A Pivotal Heme-transfer Reaction Intermediate in Cytochrome c Biogenesis

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Background: Heme attachment to cytochrome c is a catalyzed post-translational modification.

Results: We identify a ternary complex of the cytochrome c biogenesis protein CcmE, heme, and a cytochrome, and demonstrate its functional significance.

Conclusion: The complex is a trapped catalytic intermediate at the point of heme transfer from the cytochrome biogenesis apparatus to the cytochrome.

Significance: An insight into biosynthesis of heme proteins.

c-Type cytochromes are widespread proteins, fundamental for respiration or photosynthesis in most cells. They contain heme covalently bound to protein in a highly conserved, highly stereospecific post-translational modification. In many bacteria, mitochondria, and archaea this heme attachment is catalyzed by the cytochrome c maturation (Ccm) proteins. Here we identify and characterize a covalent, ternary complex between the heme chaperone CcmE, heme, and cytochrome c. Formation of the complex from holo-CcmE occurs in vivo and in vitro and involves the specific heme-binding residues of both CcmE and apocytochrome c. The enhancement and attenuation of the amounts of this complex correlates completely with known consequences of mutations in genes for other Ccm proteins. We propose the complex is a trapped catalytic intermediate in the cytochrome c biogenesis process, at the point of heme transfer from CcmE to the cytochrome, the key step in the maturation pathway.

* This work was supported in part by Biotechnology and Biological Sciences Research Council Grants BB/D019753/1 and BB/H017887/1, Wellcome Trust Grant 092532, and a Value-in-People Award.

‡ This article contains supplemental Figs. S1 and S2 and Tables S1–S4.

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FIGURE 1. Scheme illustrating the Ccm system in *E. coli* and the structures of heme attachment to *c*-type cytochromes and CcmE. All Ccm system components are membrane anchored or integral membrane proteins. Apocytochrome *c* (apo-*c*-b$_{562}$) is synthesized in the cytoplasm and transported to the periplasm (where all bacterial *c*-type cytochromes are matured and located) by the Sec apparatus. The heme-binding cysteines (highlighted in yellow) are oxidized in the periplasm by the nonspecific oxidant DsbA to form an intramolecular disulfide bond. Heme is also synthesized in the cytoplasm and transported to the periplasm by an unknown route (indicated by a question mark). Heme becomes associated with CcmC (15, 16) and is then passed to CcmE, where it binds covalently to a histidine residue (H130, lilac) (10). The disulfide bond in the apocytochrome is reduced by electron transfer from CcmG (and/or CcmH). Electrons are first passed (yellow arrows) from the cytoplasmic thioredoxin (Trx) through the transmembrane protein DsbD to CcmG (proteins in this part of the pathway colored dark gray). Release of heme-bound CcmE from a tight complex with CcmC requires the ATPase activity of CcmAB (13, 14). CcmD is implicated in formation/stabilization of the CcmCE complex. Proteins involved in heme delivery and binding to holo-CcmE are colored dark pink. Subsequent holocytochrome (holo-*c*-b$_{562}$) formation (boxed) involves heme transfer from CcmE to the reduced apocytochrome; this requires the hemoprotein CcmF and CcmH (light pink) (10, 17, 18), but the mechanism is not known. *E. coli* CcmH consists of two functionally distinct domains that are found as separate proteins (CcmH and CcmI) in most Ccm-containing bacteria (38). Key amino acids for experiments in this work are highlighted. His-130 of CcmE (lilac), and Cys-98 and Cys-101 of cytochrome *c*-b$_{562}$ (yellow), covalently bind to heme. His-102 of cytochrome *c*-b$_{562}$ is a ligand to the heme iron atom in the holocytochrome (iron ligation indicated by a dashed line and residues in green). His-60 and His-184 of CcmC have been implicated in heme provision to CcmE (15, 16). Lys-40 of CcmA (in white) is part of the Walker A motif of the protein and therefore crucial for the ATPase activity of CcmAB (13). Structures of free heme and heme as attached to cysteine thiols of the *CXCH* heme-binding motif of a typical *c*-type cytochrome are shown in the upper middle of the figure. The stereospecificity of this attachment is universally conserved. The histidine of the *CXCH* heme-binding motif becomes a ligand to the iron atom.
ing a single cysteine residue also covalently bind heme (22), but single cysteine c-type cytochromes are very poor substrates for the Ccm system (23, 24). Exceptionally, apocytochrome \( b_{562} \) is also stable (3, 22); normally apocytochromes c are subject to rapid degradation in vivo.

In this work, we describe experiments to identify novel interactions between components of the Ccm system and (apo)cytochrome c. We have used c-type cytochrome \( b_{562} \) variants to maximize the chances of isolating such interacting partners. We report a covalent, ternary complex between CcmE, heme, and cytochrome, and propose this represents a critical intermediate in the Ccm pathway, at the point of heme transfer from the biogenesis system to the product cytochrome.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids**

Plasmids used in this study are listed in supplemental Table S3. Plasmid pb562R98CStrep was produced by site-directed mutagenesis (ExSite, Stratagene) using plasmid pb562R98C (22) as the template. The same method was used to insert a thrombin cleavage site, with additional Gly residues (GLVPRGSG) into pE221 (25). The plasmid produced, pE225, expresses the periplasmic region of CcmE (from Ser-32) with an N-terminal cleavable pellB signal sequence. Plasmids pb562R98CH102R and pFR015 were produced by site-directed mutagenesis (QuikChange, Stratagene) using plasmids pb562R98C (22) and pEC86 (9) as templates, respectively. DNA manipulations were conducted using standard methods. KOD Hot Start DNA polymerase (Novagen) was used for all PCRs and all constructs were sequenced before use.

**Cell Growth and Fractionation**

Bacterial strains used in this study are listed in supplemental Table S4. Routine cell growth was conducted in 100 ml of TY medium (16 g liter\(^{-1}\) of peptone, 10 g liter\(^{-1}\) of yeast extract, 5 g liter\(^{-1}\) of NaCl) in 2.5-liter flasks. Cultures were inoculated from single colonies and incubated at 37 °C for 15–18 h with shaking at 200 rpm. Fully aerobic growth conditions prevented expression of the endogenous \( E. \) coli Ccm system. 1 mM Isopropyl 1-thio-\( \beta \)-d-galactopyranoside was added to the cultures from inoculation. 100 \( \mu \)g ml\(^{-1}\) of ampicillin and 34 \( \mu \)g ml\(^{-1}\) of chloramphenicol were used.

For the isolation of the crude membrane fraction a French press was used. Disruption of the cells was performed at 16,000 p.s.i. followed by centrifugation at 257,000 \( \times \) g for 1 h at 4 °C. The membrane fraction was resuspended in ~25 ml of 50 mM Tris-HCl, 150 mM NaCl (pH 7.5) and was re-centrifuged as above. The washed crude membrane fraction was resuspended in 1–2 ml of 50 mM Tris-HCl, 150 mM NaCl (pH 7.5).

**SDS-PAGE Analysis**

SDS-PAGE analysis was carried out on 10% Bis-Tris NuPAGE gels (Invitrogen) with prestained molecular weight markers (SeeBlue Plus 2, Invitrogen, or ColorPlus Prestained Protein Marker, New England Biolabs). Samples containing membrane fractions were denatured by incubation at 42 °C for 5 min. All other samples were denatured at 100 °C for 2 min. 2-Mercaptoethanol and urea were added at final concentrations of 5% (v/v) and 8 \( \mu \)M, respectively, where appropriate. Gel loadings were normalized according to total protein content, and determined using the Pierce BCA Reducing Agent Compatible Protein Assay Kit (ThermoScientific); 5–20 \( \mu \)g of protein were loaded per lane. Proteins with covalently bound heme were detected on gels using the method of Goodhew et al. (26) and quantification of heme-bound species was performed by densitometry using GeneSnap (SYNGENE).

Western blotting was carried out following SDS-PAGE by transferring onto nitrocellulose (Hybond C-Extra, Amersham Biosciences). Blocking was with 5% (w/v) skimmed milk powder in Tris-buffered saline (50 mM Tris-HCl, 120 mM NaCl, 0.1% (w/v) Tween 20 (pH 7.5)). The primary antibodies used (Covalab) were rabbit antiserum raised against \( E. \) coli cytochrome \( b_{562} \) (dilution 1:1000) and rabbit antiserum raised against \( E. \) coli CcmE (dilution 1:1000). In the in vitro studies antibody raised against a CcmE peptide (10) was used. Goat anti-rabbit alkaline phosphatase-conjugated antibody (Sigma) was used as secondary antibody (dilution 1:30000). Development was carried out using a SigmaFast BCIP/NBT tablet.

**Sample Preparation for Proteomics Analysis**

Proteomics analysis of the CcmE-heme-cytochrome \( c-b_{562} \) R98C complex was carried out on two different samples.

**Co-immunoprecipitation**—Crude membrane fractions from \( E. \) coli JCB387 cells transformed with plasmids pEC86 and pb562R98C were solubilized in 1% (w/v) n-dodecyl \( \beta \)-d-maltoside (DDM) (Melford) under gentle agitation at 4 °C for 1 h. Rabbit antiserum raised against \( E. \) coli cytochrome \( b_{562} \) was bound to beads from the Dynabeads-Protein G Immunoprecipitation Kit (Invitrogen). The pulled-down product was analyzed by SDS-PAGE. The gel was silver-stained using the SilverQuest Silver Staining Kit (Invitrogen) and the band corresponding to the CcmE-heme-cytochrome \( c-b_{562} \) R98C complex was excised and destained.

**Purification of CcmE-Heme-Cytochrome c-b_{562} R98C Complex Using a Streptavidin II Tag**—Crude membrane fraction from \( E. \) coli JCB387 cells, transformed with plasmids pEC86 and pb562R98CStrep, was solubilized in 20 mM Tris-HCl, 300 mM NaCl, 20% (v/v) glycerol, 1% (w/v) DDM (pH 7.5) under gentle agitation at 4 °C for 1 h at a protein concentration of 5 mg ml\(^{-1}\). Unsolubilized material was removed by centrifugation at 257,000 \( \times \) g for 30 min at 4 °C. The supernatant was diluted 10-fold with 20 mM Tris-HCl, 150 mM NaCl (pH 7.5) and applied to 7 ml of \( \text{Strep}-\text{Tactin} \) Sepharose (IBA GmbH) pre-equilibrated with 20 mM Tris-HCl, 150 mM NaCl, 0.1% (w/v) DDM (pH 7.5). The column was washed with 20 mM Tris-HCl, 1 mM NaCl, 0.1% (w/v) DDM (pH 7.5) and protein was eluted using 20 mM Tris-HCl, 150 mM NaCl, 0.1% (w/v) DDM, 2.5 mM desthiobiotin (IBA) (pH 7.5). The eluent was exchanged into 20 mM Tris-HCl, 50 mM NaCl, 0.03% (w/v) DDM (pH 7.5) and concentrated. The protein solution was analyzed by SDS-PAGE in the presence of dithiothreitol (DTT). The gel was stained using SimplyBlue Safestain (Invitrogen) and the band corresponding to the CcmE-heme-cytochrome \( c-b_{562} \) R98C complex was excised.
Proteomics Analysis

Identification of the CcmE-heme-cytochrome $c_{552}$ R98C complex components was achieved by proteomics analysis. Blue-stained gel bands were excised, destained in 25 mM ammonium bicarbonate in 50% acetonitrile, and then reduced with DTT and alkylated with iodoacetamide before being digested with trypsin overnight. Tryptic peptides were extracted and then desalted on an in-house manufactured C18 tip. Samples were analyzed on a Thermo LTO XL Orbitrap coupled to a Dionex U3000 nano-LC system run in direct injection configuration. Peptides were resolved on an in-house manufactured reverse-phase column made by packing a Picotip (New Objective) with C18 resin (Repasil-Pur, C18-Aq, 3-μm beads). Samples were typically resolved on 40- or 120-min LC-MS/MS gradients depending on sample complexity. The Orbitrap was operated in a “Top 5” method in which 1+ ions were not selected for fragmentation and dynamic exclusion was applied. Precursor mass accuracy tolerance was set at ± 20 ppm and the MS/MS fragment ion tolerance at ±0.5 Da. Data were searched using Mascot against database NCBI nr/nr version 2010.03.21, specifying E. coli as the taxonomy. The fixed modification was defined as carbamidomethyl cysteine and variable modifications were oxidized Met, N-terminal acetylation, and deamidation on Asn and Glu.

For peptide mapping, stained gel bands containing CcmE-heme-cytochrome $c_{552}$ R98C complex were subjected to in-gel digestion with trypsin as described elsewhere (27) or chymotrypsin (Roche Diagnostics) used under identical conditions. Digested samples were subjected to analysis by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) using an Ultraflex mass spectrometer (Bruker Daltonics) as described (28). The MS data (peptide mass fingerprinting) was analyzed using FlexAnalysis software version 2.4 (Bruker Daltonics) and searched against the SwissProt data base combined with the E. coli CcmE and cytochrome $c_{552}$ R98C sequences using an in-house Mascot server (Matrix-science). In addition, the same sample was also analyzed by LC-MS/MS using a U3000 nano-LC (Dionex) coupled to a high capacity trap tandem mass spectrometer (Bruker Daltonics) as described previously (27). MS/MS data were also searched using Mascot as described above, allowing for carbamidomethylation on Cys, oxidation on Met, deamidation on Glu and Asn, and heme addition on Cys and His, as modifications.

In Vitro Formation and Characterization of CcmE-Heme-Cytochrome c Complex

Soluble holo-CcmE was produced by coexpressing plasmids pE225 and pEC86 in E. coli JM109(DE3) cells. Cultures were incubated at 37 °C with shaking at 200 rpm in LB medium to mid-exponential phase and induced with 1 mM isopropyl-1-thio-β-d-galactopyranoside overnight at 30 °C. Cells were harvested and resuspended in 50 mM Tris-HCl, 300 mM NaCl (pH 7.5). The periplasmic fraction containing holo-CcmE was isolated and the protein was purified as described previously (25). Thrombin cleavage of the polyhistidine tag was performed using the Sigma CleanCleave Kit. Hydrogenobacter thermophilus apocytochrome $c_{552}$ and variants thereof were produced as described previously (29, 30). N-terminal sequencing was carried out from protein samples in SDS-PAGE gels that were electrophoretically transferred to a PVDF membrane and stained with Coomassie Brilliant Blue. The protein bands were excised and subjected to automated Edman sequencing using an Applied Biosystems 494A Procise protein sequencer. A PerkinElmer Lambda 2 spectrophotometer was used to collect absorption spectra. Pyridine hemochrome spectra were obtained according to the method of Bartsch (31) using 5 μM protein in 19% (v/v) pyridine and 0.15 mM NaOH.

H. thermophilus apocytochrome $c_{552}$, C11A, C14A, and C11A/C14A apocytochrome variants (15–25 μM) were incubated with E. coli holo-CcmE$^*$ (5–10 μM) in 50 mM potassium phosphate buffer (pH 7.0) at 25 °C for up to 20 h. Samples were reduced by the addition of disodium dithionite. Solutions contained 10 mM Tris(2-carboxyethyl)phosphine and were thoroughly sparged with humidified argon. Reactions were carried out in the dark.

Inhibition of the Ccm System

E. coli JCB387 cells were transformed with the following combinations of plasmids (supplemental Table S3): pEG278 + pb562EV; pEG278 + pb562R98C; pRZ001 + pb562EV; and pRZ001 + pb562R98C. Cell growth, fractionation, and analysis of the periplasmic fractions was performed as described in Ref. 32. Autoinduction was used for expression of cytochrome $c_{552}$ R98C and cytochrome cd$_4$. The production of both the endogenous cytochrome NrfA and the exogenous cytochrome cd$_4$ was quantified (33).

RESULTS AND DISCUSSION

A Complex between CcmE, Heme, and Cytochrome c—The Ccm system must interact with apocytochrome c to catalyze heme attachment and hence holocytochrome formation; we set out to identify such interactions using variants of cytochrome $b_{562}$ as the test cytochromes. E. coli strain JCB387 cells were co-transformed with a plasmid from which the Ccm proteins are constitutively expressed (pEC86), and plasmids encoding variants of cytochrome $b_{562}$ with CXXCH (R98C/Y101C), CXXXH (R98C), and XXXCH (Y101C) heme-binding motifs. Similar results to those in this article were also obtained using E. coli strains MC1000 and MC1061 (supplemental Table S4). In this work, we refer to the c-type cytochrome variants of cytochrome $b_{562}$ as cytochrome $c_{552}$. Cells were fractionated into soluble and membrane extracts. These fractions were analyzed by Western blotting with a cytochrome $b_{562}$ antibody. Of particular note was a band in the blot of the membrane fraction, observed at ~32 kDa (Fig. 2A, lanes 3–5). This band was not observed in the soluble fraction (not shown). The cytochrome $b_{562}$ variants themselves ran at ~12 kDa and a dimer was also observed at ~30 kDa (Fig. 2A); these bands arise from cytochrome $b_{562}$ that remains bound to the membranes even after extensive washing.

From the molecular masses of the Ccm proteins, we reasoned that the 32-kDa band might be a complex between (apo)cytochrome $c_{552}$ (molecular mass ~12 kDa) and CcmE (18 kDa), which is a membrane-anchored protein. The band was only observed when both the Ccm system and a cytochrome $c_{552}$...
Complex between CcmE, Heme, and Cytochrome c

To isolate the complex, solubilized membranes were immunoprecipitated using the cytochrome b<sub>562</sub> antibody. The 32-kDa species was visible on a silver-stained SDS-PAGE of the immunoprecipitate. The relevant band was excised from the gel, trypsin digested, and assessed by high-resolution mass spectrometry (LC-MS/MS). Peptides from both cytochrome c-b<sub>562</sub> and CcmE were detected in this analysis, including two from cytochrome c-b<sub>562</sub> and one from CcmE, each of ≥12 amino acids, and that could be assigned with high confidence (supplemental Table S1A). To verify this analysis, we co-expressed a C-terminal streptavidin II-tagged form of cytochrome c-b<sub>562</sub> R98C with the Ccm proteins. The tag facilitated small scale purification of the putative complex, which could be seen on protein-stained SDS-PAGE. Again, bands were excised, trypsin digested and analyzed by LC-MS/MS. Multiple peptides were identified from both cytochrome c-b<sub>562</sub> R98C and CcmE, each with >95% confidence (but often much higher); sequence coverage in identified peptides (supplemental Table S1A) was 64% for cytochrome b<sub>562</sub> and 44% for CcmE.

We conclude that we have resolved a stable complex containing cytochrome c-b<sub>562</sub>, CcmE, and heme, all linked covalently. This is the first complex to be identified between CcmE and a cytochrome. Such a complex is a critical intermediate in current models of the Ccm system-mediated pathway of cytochrome c biogenesis (10). The small amount of complex formed from CXXCH containing cytochrome c (Fig. 2, A and B, lane 3) presumably represents the steady-state level of the complex as a reaction intermediate in the normally functioning Ccm pathway. In contrast, with single cysteine (XXXCH or CXXXH) cytochromes, the complex accumulated as a trapped intermediate.

Organization of the Complex—What is the structural arrangement of this ternary complex between cytochrome c-b<sub>562</sub> CcmE, and heme? We investigated its formation using site-directed variants of CcmE and cytochrome c-b<sub>562</sub> R98C. No complex was observed when wild-type cytochrome b<sub>562</sub> (which contains no cysteine residues) was co-expressed with R98C variant cytochrome c-b<sub>562</sub> were therefore selected for further analysis.

The 32-kDa band remained even after treatment of the solubilized membrane proteins in harshly denaturing conditions such as boiling (supplemental Fig. S1, lanes 2 and 4) or 8 M urea (data not shown), indicating that the CcmE-heme-cytochrome c-b<sub>562</sub> R98C complex is very stable and that the components are covalently linked. The complex was insensitive to treatment with 2-mercaptoethanol, which would reduce any disulfide bonds present (supplemental Fig. S1, lanes 3 and 4). Following treatment with 2-mercaptoethanol, the intensity of the heme-staining bands arising from each of the complex, holo-CcmE and holocytochrome c-b<sub>562</sub> R98C, decreased (supplemental Fig. S1C, compare lane 1 with 3). Such reducing agents cause a portion of the iron to dissociate from heme, which reduces the intrinsic peroxidase activity of the heme that gives rise to the heme stain. However, it is clear from the Western blots using both cytochrome b<sub>562</sub> and CcmE antibodies (supplemental Fig. S1, A and B, compare lanes 3 and 4 with lane 1) that treatment with 2-mercaptoethanol did not decrease the total amount of complex.

To establish whether heme was involved in the complex with CcmE and cytochrome c-b<sub>562</sub>, we ran the cell membrane fractions on denaturing SDS-PAGE, which was stained for proteins containing covalently bound heme (Fig. 2C). Once again, the 32-kDa band was apparent. Densitometry measurements of the heme-stained gel (Fig. 2C), which was normalized for total protein loading, indicate the largest amount of the complex formed with the R98C (CXXXH) variant cytochrome (lane 4). The relative yield was ~70% lower with the Y101C (XXXXH) protein (lane 5). With the CXXCH variant, no band was visible on the heme-stained gel (Fig. 2C, lane 3) (sensitivity ≥ 1 pmol of holocytochrome c (3)); it was barely detectable in the Western blots (Fig. 2, A and B, lanes 3). Membranes from cells expressing the
the Ccm system (Fig. 3, A and B, compare lane 3 with lane 4). Similarly, no complex was observed when His-102, the proximal heme iron ligand in cytochrome c-b-562 R98C (i.e. the histidine residue of the heme-binding motif, which binds noncovalently and axially to the heme iron atom), was mutagenized to produce cytochrome c-b-562 R98C/H102R (Fig. 3C, lane 2). The histidine of the CXXCH motif has previously been shown to be essential for c-type cytochrome maturation by the Ccm system (34, 35). E. coli CcmE binds heme covalently through residue His-130 as an intermediate in Ccm system-dependent cytochrome c biogenesis (Fig. 1) (10). No complex between CcmE, heme, and cytochrome c-b-562 R98C was observed for the H130A variant of CcmE (Fig. 3C, lane 3). As anticipated, this mutation also prevented accumulation of holo-CcmE. Apo-CcmE was clearly abundant on Western blots (not shown).

To investigate the heme-binding sites in greater detail, we used a peptide mapping approach. CcmE-heme-cytochrome c-b-562 R98C complex was purified using the streptavidin tag, separated by SDS-PAGE, and excised gel slices were digested using trypsin or chymotrypsin. Peptide mass fingerprinting by mass spectrometry identified a heme-binding peptide fragment of CcmE (residues 114–141), which included His-130 (supplementary Table S1B and Fig. 4A). In addition, we detected four heme-binding peptide fragments from cytochrome c-b-562 R98C comprising residues 96–103, 86–103, 95–101, and 106–114, three of which included Cys-98 (supplementary Table S1B and Fig. 4, A and B). Heme binding to the latter peptide (cytochrome c-b-562 R98C residues 106–114) is very likely to be an artifact because residues 107–114 are part of the streptavidin II tag we added to the C terminus of cytochrome c-b-562 R98C to facilitate purification of the complex. We have only used streptavidin-tagged protein to isolate CcmE-heme-cytochrome c-b-562 for mass spectrometry analysis; all other experiments in this article used untagged protein. Thus formation of the CcmE-heme-cytochrome c-b-562 complex is independent of the streptavidin tag (e.g. Figs. 2, 3, and 5). The trypsin and chymotrypsin-based peptide mapping did not definitively determine which residues in the various fragments had heme bound. Therefore, we further analyzed the peptides obtained from the digests by LC-MS/MS. Despite the presence of the heme group reducing ionization and fragmentation efficiency, MS/MS analysis showed a peptide fragment consisting of residues 95–103 from cytochrome c-b-562 R98C had heme bound to Cys-98, and a fragment consisting of residues 107–130 from CcmE had heme attached to His-130 (Fig. 4, C and D). We were unable to identify a single cross-linked fragment containing heme and a peptide from both CcmE and cytochrome c-b-562 R98C possibly because the ion intensity of such a species would be impaired by the presence of the heme group as well as its expected size (>4000 Da), where the sensitivity of mass spectrometry-based detection is suboptimal.

The simplest interpretation of our mutagenesis and mass spectrometry data is that the CcmE-heme-cytochrome c-b-562 R98C complex consists of two proteins cross-linked by heme covalently bound to both His-130 of CcmE and Cys-98 of cytochrome c-b-562 R98C (Fig. 4E). It has recently been suggested (6) that heme might be released from holo-CcmE by a reverse Michael reaction before becoming attached to apocytochrome c. This would restore free heme, with the vinyl group formerly bound to CcmE once again unsaturated. Our data, showing a heme transfer intermediate with heme bound simultaneously to both CcmE and the cytochrome casts doubt on this proposal, although a reverse Michael reaction could release the heme vinyl group from CcmE once the other vinyl group has reacted with the apocytochrome.

### Complex between CcmE, Heme, and Cytochrome c

**Complex Reflects Phenotypes of Mutants of the Ccm System**—If the complex described above is an intermediate in the Ccm pathway, one would expect its abundance to change predictably in characterized variants of the Ccm system. CcmF and -H function late in the pathway after heme becomes attached coval-
lently to CcmE and are involved in transferring heme from holo-CcmE to apocytochrome c to form the holocytochrome (Fig. 1) (10, 17, 18). When cytochrome c-b562 R98C was co-expressed in E. coli with a plasmid encoding a partial Ccm system with ccmFGH deleted (i.e., expressing ccmABCDE), more CcmE-heme-cytochrome c-b562 complex was observed than when CcmFGH were present (Fig. 5, compare lane 1 with lane 3). When cytochrome c-b562 R98C/Y101C (i.e., with the CXXCH heme-binding motif) was co-expressed with the Ccm system lacking ccmFGH, the complex also accumulated and became visible on a heme-stained gel (Fig. 5, lane 4). In contrast, it was undetectable by heme stain when cytochrome c-b562 R98C/Y101C was co-expressed with the entire Ccm system (Figs. 2C, lane 3, and 5, lane 4), although low levels were observed on Western blots (Fig. 2, A and B, lane 3). Western blots of membranes from cells expressing the ΔccmFGH Ccm system (not shown) confirmed the results from the heme-stained gel. Note that, as expected (10), holo-CcmE accumulates in the ΔccmFGH cells (Fig. 5, compare lanes 3 and 4 with

FIGURE 5. More CcmE-heme-cytochrome c-b562 complex accumulates in cells where ccmFGH are not expressed. SDS-PAGE of cell membranes stained for proteins containing covalently bound heme. The lane order is: M, molecular mass markers (as indicated, in kDa); lanes 1, cells expressing the whole Ccm system (from pEC86) and cytochrome c-b562 R98C; 2, cells expressing the whole Ccm system and cytochrome c-b562 R98C/Y101C; 3, cells expressing ccmABCDE and cytochrome c-b562 R98C; 4, cells expressing ccmFGH and cytochrome c-b562 R98C/Y101C. An equal amount of total protein was loaded in each lane.
Complex between CcmE, Heme, and Cytochrome c

A further prediction is that if the CcmE-heme-cytochrome c-b562 complex is an intermediate in cytochrome c maturation by the Ccm system, the system overall will be inhibited if the complex accumulates (because at least some CcmE will be blocked and unavailable for cytochrome c maturation). To test this, we co-expressed cytochrome c-b562 R98C (to accumulate the complex) and another c-type cytochrome, Paracoccus pantotrophus cytochrome cd$_1$. Control experiments were also performed using appropriate combinations of plasmids, including otherwise identical expression vectors lacking the cytochrome genes. The cells were grown anaerobically on a nitrate-rich medium to induce expression of the endogenous E. coli ccm genes (pEC86 was not used in this experiment). The endogenous E. coli c-type cytochrome NrfA (a nitrite reductase) is also induced in these conditions. We assessed the yields of cytochrome cd$_1$ and NrfA for each combination of plasmids using densitometry of heme-stained SDS-PAGE (e.g. supplemental Fig. S2). The relative holocytochrome yields are given in Table 1. The yields of both cytochrome cd$_1$ and NrfA were significantly reduced when co-expressed with cytochrome c-b562 R98C (supplemental Fig. S2), but not when the equivalent expression vector lacking the cytochrome c-b562 gene was used instead. Thus, consistent with the prediction, accumulation of the CcmE-heme-cytochrome c-b562 complex does inhibit overall flux through the Ccm system.

In Vitro Formation of a CcmE-Heme-Cytochrome c Complex—Previous work has investigated cytochrome c biogenesis by exploring the reactions of apocytochromes c, CcmE, and heme in vitro, using purified proteins and in the absence of other Ccm system components (25, 29, 30, 36, 37). In the light of the results above, we have investigated in vitro formation of a complex involving apocytochrome c, CcmE, and heme. We were not able to form a complex in vitro using cytochrome c-b562 or variants. We believe this is because the apocytochrome is fully folded in vitro (as judged by NMR). We were, however, able to investigate in vitro formation of the complex using H. thermophilus apocytochrome c$_{552}$, which forms a molten globule. This apocytochrome has been used extensively in previous in vitro studies of cytochrome c biogenesis (30, 36, 37).

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7 D. A. I. Mavridou, S. J. Ferguson, and C. Redfield, unpublished data.
8 S. J. Ferguson and C. Redfield, unpublished data.
Holo-CcmE lacking its membrane anchor and with its C-terminal polyhistidine purification tag cleaved (denoted CcmE\textsuperscript*a in this work) was mixed with \textit{H. thermophilus} apocytochrome \textit{c\textsubscript{552}}, which has a typical CXXCH heme-binding motif, in reducing conditions. Over several hours, a change in the visible absorption spectrum of the mixture was observed, indicating that a reaction was taking place (Fig. 7A and supplemental Table S2). The pyridine hemochrome and absorption maxima blue-shifted (supplemental Table S2), as would be expected for saturation of the free vinyl group of the heme bound to CcmE (Fig. 1) if it were forming an adduct with cytochrome \textit{c\textsubscript{552}}.

Supporting this interpretation, when the mixture was analyzed by denaturing SDS-PAGE stained for proteins with covalently bound heme, an additional band appeared compared with that from holo-CcmE\textsuperscript*a alone, its mass (\sim 26 kDa) corresponding to that of holo-CcmE\textsuperscript*a (16 kDa) plus apocytochrome \textit{c} (8 kDa) (Fig. 7B, lane 2). The additional band (from an equivalent protein-stained gel) was subjected to N-terminal sequencing; the band contained the sequences of both \textit{H. thermophilus} cytochrome and \textit{E. coli} CcmE\textsuperscript*a in a 1:1 ratio (Table 2). The presence of CcmE\textsuperscript*a in this complex was further confirmed by Western blotting (Fig. 7C).

The results from the optical spectroscopy, SDS-PAGE, Western blotting, and Edman sequencing therefore all indicate formation \textit{in vitro} of a covalent, ternary complex between CcmE, heme, and cytochrome. Surprisingly, in these experiments little heme transfer to the apocytochrome \textit{c} (to produce holocytochrome) was observed. This is in contrast to the observation that a His-tagged version of holo-CcmE used in previous studies was capable of transferring most of its heme to yield holocytochrome \textit{c} (25). Isolation of this stable CcmE-heme-cytochrome \textit{c\textsubscript{552}} complex \textit{in vitro} is also consistent with the role of CcmE, -G and/or -H in breaking down the complex, which was apparent in our experiments \textit{in vivo} (Fig. 5).

No complex formed when a C11A/C14A variant of \textit{H. thermophilus} apocytochrome \textit{c\textsubscript{552}}, which lacks the heme-binding cysteines, was used in experiments similar to those above (Fig. 7, B and C, lane 8). However, a complex did form when either the C11A (XXXCH heme-binding motif) or C14A (CXXXH) variants were used (Fig. 7, B and C, lanes 4 and 6), indicating that both cysteines of the apocytochrome are capable of binding covalently to CcmE\textsuperscript* \textit{in vitro}. In structurally characterized \textit{c}-type cytochromes, the stereochemistry of heme attachment is invariant; the N-terminal cysteine of the CXXCH motif is bound covalently to the 2-vinyl group of the heme, and the C-terminal cysteine to the 4-vinyl group (1) (Fig. 1). The structure of heme attachment to His-130 of CcmE has been determined from a heme-containing fragment of the protein (Fig. 1), but the heme vinyl group forming the covalent bond was not identified (11). It is often assumed that heme binding to CcmE is stereo- and regiospecific, leaving one heme vinyl group free and oriented for stereospecific attachment to cytochrome \textit{c}. However, the CcmE-heme-cytochrome complex reported here formed from apocytochromes with both XXXCH and CXXXH heme-binding motifs, both \textit{in vivo} and \textit{in vitro} (Figs. 2 and 7). It may be that stereochemical control is favored \textit{in vivo} for a cytochrome with a CXXCH heme-binding motif, \textit{i.e.} for the genuine substrate of the Ccm system, but not for the single cysteine.

### TABLE 1

| Combination of cytochromes expressed | Relative yield of NrfA | Relative yield of cytochrome \textit{cd\textsubscript{i}} |
|-------------------------------------|-----------------------|-----------------------------|
| NrfA only (dummy cytochrome \textit{c\textsubscript{552}} and cytochrome \textit{cd\textsubscript{i}}, expression vectors) | 100% | NA* |
| NrfA and cytochrome \textit{cd\textsubscript{i}} (dummy cytochrome \textit{c\textsubscript{552}}, expression vector) | 88% | 100% |
| NrfA and cytochrome \textit{c\textsubscript{552}} \textit{R596} (dummy cytochrome \textit{cd\textsubscript{i}}, expression vector) | 56% | NA |
| NrfA, cytochrome \textit{cd\textsubscript{i}} and cytochrome \textit{c\textsubscript{552}} \textit{R596} | 40% | 18% |

* NA = not applicable.

\[ \text{FIGURE 7. The \textit{in vitro} reaction between \textit{E. coli} holo-CcmE\textsuperscript*a and \textit{H. thermophilus} apocytochrome \textit{c\textsubscript{552}}.} \]

- **A.** Visible absorption spectra of reduced holo-CcmE\textsuperscript*a with and without addition of apocytochrome \textit{c\textsubscript{552}}. Dashed line, reduced holo-CcmE\textsuperscript*a. Solid line, the spectrum of the holo-CcmE\textsuperscript*a-cytochrome \textit{c} mixture obtained after a 10-min incubation in reducing conditions. Dotted line, the reaction product from holo-CcmE\textsuperscript*a and apocytochrome \textit{c} after incubation for 10 h. All samples were in 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM Tris(2-carboxyethyl)phosphine and disodium dithionite. B. SDS-PAGE stained for proteins containing covalently bound heme, and C. Western blot using a CcmE antibody showing various forms of \textit{H. thermophilus} cytochrome \textit{c} with and without incubation with \textit{E. coli} holo-CcmE\textsuperscript*a. The lane order for both panels \textit{M}: molecular mass markers (as indicated, in kDa); \textit{lanes 1, wild-type \textit{H. thermophilus} apocytochrome \textit{c\textsubscript{552}}; 2, the reaction mixture of wild-type apocytochrome \textit{c\textsubscript{552}} with holo-CcmE\textsuperscript*a; 3, C11A variant apocytochrome \textit{c\textsubscript{552}}; 4, the reaction mixture of C11A variant apocytochrome \textit{c\textsubscript{552}} with holo-CcmE\textsuperscript*a; 5, C14A variant apocytochrome \textit{c\textsubscript{552}}; 6, the reaction mixture of C14A variant apocytochrome \textit{c\textsubscript{552}} with holo-CcmE\textsuperscript*a; 7, C11A/C14A variant apocytochrome \textit{c\textsubscript{552}}; 8, the reaction mixture of C11A/C14A variant apocytochrome \textit{c\textsubscript{552}} with holo-CcmE\textsuperscript*a; 9, holo-CcmE\textsuperscript*a incubated by itself under otherwise identical reaction conditions. All reactions were carried out in 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM Tris(2-carboxyethyl)phosphine and disodium dithionite. The incubation time was 20 h.\]
cytochromes, which are poor substrates. We have been unable to produce enough CcmE-heme-cytochrome c-b_562 R98C/Y101C complex to investigate this point (Fig. 2, lane 3). It may also be that, contrary to expectations, heme binding to CcmE is not the site of stereochromical control by the Ccm system.

**Concluding Remarks**—Here we describe detection and analysis of the first identified complex between CcmE and cytochrome c. This membrane-bound complex includes the two proteins both covalently linked to heme, via the heme-binding histidine of CcmE and a heme-binding cysteine of cytochrome c. Multiple lines of evidence suggest this CcmE-heme-cytochrome complex is an intermediate in the catalytic pathway of the Ccm system. Identification of this complex provides a new focus for experiments on the Ccm apparatus. Analysis of formation and breakdown of the complex is also a means by which the phenotypes of new Ccm system mutants can be assessed.

**Acknowledgments**—We thank Feng Rao for the use of the plasmid pFR015. We are grateful to Nicola Ternette (Nuffield Dept. of Clinical Medicine, Oxford), Benjamin Thomas (Dunn School of Pathology, Oxford) and Antony C. Willis (MRC Immunochemistry Unit, Oxford) for assistance with proteomics analyses. We acknowledge the Computational Biology Research Group, Medical Sciences Division, Oxford for use of their services in this project.

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