The Heme Chaperone ApoCcmE Forms a Ternary Complex with CcmI and Apocytochrome c

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Background: Cytochrome c maturation (Ccm) is the covalent ligation of heme b to an apocytochrome c.
Results: CcmE forms a stable ternary complex with CcmI and apocytochrome c.
Conclusion: Together with CcmFHI, the heme chaperone CcmE is a part of the heme ligation complex that matures apocytochromes c.
Significance: These findings contribute to our mechanistic understanding of how the Ccm process occurs in cells.

Cytochrome c maturation (Ccm) is a post-translational process that occurs after translocation of apocytochromes c to the positive (p) side of energy-transducing membranes. Ccm is responsible for the formation of covalent bonds between the thiol groups of two cysteines residues at the heme-binding sites of the apocytochromes and the vinyl groups of heme b. Oxidized heme (Fe3+/H11001) covalently bound to the His residue of CcmH is different and has a single transmembrane helix although CcmF is similar to its periplasmic domain with a thioredoxin-like motif (8, 9). It belongs to the oligo-binding-fold family of proteins in which the C-terminal helix is often involved in protein-protein interactions (10).

Previously, CcmE was shown to form a complex with CcmC and CcmD in the absence of CcmAB. This complex contains oxidized heme (Fe3+) covalently bound to the His residue of CcmE, with CcmC providing two additional His residues as axial ligands of the heme iron (11). Besides CcmC and CcmD, CcmF that forms together with CcmH the heme ligation core complex in E. coli was shown to interact with CcmE (12, 13). CcmF is a large multispan membrane protein with a b-type heme (11) and belongs to the heme handling protein (HHP) family like CcmC (supplemental Table S1). Recently, it has been suggested that CcmF reduces the heme of holoCcmE to facilitate its transfer to the apocytochrome c (11, 15). In α-proteobacteria (e.g. R. capsulatus), although CcmF is similar to its Escherichia coli counterpart, CcmH is different and has a single transmembrane helix attached to a periplasmic domain with a thioredoxin-like motif (16). However, these species contain an additional component.

The abbreviations used are: Ccm, cytochrome c maturation (Ccm); DDM, n-dodecyl-β-D-maltoside.

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2 The abbreviations used are: Ccm, cytochrome c maturation; DDM, n-dodecyl-β-D-maltoside.
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FIGURE 1. Overall organization of R. capsulatus Ccm system 1. After translocation to the periplasm via the SEC system, a pre-apocytochrome c is processed to an apocytochrome c that undergoes a series of thio-redox reactions. The heme-binding site cysteines can be oxidized by DsbA to form an intramolecular disulfide bond, rendering the apocytochrome ligation-competent (left panel). Heme b is produced in the cytoplasm, and CcmABCDE proteins mediate its translocation and relay to the ligation site. CcmE is a heme chaperone that binds heme covalently via a conserved His residue. Heme-loaded holoCcmE is released from its partners (right panel) to transfer heme to apocytochrome c together with the heme ligation core complex CcmFHI (middle panel). CcmI binds to the C-terminal portion of the apocytochrome c substrates via its large periplasmic domain. CcmE is a heme b-containing protein that is thought to reduce the heme in holoCcmE to facilitate its release to the apocytochrome c. WWD refers to a tryptophan (W) rich domain.

CcmI (supplemental Table S1), which is a bipartite protein with a membrane integral domain (CcmI-1) composed of two transmembrane helices linked through a cytoplasmic loop with a leucine zipper-like motif. Interestingly, the second domain (CcmI-2) of CcmI, which has three tetratricopeptide (TPR) repeats, is homologous to the C-terminal periplasmic extension of E. coli CcmH (17), rendering E. coli CcmFH and R. capsulatus CcmFHI heme ligation core complexes highly similar.

Recently, a ternary complex formed by CcmE, heme, and a C2×XXXH-containing c-type variant of E. coli cytochrome b562 has been identified. This heme was covalently ligated to both CcmE and apocytochrome b562 variant via the His and Cys residues, respectively. A similar complex was also obtained in vitro using the Hydrogenobacter thermophilus apocytochrome c552 (18).

Previously, we showed that CcmI functions as an apocytochrome c chaperone as it binds tightly to the C-terminal helical portion of apocytochrome c2 via its periplasmic CcmI-2 domain. CcmI was suggested to capture apocytochrome c and to facilitate heme ligation carried out by CcmFHI (19). We pursued our studies by investigating how CcmE recognizes the apocytochromes using the apocytochrome form of R. capsulatus cytochrome c552 as a substrate and how it interacts with the components of the heme ligation core complex. In the present study we expressed in E. coli and affinity-purified the R. capsulatus CcmE, CcmI, and apocytochrome c2 proteins and their appropriate mutant derivatives. Using reciprocal in vitro protein-protein interaction assays combined with size exclusion chromatography, we showed for the first time that R. capsulatus apoCcmE interacts directly with apocytochrome c2 in the absence of heme. In contrast with CcmI, which recognizes the C-terminal helical region of apocytochrome c2, apoCcmE has higher affinity for the apocytochrome c2 when a disulfide is present at its heme-binding site. In addition, apoCcmE also binds CcmI, and together with apocytochrome c2 they form a stable ternary complex in vitro. Finally, using detergent-dispersed membranes, we showed that R. capsulatus apoCcmE interacts with the heme ligation core complex components CcmH and CcmI and discuss these findings in the context of the Ccm process.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—The bacterial strains and plasmids used in this work are described in Table 1. R. capsulatus strains were grown chemoheterotrophically (i.e. by respiration) at 35 °C on enriched mineral-peptone-yeast extract medium supplemented with tetracycline or spectinomycin at 2.5 and 10 μg/ml final concentration, respectively (20). E. coli strains were grown aerobically at 37 °C and 200 rpm in Luria-Bertani broth medium supplemented with ampicillin (100 μg/ml). Cultures were induced at A600 of 0.6-0.8 by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside for 4–6 h at 37 °C for FLAG-CcmI, His-CcmI, and Strep-apocytochrome c2, and for 16–18 h at room temperature for His-apoCcmE production.

Plasmids—Molecular genetic techniques were performed according to Sambrook et al. (21). All constructs were confirmed by DNA sequencing and analyzed using the Serial Cloner 2.1 and BLAST software. Nucleotide sequences of the primers used are described in supplemental Table S2). The full-length ccmE gene was PCR-amplified from R. capsulatus MT1131 (Table 1) genomic DNA using the primers CcmE-Ndel-Fw and CcmE-BamHI-Rv, inserting at the N and C terminus of CcmE the Ndel and BamHI restriction sites, respectively. This PCR product was then cloned into pCS1303 (19)
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TABLE 1
Strains and plasmids used in this work

| Bacteria          | Strains/Plasmids                                             | Relevant characteristics                                                                 | References |
|-------------------|-------------------------------------------------------------|------------------------------------------------------------------------------------------|------------|
| *R. capsulatus*   |                                                             |                                                                          |            |
| MTSTRP1.r1        | Δ(*ccm*-kan) G448A in promoter of *ccmFH; Res⁺ Nadi⁺ Ps⁺, cyt c⁺ on MedA |                                                                          | (46)       |
| MT1131            | Wild type, ctsD121 Rif, Res⁺ Nadi⁺ Ps⁺                        |                                                                          | (47)       |
| MD2               | Δ(*ccmEspect); Res⁺ Nadi⁺ Ps⁺                                |                                                                          | (48)       |
| *Escherichia coli*|                                                             |                                                                          |            |
| HB101             | F⁻ Δ(gpt-proA)Δ62 araC14 leuB6(Am) gluV44(As) galK2(Oc) lacY1 Δ(mcrC-mrr) rpsL20(St') ysl54 mitl-1 thi-1 |                                      | Stratagene |
| XLI-Blue          | endA1 gyrA96(Na) thi-1 relA1 lacY44 F:[::Tn10 proAB⁺ lacI² Δ(lacZ)M15] |                                      | Stratagene |
| RP4182            | trp, gal, rpsL Δ(supE, dcm, fla)                              |                                                                          | (49)       |
| **Plasmids**      |                                                             |                                                                          |            |
| pRK415            | Broad host-range vector, gene expression supported by *E. coli lacZ* promoter, Tet’ |                                                                          | (50)       |
| pAV1              | *R. capsulatus* cyoA encoding mature cytochrome c₂ with a N-terminal Strep tag, Amp’ |                                                                          | (19)       |
| pAVIC13S          | Cys13 of *R. capsulatus* cyoA in pAV1 mutated to Ser, Amp’ |                                                                          | (19)       |
| pAVIC16S          | Cys16 of *R. capsulatus* cyoA in pAV1 mutated to Ser, Amp’ |                                                                          | (19)       |
| pAVIH17S          | His17 of *R. capsulatus* cyoA in pAV1 mutated to Ser, Amp’ |                                                                          | (19)       |
| pAVIM96S          | Met96 of *R. capsulatus* cyoA in pAV1 mutated to Ser, Amp’ |                                                                          | (19)       |
| pAVIC15SC16S      | Cys13 and Cys16 of *R. capsulatus* cyoA in pAV1 mutated to Ser, Amp’ |                                                                          | (19)       |
| pAV2              | pAV1 derivative with an in-frame stop codon (TAA) 78 bp upstream of the TAG codon, deleting the 26 last amino acids of cyoA, Amp’ |                                                                          | (19)       |
| pAV2C15SC16S      | Cys-13 and Cys-16 of *R. capsulatus* cyoC in pAV2 mutated to Ser, Amp’ |                                                                          | (19)       |
| pCS1303           | His₁₀ tag sequence fused to GFP, rendering GFP replaceable by cloning any gene of interest in-frame into NdeI and BamHI sites, Amp’ |                                                                          | (19)       |
| pMADO5            | *R. capsulatus* ccmI encoding full length CcmI with a N-terminal His₁₀ tag sequence, Amp’ |                                                                          | (19)       |
| pFLAG-CcmI        | pFLAG005 derivative, with a N-terminal Flag tag sequence instead of the His₁₀ tag, Amp’ |                      This work                      |            |
| pAV4              | pCS1303 derivative containing *R. capsulatus* ccmI encoding full length CcmI with a N-terminal His₁₀ tag sequence, Amp’ |                      This work                      |            |
| pAV4H123A         | His₁₂₃ of *R. capsulatus* ccmE in pAV4 mutated to Ala, Amp’ |                                                                          | (17)       |
| pNJ2              | ccmI::FLAG expressed from its own promoter in pRK415, Tet’ |                                                                          |            |

Using the same restriction sites. The plasmid pAV4 thus obtained contains an in-frame 10 histidine-long (His) epitope tag followed by the Factor Xa cleavage site fused to the N terminus of CcmE. Plasmid pAV4H123A, containing the His-123 to Ala substitution, was generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with pAV4 as a template and CcmEH123A-Fw and CcmEH123A-Rv as mutagenic primers. A FLAG-tagged version of full-length CcmE was obtained by site-directed mutagenesis using the primers FLAG-Fw and FLAG-Rv and pMADO5 as a template (19). The plasmid pFLAG-CcmE thus constructed contained a FLAG epitope tag followed by the Factor Xa cleavage site fused to the N terminus of CcmI. Plasmid pMADO5 was used for production and purification of a His-tag version of CcmE, as described earlier (19). The apocytochrome c₂ and its derivatives were obtained as described previously (19), and all contained an N-terminal in-frame Streptag-II-epitope tag followed by the Factor Xa cleavage site.

Preparation of *R. capsulatus* or *E. coli* Detergent-solubilized Membrane Proteins—*R. capsulatus* or *E. coli* solubilized membrane proteins were obtained as described previously (19). Briefly, intracytoplasmic membrane vesicles were prepared using a French pressure cell. The membrane pellets were obtained after 2 h of centrifugation at 4 °C and 138,000 × g. Membranes were dispersed by the addition of n-dodecyl-β-D-maltoside (DDM) (Anatrace, Inc.) at a protein:detergent ratio of 1:1 (w/w) under continuous stirring for 1 h at 4 °C followed by another high speed centrifugation to remove non-solubilized proteins.

Protein Purification—For purification of His-CcmI (19), HisapoCcmE and its H123A mutant detergent-solubilized *E. coli* membranes were loaded onto a Ni²⁺-Sepharose high performance column (GE Healthcare) equilibrated with a buffer containing 25 mM Tris-HCl, pH 7.4, 500 mM NaCl, 40 mM imidazole, and 0.01% DDM. Elution of the His-tagged-proteins was done using the same buffer with 500 mM imidazole. Purification of FLAG-CcmI from *E. coli* solubilized membranes was done using the Anti-FLAG® M2 affinity gel (Sigma) equilibrated with a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.01% DDM. Elution was done with a buffer containing 100 mM glycine, pH 3.5, and 0.01% DDM. After elution, samples were immediately neutralized by the addition of a few μL of 1 M Tris-HCl, pH 8.0. Purifications of Strep-apocytochrome c₂ and its mutant derivatives were performed as described previously (19). After SDS-PAGE analysis, protein fractions were concentrated and desalted with a PD-10 column (GE Healthcare) using a buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl with 0.01% DDM. The identity of purified proteins was confirmed routinely by nLC-MS/MS spectroscopy as described earlier (19).

Protein-Protein Interaction Studies Using Reciprocal Co-purification Assays—Protein-protein interactions between HisapoCcmE, Strep-apocytochrome c₂, FLAG-CcmI, and their respective derivatives were assayed as described before (19). Briefly, purified His-apoCcmE (10 μg), Strep-apocytochrome c₂ (15 μg), and FLAG-CcmI (10 μg) were mixed as appropriate in a binding/wash buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM imidazole, and 0.01% DDM (final assay vol-
Heterologous Expression and Purification of ApoCcmE and Apocytochrome c2

To investigate whether and, if so, how the heme chaperone apoCcmE interacts with apocytochrome c and other Ccm components, including the CcmFHI complex, we produced in E. coli an N-terminal His epitope-tagged full-length R. capsulatus CcmE (His-apoCcmE) and its H123A mutant (His-apoCcmE-H123A) (Fig. 2A) (“Experimental Procedures” and Table 1). These proteins were purified from the membrane fractions of E. coli cells grown aerobically to avoid holoCcmE production by the host Ccm machinery. Purified materials were assessed by SDS-PAGE and immunoblot analyses using specific rabbit polyclonal antibodies. By Coomassie staining, His-apoCcmE (Fig. 2B) and its His-apoCcmE-H123A mutant (data not shown) showed a major band at ~18 kDa and a minor band (roughly 15%) around 37 kDa. Purified proteins did not contain any heme, based on SDS-PAGE/tetramethylbenzidine staining and visible spectroscopy (data not shown). The 37-kDa band was attributed to a dimeric form of His-apoCcmE that persisted under reductive SDS-PAGE conditions, as identified using spe-
cific anti-apoCcmE polyclonal antibodies (Fig. 2C) and nanoLC-tandem mass spectrometry (data not shown). A tiny amount of His-apoCcmE degradation product (~15 kDa, not visualized by SDS-PAGE) was also detected by immunoblot (Fig. 2C). A similar, highly stable holocmmE dimer has been reported previously (18, 24), and our data with the apoCcmE derivative indicated that the presence of heme is not required for this oligomerization.

We also produced in E. coli cytoplasm the N-terminally Strep-epitope tagged full-length R. capsulatus apocytochrome c2 (Fig. 2D) as well as its various mutant derivatives as Ccm substrates (Table 1). These Ccm substrates were purified using Strep-Tactin-Sepharose affinity chromatography as described previously (19). Like many apocytochromes, full-length Strep-apocytochrome c2 was highly prone to degradation during its production and purification even in the presence of protease inhibitors (19). Purified Strep-apocytochrome c2 showed a major band of 13.5 kDa and a minor band of 12.9 kDa (roughly 20%) (Fig. 2E, lane 1), identified using anti-cytochrome c2 polyclonal antibodies and nanoLC-tandem mass spectrometry (data not shown) as a C-terminally truncated derivative with an intact N-terminal Strep tag. This degradation product was not detectable in the case of the t26-Strep-apocytochrome c2 variant of 11.5 kDa, which lacks the last C-terminal 26 amino acid residues (Fig. 2E, lane 2). His-apoCcmE and Strep-apocytochrome c2 proteins thus purified as well as their mutant derivatives were of sufficient purity (over 95%) to carry out reliably co-purification assays analyzed by SDS/PAGE-Comassie staining and did not contain any unrelated protein.

**ApoCcmE Interacts Directly with Apocytochrome c2 in the Absence of Heme**

Direct physical interactions between apoCcmE and apocytochrome c2 in the absence of heme were probed using in vitro reciprocal co-purification assays, as carried out previously with CcmI (19). Purified His-apoCcmE (or His-apoCcmE-H123A mutant) was incubated with Strep-apocytochrome c2 and subjected to co-purification assays using tag specific affinity chromatography, and the elution fractions were analyzed by SDS-PAGE as described under “Experimental Procedures.” Control experiments showed no unspecific binding of His-apoCcmE to the Strep-Tactin or of Strep-apocytochrome c2 to Ni2+-Sepharose columns under the conditions used (Fig. 3A, lane 2, and B, lane 4). In contrast, upon incubation of His-apoCcmE with Strep-apocytochrome c2, the two proteins were co-eluted from either of the tag-affinity columns (Fig. 3A, lane 3, and 3B, lane 2), indicating that apoCcmE and apocytochrome c2 interacted with each other to form a stable binary complex. We noted that the minor band corresponding to the C-terminally truncated form of apocytochrome c2 also co-purified with apoCcmE, suggesting that the C-terminal portion of apocytochrome c2 was not critical for these interactions (Fig. 3A, lane 3). Moreover, co-purification of Strep-apocytochrome c2 with the His-apoCcmE-H123A mutant derivative indicated that these interactions were not abolished by the absence of the heme binding His residue (Fig. 3B, lane 3), further confirming their independence of heme.

We also carried out binding assays under various salt concentrations to characterize the nature of the apoCcmE and apocytochrome c2 interactions. Increasing NaCl concentrations from 50 to up to 250 mM decreased substantially the amount of His-apoCcmE that co-purified with wild type Strep-apocytochrome c2 (lane 1) was taken as 100% for image quantification using Image J program and compared with the amounts seen with different salt concentrations. D. R. capsulatus holocmmE c2 does not co-purify with His-apoCcmE using Ni2+-Sepharose resin. Note the presence in the flow-through (FT; lane 1) and the absence in the elution fraction (E; lane 2) of holocytochrome c2. All co-purification assays were done using the standard assay conditions described under “Experimental Procedures.” Panel C and D show only the regions of the gel containing the two forms of apocytochrome c2 or holocytochrome c2, and the major monomeric form of apoCcmE for the sake of clarity. Molecular markers (kDa) are shown as needed.

Next, we investigated whether apoCcmE could also recognize mature cytochrome c2 with its covalently bound heme. Under the standard assay conditions, when His-apoCcmE was incubated together with cytochrome c2 purified from **R. capsula-**

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![FIGURE 3. Co-purification of R. capsulatus apoCcmE with apo and holoforms of cytochrome c2. Shown are co-purification of Strep-apocytochrome c2 with His-apoCcmE using Ni2+-Sepharose resin (lane 3) (A) and reciprocal co-purification of His-apoCcmE (lane 2) or His-apoCcmE-H123A mutant variant (lane 3) with Strep-apocytochrome c2 using Strep-Tactin-Sepharose resin (B). Strep-apocytochrome c2 (panel A, lane 2) and His-apoCcmE (panel B, lane 4) are not retained unspecifically with Ni2+-Sepharose and Strep-Tactin-Sepharose resins, respectively. Both the intact and the C-terminally truncated forms of apocytochrome c2 are indicated. C. binding of His-apoCcmE to wild type Strep-apocytochrome c2 is salt-dependent. Different concentrations of NaCl, 50 mM (lane 1), 150 mM (lane 2), and 250 mM (lane 3) were used during the incubation mixture before co-purification using the Strep-Tactin-Sepharose resin. The relative amount of His-CcmE that co-purified with wild type Strep-apocytochrome c2 (lane 1) was taken as 100% for image quantification using Image J program and compared with the amounts seen with different salt concentrations. D. R. capsulatus holocmmE c2 is salt-dependent. Different concentrations of NaCl, 50 mM (lane 1), 150 mM (lane 2), and 250 mM (lane 3) were used during the incubation mixture before co-purification using the Strep-Tactin-Sepharose resin. The relative amount of His-CcmE that co-purified with wild type Strep-apocytochrome c2 (lane 1) was taken as 100% for image quantification using Image J program and compared with the amounts seen with different salt concentrations. D. R. capsulatus holocmmE c2 does not co-purify with His-apoCcmE using Ni2+-Sepharose resin. Note the presence in the flow-through (FT; lane 1) and the absence in the elution fraction (E; lane 2) of holocytochrome c2. All co-purification assays were done using the standard assay conditions described under “Experimental Procedures.” Panel C and D show only the regions of the gel containing the two forms of apocytochrome c2 or holocytochrome c2, and the major monomeric form of apoCcmE for the sake of clarity. Molecular markers (kDa) are shown as needed.
The data indicating that apoCcmE interacted with apocytochrome c2 in vitro in the absence of heme led us to inquire the molecular determinants responsible for these interactions. We thought that the specific regions of these proteins involved in these interactions might be important for transferring heme from CcmE to apocytochrome c. R. capsulatus cytochrome c2, like the H. thermophilus cytochrome c552, recently shown to form a ternary complex in vitro with CcmE and heme (18), belongs to the Class I of c-type cytochromes (26). These c-type cytochromes have a N-terminally located heme binding (C1XXC2H) motif, a C-terminally located Met residue acting as the sixth axial ligand of the heme-iron, and a general globular fold with interacting N- and C-terminal helices (27, 28). These salient features of apocytochrome c2 were probed for their role(s) in the interactions with apoCcmE.

**Heme-binding Site Cys Residues**—Participation of the heme-binding site (C1XXC2H) Cys residues of apocytochrome c2 in apoCcmE interactions was examined by using appropriate Strept-apocytochrome c2 mutants. Binding of purified Strept-apocytochrome c2 variant proteins (i.e. -C13S, -C16S, -C13S/C16S) to His-apoCcmE was probed by affinity co-purification using the Strept-Tactin-Sepharose resin as done with the native Strept-apocytochrome c2. Image analyses of Coomassie Blue-stained gels indicated that apoCcmE still co-purified with apocytochrome c2 mutants lacking either one of the heme-binding site Cys residues (Fig. 4A, lanes 3 and 7), but in the absence of both Cys residues the amount of apoCcmE that co-purified decreased drastically to ~15% that determined for the native apocytochrome c2 (Fig. 4A, lane 5). Moreover, with native apocytochrome c2 or its single cysteine mutants treated with DTT/iodoacetamide, we observed that alkylation of the Cys residues is as damaging for apoCcmE-apocytochrome c2 interactions (Fig. 4A, lanes 2, 4, and 8) as the absence of the Cys residues (Fig. 4A, lane 5). Thus, at least one reactive Cys residue at apocytochrome c2 heme-binding site appeared critical for its recognition by apoCcmE. Considering that the single Cys mutants of apocytochrome c2 can still form intermolecular disulfides, we carried out the co-purification assays in the presence of either reducing or oxidizing agents. In the presence of DTT the amount of apoCcmE that co-purified with apocytochrome c2 decreased to ~35% that observed in the absence of a reductant (Fig. 4B, lanes 1 and 2). On the other hand, in the presence of diamide, CuCl2, or H2O2, this amount remained unchanged or increased slightly (Fig. 4B, lanes 3–6). The data therefore suggested that apoCcmE preferred to bind to an apocytochrome c2 with a disulfide bond rather than reduced thiols at its heme-binding site.

**Heme-iron Atom Axial Ligands His-17 and Met-96**—We next tested the interactions of Strept-apocytochrome c2 derivatives lacking the heme-iron axial ligands His-17 or Met-96 with His-apoCcmE. Co-purification data showed that apoCcmE co-purified with these mutants at amounts comparable with those observed with the native apocytochrome c2 (Fig. 4C, lanes 1–3). Thus, the heme-iron axial ligands were not important for apoCcmE-apocytochrome c2 interactions.

**The C-terminal α-Helix of Apocytochrome c2**—Previously, we found that the C-terminal helix of apocytochrome c2 was critical for its recognition by the apocytochrome c chaperone CcmI (19). We tested whether a truncated apocytochrome c2 derivative lacking its last C-terminal 26 amino acids residues and its variant lacking the heme-binding site Cys also interacted with apoCcmE. Co-purification assays similar to those carried out with native Strept-apocytochrome c2 indicated that the amounts of His-apoCcmE that co-purified with truncated Strept-t26-apocytochrome c2 and its Cys-less derivative decreased to ~75% and ~15% that observed with native Strept-apocytochrome c2, respectively (Fig. 4D, lanes 4–6). The data demonstrated that the C-terminal portion of apocytochrome c2 is important for transferring heme from CcmE to apocytochrome c2.
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Binary interactions detected between apoCcmE-CcmI, apoCcmE-apocytochrome c2, and CcmI-apocytochrome c2 suggested that these proteins might form a stable ternary complex in vitro. Incubation mixtures containing His-apoCcmE, Strep-apocytochrome c2, and FLAG-CcmI altogether were loaded onto either Strep-Tactin- or Ni2+-Sepharose resin-containing columns. Co-purification assays showed that these three proteins co-eluted together (Fig. 6, A and B). Comparison of the amount of the ternary complexes formed by these three proteins (Fig. 6, A and B) with the corresponding appropriate binary complexes (Fig. 3, A and B, versus Fig. 5, D and E) further supported the finding that apoCcmE and CcmI do not compete directly with each other for the same structural elements of apocytochrome c2.

Size exclusion chromatography was used to confirm the formation of the ternary complex of apoCcmE, CcmI, and apocytochrome c2. After incubation of the three proteins under the standard assay conditions, the mixture was loaded onto a previously calibrated Sephacryl-S200 column. The elution profile was monitored at 280 nm, and selected elution fractions were subjected to SDS-PAGE (Fig. 6, C and D). The bulk of apoCcmE was found in the first peak eluted at the column dead volume corresponding to a molecular mass larger than 250 kDa, where CcmI and apocytochrome c2 were also present, albeit at lower amounts (Fig. 6D, fractions 35–40). This observation suggested that the three proteins formed a multisubunit complex(es) containing large amounts of apoCcmE. The next peak covering a range of 110 to 65 kDa (Fig. 6D, fractions 48–55) contained CcmI, apoCcmE, and apocytochrome c2. Roughly at stoichiometric ratios, considering molecular masses of 50, 18, and 13.5 kDa for CcmI, apoCcmE, and apocytochrome c2, respectively. Finally, fractions 65–70 contained mainly excess apocytochrome c2 not retained by apoCcmE or CcmI. Overall data, therefore, further supported the formation in vitro of a ternary complex composed of apoCcmE, CcmI, and apocytochrome c2.
**CcmE-CcmI-Apocytochrome c Ternary Complex**

*ApoCcmE Interacts Directly with the CcmI and CcmH Components of the Heme Ligation Complex CcmFHI in *R. capsulatus* Membranes*

In light of the stable ternary complex observed in vitro between apocytochrome *c*$_2$, apoCcmE, and CcmI, we examined the interactions of these proteins with the heme ligation core complex CcmFHI of *R. capsulatus*. Co-purification assays were conducted using 500 μg of DDM-solubilized membranes from an *R. capsulatus* strain overproducing the CcmFHI complex (MTSRP1.r1/pNJ2, Table 1) supplemented with purified His-apoCcmE or His-CcmI under the assay conditions used in vitro with purified proteins. The interacting partners were identified by immunoblot analysis of the elution fractions from the Ni$_2^+$-Sepharose columns. Upon incubation of the solubilized membranes with purified His-apoCcmE, we observed that mainly the CcmI and CcmH, but not CcmF, co-purified with His-apoCcmE (Fig. 7A, lane 3). Similarly, when purified His-CcmI was used, co-purification of CcmF and CcmH with His-CcmI was observed as seen earlier (7) (Fig. 7B, lane 3). In addition, the anti-apoCcmE antibodies recognized in the elution fraction a band of 18 kDa, reminiscent of the size of native CcmE. This suggested that CcmE might interact directly with the CcmFHI complex (Fig. 7B, lane 3). Initially, unambiguous identification of CcmE in *R. capsulatus* solubilized membranes was challenging as the available anti-apoCcmE polyclonal antibodies identifies multiple bands with apparent molecular masses of 37, 30, 28, 27, and 18 kDa (Fig. 7B, lane 1). However, based on the CcmE amino acid sequence, the use of a CcmE knock-out strain (MD2, Table 1) and *R. capsulatus* His-apoCcmE protein produced and purified from *E. coli* membranes (Fig. 2, B and C), we attributed the ~18- and ~37-kDa bands to *R. capsulatus* CcmE and its dimeric form, respectively. The intense band with a molecular weight of 28 kDa, detected in solubilized membranes of *R. capsulatus* wild type (MT1131, Table 1) and CcmE knock out strains (data not shown), was considered to be a nonspecifically recognized protein, and the bands located between the 25 and 37-kDa molecular mass markers were attributed to peroxidase activity-containing proteins like the *c*-type cytochromes (note that *R. capsulatus* has several membrane-bound *c*-type cytochromes) detected with the SuperSignal West Pico Chemiluminescent Substrate. The latter bands were absent when the anti-rabbit alkaline phosphatase conjugate was used as the secondary antibody (data not shown). The data, therefore, indicated convincingly that apoCcmE is associated with the heme ligation complex in a manner independent of the presence of heme. We, therefore, concluded that CcmE together with CcmF, CcmH, and CcmI form a multisubunit maturation complex that recognizes apocytochrome *c*$_2$.

**DISCUSSION**

Earlier studies using co-immunoprecipitation assays and native gel electrophoresis documented physical interactions between the Ccm components, showing that they form various multisubunit complexes (e.g. CcmABCD, CcmCDE, CcmFHI (7, 29–31)). Similarly, direct interactions between the appropriate Ccm components and the apocytochrome substrates were also reported. For example, genetic approaches, like the
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A

| Mb | apoCcmE |
|----|---------|
| CcmF | BC |
| CcmI | Mb |
| CcmH | 50 |
| CcmI | 50 |

B

| Mb | CcmI |
|----|------|
| CcmF | BC |
| CcmH | Mb |
| CcmE | 50 |
| CcmE | 50 |

\(\text{Ni Sepharose}\)

**FIGURE 7.** ApoCcmE forms a multisubunit complex with the heme ligation components CcmI and CcmE in *R. capsulatus* membranes. *A*, shown is an immunoblot analysis of the elution fraction obtained using DDM-dispersed *R. capsulatus* membranes incubated with purified His-apoCcmE and loaded onto a Ni\(^{2+}\)-Sepharose column. Lane 1, \(-50 \mu\)g of the protein mixture after incubation and before loading onto the column (BC); lane 2, elution fraction of a control experiment performed just with *R. capsulatus* membranes shows that none of the proteins of interest is retained unspecifically by the Ni\(^{2+}\)-Sepharose resin (Mb); lane 3, an elution fraction shows that CcmI and CcmH, but not CcmF, are co-purified together with His-apoCcmE under the conditions used (Mb + apoCcmE). *B*, shown is an immunoblot analysis of the elution fractions obtained using DDM-dispersed *R. capsulatus* membranes incubated with purified His-CcmI and loaded onto a Ni\(^{2+}\)-Sepharose column. Lane 1 as in panel *A*; lane 2 is as in panel *A*; lane 3, the elution fraction shows that CcmF, CcmH, and native CcmE are co-purified together with His-CcmI (Mb + CcmE). Note that CcmE detection shows several bands (marked with a \(\times\)) between the 25- and 37-kDa marker that are either unspecifically recognized by the antibodies or are heme-dependent peroxidase activity exhibiting c-type cytochromes due to the detection system. The band corresponding specifically to CcmE co-purifying with His-CcmE is seen at 18 kDa.

yeast two hybrid screens, revealed that in *Arabidopsis thaliana* CcmFN2 and CcmH interacted with apocytochrome \(c_2\) (32, 33). *E. coli* CcmH could reduce the disulfide bond of a cytoplasm \(c\)-like peptide in *vitro* (16), and *R. capsulatus* Cys-less CcmG could co-purify with apocytochrome \(c_2\) (34). Previously, we showed that the large periplasmic domain of CcmI with its TPR motifs interacted strongly with the C terminus of *R. capsulatus* apocytochrome \(c_2\), whereas its membrane integral domain was needed for the integrity of the CcmFHI complex (19). In this work we extended these protein-protein interactions studies to other Ccm components. In particular, we inquired whether, and if so how, the heme chaperone apoCcmE recognizes the apocytochromes \(c\) substrates and the heme ligation complex CcmFHI.

First, we found that, like CcmI, apoCcmE interacted directly with the apo- and not with the holo-cytochrome \(c_2\) of *R. capsulatus*. However, unlike CcmI, the apoCcmE-apocytochrome \(c_2\) interactions involved the N-terminal, and not the C-terminal, portion of the apocytochrome. Moreover, these interactions were sensitive to ambient ionic strength and were stronger when a disulfide bond was present at the apocytochrome heme-binding site. Although no three-dimensional structure of an apocytochrome \(c\) is yet available, conceivably, *R. capsulatus* apocytochrome \(c_2\) might adopt different conformations according to the redox state of its \(C_{\text{II}}\) heme-binding site Cys residues. Besides forming disulfide bonds, the free thiols (or thiolates) of the Cys residues can also interact with nearby charged residues (35) and affect protein-protein interactions (36, 37). The three-dimensional structure of apoCcmE (8, 9) shows at the top of the \(\beta\)-barrel fold a surface-exposed region composed of hydrophobic and basic residues. This region is near the His residue that binds heme covalently and is thought to act as the heme platform. Assuming that this area may be a docking site for the apocytochrome \(c_2\) carrying a disulfide bond, reduction of this bond might alter the environment of the heme-binding site of apocytochrome \(c_2\) and consequently affect its interactions with apoCcmE.

In *E. coli* cells a cytochrome \(b_{562}\) derivative with a \(C_{\text{II}}\) binding motif forms a complex with heme and CcmE (18). The heme was covalently bound to the Cys-98 of this \(c\)-type cytochrome mimic and the His-130 of CcmE. This complex was observed *in vitro* using the purified *H. thermophilus* apocytochrome \(c_{552}\) but not the cytochrome \(b_{562}\) variant. Interestingly, before heme attachment, the apocytochrome \(b_{562}\) derivative is fully folded as a four-helical bundle, whereas the apocytochrome \(c_{552}\) forms a molten globule (18). In fact, *H. thermophilus* apocytochrome \(c_{552}\) is notorious in acquiring heme independently of the Ccm machinery in *E. coli* cytoplasm and also *in vitro* (38, 39). Thus, the global conformation of an apocytochrome is important for the Ccm process. With the exception of a few cases like *H. thermophilus* apocytochrome \(c_{552}\), Class-I apocytochromes are usually unfolded before heme binding and do not bind heme readily unless they are assisted by the Ccm machinery. Remarkably though, our data indicate that, like CcmI, apoCcmE can recognize and bind to apocytochrome in the absence of its covalently bound heme. Whether similar interactions also occur with holoCcmE awaits the purification of *R. capsulatus* holoCcmE.

Second, we found that purified apoCcmE (and its H123A mutant) also interact with purified CcmI, which is a subunit of the CcmFHI complex. Moreover, apoCcmE formed a stable ternary complex together with CcmI and apocytochrome \(c_2\). The high propensity for oligomerization shown by the members of the oligo-binding-fold family proteins like CcmE plus the highly hydrophobic nature of CcmI and apoCcmE precluded us from determining a precise subunit stoichiometry for the ternary complex. Nonetheless, the occurrence of this complex strongly suggested that apoCcmE interacted closely with the heme ligation complex CcmFHI. Indeed, the data obtained using DDM-dispersed *R. capsulatus* membranes indicated that CcmI and CcmH copurified with apoCcmE. Similarly, CcmF, CcmH, and CcmE co-purified with CcmI, providing further evidence that apoCcmE interacted closely with the heme ligation components CcmFHI (Fig. 8A). However, considering that
CcmF did not co-purify with apoCcmE, these two proteins might not interact directly within the CcmFHI complex, as suggested (15). Earlier co-immunoprecipitation data using anti-CcmF antibodies indicated that apoCcmE interacted with CcmF in E. coli, although the reciprocal experiments using anti-CcmE antibodies remained inconclusive (41). However, no protein-protein interaction(s) between CcmE and CcmH has been reported before this study. Furthermore, CcmI might be the component linking the heme ligation complex, the heme chaperone CcmE, and the apocytochrome c substrates altogether, as depicted in Fig. 8A, and inferred based on earlier genetic studies (40).

The finding that CcmI-apocytochrome c2-apoCcmE forms a stable ternary complex in vitro together with the data obtained using solubilized membranes raises the possibility of whether or not CcmABCD, CcmECcmFHI, and apocytochrome c form altogether a very large molecular complex (Fig. 8B). Earlier, co-localization of CcmF, CcmI, and CcmH in high molecular weight R. capsulatus membrane fractions (~800 kDa) has been reported (7). Similarly, high molecular weight complexes containing CcmF and CcmH were also observed in A. thaliana (~500 kDa) (32) and Triticum aestivum (~700 kDa) (42). Excitingly, ongoing co-purification assays using similar R. capsulatus DDM-dispersed membranes indicate that all components of the heme ligation CcmFHI and the heme chaperone CcmE co-elute together when membranes are supplemented with exogenous apocytochrome c2. It now remains to be seen which one(s) of the remaining Ccm components is also located in these large molecular entities.

Finally, recognition of an apocytochrome c by the apoCcmE might appear counterintuitive at first, as only the holoCcmE would be suitable for the Ccm process. However, provided that a multisubunit maturation complex composed of CcmABCD-CcmE-CcmFHI exists in R. capsulatus membranes (Fig. 8B), one might wonder whether in the absence of heme apoCcmE associates more tightly with CcmFHI than CcmABCD. If so, once apocytochrome c is trapped by CcmI of the CcmFHI complex, then apoCcmE would assist apocytochrome to position correctly for reduction of the disulfide bond at its heme-binding site, possibly via CcmH. According to this hypothesis, only when CcmC is ready to load heme into apoCcmE will stable CcmE-CcmFHI complex be formed (31). And only upon ATP hydrolysis by the CcmAB complex (43, 44) will the holoCcmE thus formed be released to transfer heme to the apocytochrome c trapped by CcmFHI (45) (Fig. 8B). Future experiments undoubtedly will further address these issues.

In summary, this study established that apoCcmE interacts directly not only with an apocytochrome substrate but also with the CcmH and CcmI components of the heme ligation complex CcmFHI. Clearly, these interactions occur in the absence and possibly in the presence of apocytochrome c2 and heme. These findings now provide important insights into the process of how CcmE recognizes the apocytochrome c substrates together with the CcmFHI complex for heme ligation during the maturation of the c-type cytochromes.

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