains, the reduced minus the oxidized absorption spectra of the periplasmic fractions were recorded. The difference of the absorption at 550 and 536 nm is proportional to the amount of *c*-type cytochromes. The value 100% was assigned to the wild type, and the relative levels of cytochromes formed by the mutants were calculated accordingly. All measurements were performed with at least two independent experiments. The values obtained from the spectra corresponded well to the intensities of the heme staining bands (Fig. 2).

The CcmE H130C mutant can bind heme but does not form *c*-type cytochromes. H130 is the only residue of CcmE which is essential for heme binding. In mutant H130A, the lack of heme binding (Fig. 1, lane 16) abolishes cytochrome *c* biosynthesis (Fig. 2, lane 16), suggesting that heme binding is an essential step during the maturation pathway. H130 binds heme covalently, but the nature of this bond is not known (18). We speculate that the binding of the histidine side chain occurs at one of the two heme vinyl groups, which is somewhat reminiscent of the heme-cysteine bond of *c*-type cytochromes. If the formation of this bond is based mainly on a correct positioning of heme and the polypeptide, one might expect that replacement of H130 by a cysteine can result in the formation of a thioether bond, like in cytochrome *c*. Thus, we constructed an H130C version of CcmE and analyzed heme binding and heme transfer. In fact, a faint heme staining band was present in the membranes of the H130C mutant (Fig. 3A), whereas the level of expressed protein was comparable to that of the wild type (Fig. 3B). However, no *c*-type cytochromes were detected (Fig. 3C).

Purification and characterization of soluble CcmE-H_6. To further characterize the CcmE heme chaperone, large-scale purification of the protein was necessary. The membrane anchor of CcmE was replaced by a cleavable signal sequence to produce soluble, periplasmic CcmE (18). In addition, a hexahistidine tag was added to the C terminus. The resulting soluble CcmE-H_6 protein was purified in three forms: as apo-CcmE without heme, as holo-CcmE with heme, and as a mixture of these two forms. When *ccmE* was expressed in a Δ*ccmA-H* background, only apo-CcmE was produced because the covalent attachment of heme requires the presence of CcmC (17). When plasmid pEC101 carrying *ccmABCD* was coexpressed, a mixture of apo- and holo-CcmE in a ratio of about 10:1 was obtained. The proteins were purified by affinity chromatography on nickel-NTA-agarose (Fig. 4), yielding 20 mg of pure protein per liter of cells. Further separation of apo- and holo-CcmE was achieved by hydrophobic interaction chromatography. This step repeatedly produced degradation products. Four fragments with masses of 13,036, 12,851, 12,591, and 12,391 *m/z* were detected by matrix-assisted laser desorption ionization mass spectroscopy. They corre-

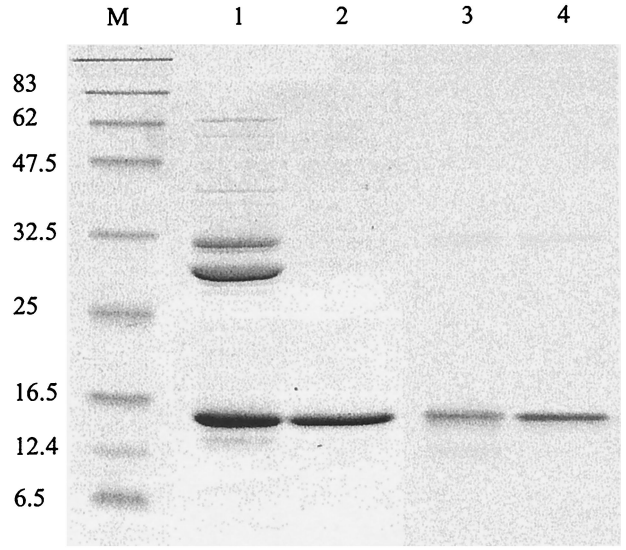

FIG. 4. Purification of soluble CcmE-H_6. Twenty-five micrograms of periplasmic proteins (lane 1) and 5 μg of purified CcmE-H_6 (lane 2) were stained with Coomassie brilliant blue, and one-third of the same samples was heme-stained (lanes 3 and 4). Proteins were separated on an SDS–15% polyacrylamide gel. The numbers on the left are molecular masses of marker proteins given in kilodaltons.

sponded to the following degradation products (theoretical masses are given in parentheses): apo-CcmE$^{30\text{-}146}$ (13,037 *m/z*), apo-CcmE$^{30\text{-}144}$ (12,852 *m/z*), apo-CcmE$^{30\text{-}142}$ (12,591 *m/z*), and apo-CcmE$^{30\text{-}140}$ (12,392 *m/z*), respectively. For three of them, the heme-binding forms were also found: 13,655 *m/z* for holo-CcmE$^{30\text{-}146}$ (13,654 *m/z*), 13,468 *m/z* for holo-CcmE$^{30\text{-}144}$ (12,468 *m/z*), and 13,208 *m/z* for holo-CcmE$^{30\text{-}142}$ (13,208 *m/z*). These fragments are lacking their C-terminal His tags. In order to eliminate the degradation products, the fractions containing only holo-CcmE, as detected by reverse-phase HPLC, were subjected a second time to nickel-NTA affinity chromatography. The eluted, nondegraded holo-CcmE was found to be stable for several weeks at 4°C if kept in 300 mM NaCl and 50 mM sodium phosphate (pH 7.2). Lower salt concentrations (100 mM NaCl) caused rapid degradation of the protein.

Visible absorption spectra of holo-CcmE-H_6 and the holo-CcmE–apo-CcmE mixture were identical. Characteristics of the spectra from the mixture are presented in Fig. 5. Maxima for the difference spectrum (Fig. 5C) of the reduced (Fig. 5B) minus oxidized (Fig. 5A) holo-CcmE correspond well with the previously published ones of membrane bound holo-CcmE (18). The α bands of the reduced sample (Fig. 5B) at 556 nm and of the pyridine hemochrome (Fig. 5E) at 551 nm are in agreement with saturation of a heme vinyl by a covalent modification. Similar bands have been found in the holo-CcmE preparation of Daltrop et al. (5). The minimum at 555 nm in the reduced CO complex (Fig. 5D) suggests that the heme iron is available for axial ligation with CO. The maximum at 620 nm in the oxidized spectrum (Fig. 5A) indicates that iron is present in the high-spin state (3).

CcmE H130C requires CcmABCD for heme binding. To facilitate purification of CcmE H130C, the soluble, His-tagged version of this mutant was constructed. As for the wild type, heme binding of this mutant was observed only in the presence

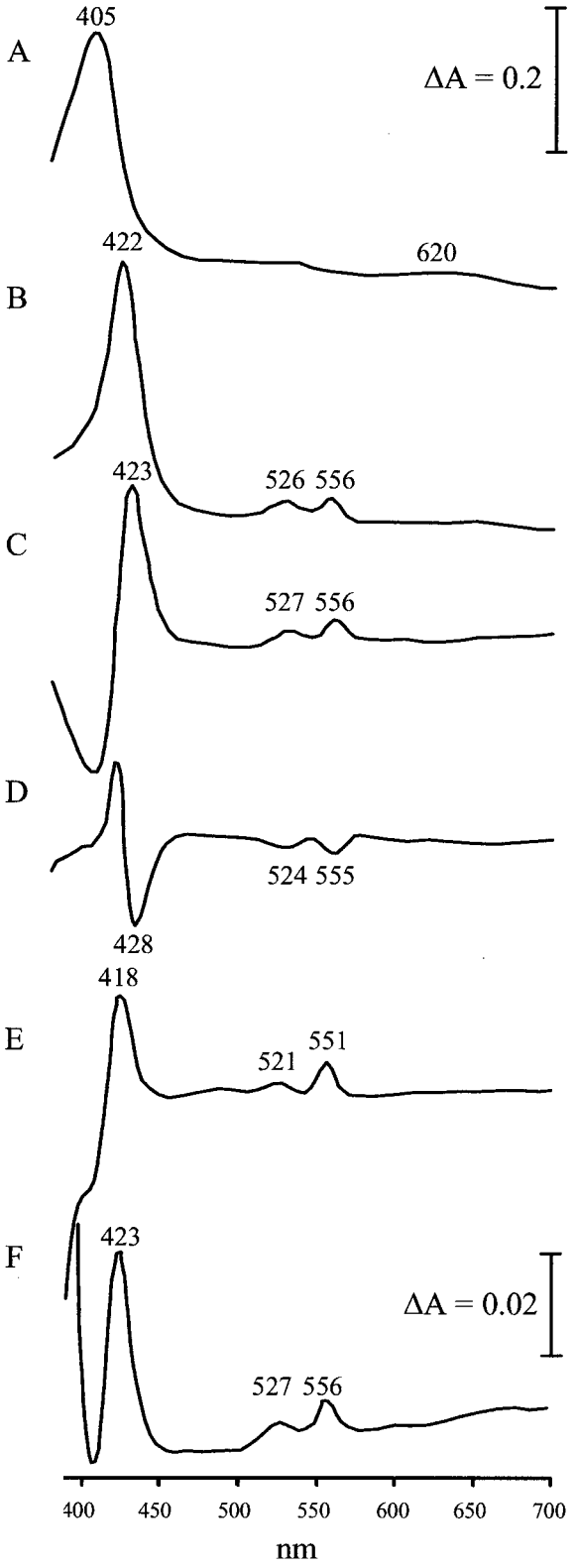

FIG. 5. Absorption spectra of soluble CcmE-H_6. (A) oxidized; (B) reduced; (C) reduced minus oxidized; (D) CO reduced minus reduced; (E) pyridine-hemochrome, reduced minus oxidized; (F) H130C mutant, reduced minus oxidized. Maxima and minima are indicated.

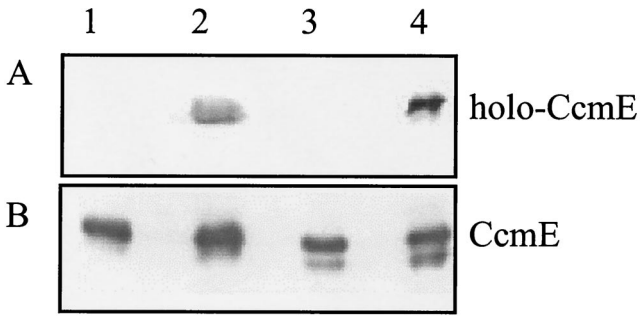

FIG. 6. CcmABCD-dependence of heme binding to CcmE. Periplasmic fraction of the Δ*ccmA-H* strain EC06 plus soluble, His-tagged *ccmE* H130C (lane 1), *ccmE* H130C and *ccmABCD* (lane 2), *ccmE* (lane 3), and *ccmE* and *ccmABCD* (lane 4). (A) Three milligrams of trichloroacetic acid-precipitated proteins was loaded in lanes 1 to 3, and 30 μg were loaded in lane 4. Proteins were separated on an SDS–15% polyacrylamide gel and heme stained. (B) An identical Western blot probed with antiserum directed against CcmE is shown, except that 20 μg of the same samples were loaded in all lanes.

of coexpressed *ccmABCD* (Fig. 6A). While the H130C mutant protein was expressed equally well as the wild-type protein (Fig. 6B), only a small fraction bound heme.

To address the nature of the newly formed heme-cysteine bond, the soluble holo-CcmE-H_6 H130C protein was purified. Optical spectra of the purified H130C mutant protein showed the same characteristics as wild-type CcmE (Fig. 5F); however, at least 200 times less of the holo-CcmE form was present.

Tryptic peptides of CcmE bind heme covalently. Purified wild-type CcmE and CcmE H130C were digested with trypsin. From wild-type CcmE, two heme-binding peptides were isolated by reverse-phase HPLC and analyzed by mass spectroscopy. The average mass of 2,074.1 *m/z* of the first peptide corresponded exactly to the sum of that of the expected 12mer peptide ^{130}HDENYTPPEVEK^{141} (1,457.5 *m/z*) plus heme (616.5 *m/z*). The second isolated peptide had a mass of 2,614.5 *m/z* coming from heme plus the 17mer ^{125}EVLAKHDENYT PPEVEK^{141} (1,998.2 *m/z*) resulting from a partial digest. We thus speculate the reaction of heme with the histidine to be an addition, whereby one of the heme vinyl side chains reacts with a nitrogen of the histidine imidazole ring. A substitution would lead to the loss of two hydrogen atoms.

After the tryptic digest of CcmE H130C and separation by reverse-phase HPLC, a peptide absorbing at 398 nm was isolated. The identified mass of 2,040.3 *m/z* corresponds to the dodecamer peptide ^{130}CDENYTPPEVEK^{141} (1,423.5 *m/z*) plus heme. This indicates that heme is bound covalently to the mutated cysteine 130.

DISCUSSION

The heme chaperone CcmE is conserved in α- and γ-proteobacteria, deinococci, and plant mitochondria. In an alanine scanning mutagenesis approach, we investigated the role of individual, highly conserved amino acids in heme binding and delivery, the two functions of the CcmE heme chaperone. Fourteen of the best conserved amino acids with charged, polar, or hydrophobic side chains were changed to alanine and checked for their ability to bind heme and transfer it to *c*-type cytochromes. We expected to find two classes of phenotypes: mutants affected in heme binding and mutants that can bind heme but are blocked in its release. However, the results of our study revealed that, with the exception of His130, none of the conserved residues was irreplaceable, neither for heme binding nor for heme delivery. Surprisingly, the mutations of charged residues had the least effect. Even a triple mutant was able to bind and transfer heme. This result indicates that the charged amino acids are not required specifically for the mechanism of heme binding or release. The heme chaperone may act mainly as a transient heme storage protein by providing a suitable binding site. The suggested binding pocket for the hydrophobic heme molecule was assumed to be formed mainly of hydrophobic residues. Heme titration experiments with apo-CcmE suggest that heme is initially bound noncovalently to CcmE, perhaps by hydrophobic interactions (5, 6). Alterations of hydrophobic amino acids should therefore impair CcmE function. In fact, the strongest effects were found for mutants with altered hydrophobic residues. Although none of these mutants had lost function completely, heme binding and the biogenesis of cytochrome *c* were affected more than in the case of the mutants of charged residues. This finding is in agreement with the view that CcmE displays a hydrophobic surface or pocket for heme binding close to His130. In fact, nuclear magnetic resonance structural data support this hypothesis (6) and indicate the presence of a hydrophobic interface formed by F37, L127, and Y134. These residues have strong effects on heme binding and cytochrome *c* formation when mutated to alanine. F103, the fourth amino acid with a strong influence on heme binding and delivery, is buried inside the protein. Changing this residue to alanine might alter the overall structure of CcmE. The same may be true for S70 and Y95, whose side chains are oriented towards the inside of the protein core (6). Mutation of S70 and Y95 to alanine led to decreased levels of the CcmE polypeptide in the membrane. Although these mutants showed only residual heme binding, they were capable of transferring heme to cytochrome *c*. Hence, these residues are involved in protein folding and/or stability rather than in heme transfer.

Heme binding to and release from CcmE is catalyzed in vivo by the membrane proteins CcmC and CcmF, respectively, which have been demonstrated to interact directly with CcmE (13, 14). D86, the amino acid with the strongest effect on cytochrome *c* formation upon mutation among the charged residues is located at the surface of CcmE, distant from the putative heme binding pocket. D86 may be responsible for protein-protein interactions, either with CcmC or CcmF. Both proteins contain a number of conserved basic residues in periplasmic domains which are candidates for interaction with D86 of CcmE: R55 and R128 for CcmC, and R84, R149, R269, K273, R295, R311, K564, and R565 for CcmF.

H130 is the only amino acid that is absolutely required for CcmE function. CcmE H130A neither binds heme nor supports production of *c*-type cytochromes (18). When H130 was mutated to a cysteine, residual heme binding to CcmE was detected, but it was more than 200-fold decreased. The resulting H130C protein showed an absorption spectrum indistinguishable from that of wild-type CcmE. In *c*-type cytochromes, cysteine reacts with heme by forming a thioether bond. As for the heme-histidine bond formed in the wild type, the heme-

cysteine bond of the mutant CcmE was formed in vivo only in the presence of other proteins of the Ccm complex. CcmC has been shown to be necessary and sufficient to transfer heme to CcmE (19). It either catalyzes the formation of the covalent bond or presents heme in the correct spatial orientation to CcmE. The H130C mutant was blocked in cytochrome *c* synthesis. Apparently, the transfer of heme that is bound to a cysteine cannot occur due to the stability of the thioether bond.

We suspect that, in the wild-type CcmE, heme binding to H130 occurs at one of the two vinyl groups for the following reasons. (i) Mass spectra of the heme peptides showed that no protons are lost when heme is bound, indicating an addition rather than a substitution. (ii) The optical spectrum of holo-CcmE has an α maximum at 556 nm under conditions where the spectrum of apo-CcmE plus heme has a maximum at 559 nm (6). The shift of the α band to a shorter wavelength in the holo form is in agreement with the presence of a covalent bond removing one of the two heme vinyl groups (11). (iii) Our idea to mimic the covalent attachment of heme by the formation of a thioether bond like that found in *c*-type cytochromes in an H130C mutant proved to be feasible, although heme binding was very inefficient. In a parallel study, Daltrop et al. found that soluble CcmE-H_6 can bind ferric heme in vitro. Upon reduction to the ferrous form, heme was bound covalently to the polypeptide when protoheme was used but not when mesoheme lacking the vinyl groups was used (5). These data support the idea that the heme-histidine bond is the product of an addition to the vinyl double bond.

With the protocol for purifying soluble apo-CcmE and holo-CcmE, it should now be possible to elucidate the most interesting features of CcmE: the nature of the heme-histidine bond and the mechanism by which this bond is formed and subsequently broken to transfer heme. We speculate that CcmE selects one of the two heme vinyl groups to form a covalent bond, thus implementing a stereospecifically correct and efficient delivery of heme to *c*-type cytochromes.

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