Cytochrome c maturation (Ccm) is a post-translational and post-export protein modification process that involves ten (CcmABCDEFGHI and CcdA or DsbD) components in most Gram-negative bacteria. The absence of any of these components abolishes the ability of cells to form cytochrome c, leading in the case of *Rhodobacter capsulatus* to the loss of photosynthetic efficiency and respiratory cytochrome oxidase activity. Based on earlier molecular genetic studies, we inferred that *R. capsulatus* CcmF, CcmH, and CcmI interact with each other, indicating that these Ccm components form a membrane-integral complex. Under the conditions used, the CcmFHI complex does not contain CcmG, suggesting that the latter thio-reduction component is not always associated with the heme ligation components. The findings are discussed with respect to defining the obligatory components of a minimalistic heme-apocytochrome c ligation complex in *R. capsulatus*.

Cytochromes (cysts) are ubiquitous hemoproteins primarily functioning as electron carriers between energy-transducing membrane complexes of photosynthesis (Ps) and respiration (Res). They contain iron-protoporphyrin IX (heme) molecules that are covalently and stereospecifically ligated via thioether bonds formed between the heme vinyl groups and the cysteine thiol groups of Cys1-XX-YY-Cys2-His (C1XXC2H) motifs within the apocysts (1). A complex cyt c maturation (Ccm) pathway is found in α- and γ-proteobacteria, deinococci, and mitochondria of plants and protozoa (Ccm-system I) (2). In most Gram-negative bacteria, Ccm-system I consists of ten components, CcmABCDEFGHI and CcdA (or DsbD), acting on the outer side of the cytoplasmic membrane (3), and ligating heme molecules to apocysts c following their cytoplasmic syntheses.

Of the Ccm components, CcmA, CcmB, CcmC, and CcmD form an ATP-binding cassette-containing transporter complex thought to translocate heme across the cytoplasmic membrane, load it to the heme chaperone CcmE, and subsequently release holoCcmE from this complex (4). CcdA (or DsbD) together with CcmG are implicated in the reduction of the disulfide bond thought to be formed between the cysteine thiols within apocyt c heme binding motifs by the DsbA-dependent thio-oxidative protein-folding pathway (5, 6). This thio-oxidative pathway is not essential for Ccm per se (7, 8), although its inactivation reduces Ccm efficiency significantly (39). Interestingly though, a defective thio-oxidative branch readily compensates Ccm deficiency in the absence of CcdA and CcmG (7, 9), suggesting that the thio-reduction process during Ccm is only required for cyt c production when a thio-oxidative branch is functional. Moreover, recent data indicate that the compensatory thio-redox interactions involve only CcdA and CcmG, and not CcmH, which is also thought to be part of the apocyst c thio-reduction process (39).

CcmH is comprised of a N-terminal signal peptide, an extracytoplasmic loop with a redox-reactive C2XXC2 motif and a C-terminal transmembrane domain (10, 11). It has an unusual thioerdoxin-like structure (12), and interacts physically with CcmF in *Escherichia coli* (13). Based on genetic studies using *Rhodobacter capsulatus*, we inferred that CcmH associates not only with CcmF, but also with CcmI (14). CcmF is a member of the Heme Handling Protein (HHP) family with multiple transmembrane segments (15). It has a tryptophan-rich (WWD) signature motif and four conserved histidine residues facing the periplasm (16) and is proposed to ligate heme delivered by CcmE to apocysts c (13, 16). However, direct association of CcmF with apocyst c has not yet been shown experimentally in bacteria (13). CcmI is thought to chaperone the apocyst c to the heme ligation site (17). In *R. capsulatus* and many other species, it contains two N-terminal transmembrane helices encompassing a leucine zipper-like motif in its cytoplasmic loop (i.e. CcmI-1) and a large periplasmic C-terminal extension (i.e. CcmI-2) with multiple tetratricopeptide repeat (TPR)-like motifs (18). Recent genetic studies indicated that the CcmI-1...
and CcmI-2 domains play distinct roles during Ccm with the former being functionally interconnected with CcmF and CcmH, and the latter with CcmG (14, 19).

In this work, using combinations of reciprocal affinity and size exclusion chromatography, we provide the first direct biochemical evidence that *R. capsulatus* CcmF, CcmH, and CcmI interact with each other to form a stable, multisubunit membrane protein complex. Implications of this CcmmFH1-containing heme ligation complex lacking CcmG for the heme-apocytochrome c ligation process during Ccm are discussed.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**—The bacterial strains and plasmids are listed in Table 1. *R. capsulatus* strains were grown at 35 °C on enriched (MPYE) or minimal (MedA) media (20), supplemented with appropriate antibiotics (tetracycline, kanamycin, and spectinomycin at final concentrations of 2.5, 10, and 10 μg/ml, respectively), either chemoheterotrophically (Res growth) or photoheterotrophically (Ps growth), as described earlier (21). *E. coli* strains were grown on Luria Bertani (LB) broth supplemented with appropriate antibiotics (tetracycline, kanamycin, spectinomycin, and ampicillin at final concentrations of 12.5, 50, 10, and 100 μg/ml, respectively) (21, 22). The Δ*ccmF*::spe and Δ*ccmH*::spe (pYZ12) alleles (23) were introduced by interposon mutagenesis (21) into the CcmI-null mutant MT-SRP1 Δ*ccmI*::kan (17) using the gene transfer agent (GTA) (24) to yield the *R. capsulatus* CcmF-null, CcmH-null (MD13), and CcmH-null CcmI-null (MD15) double mutants, respectively (Table 1).

**Molecular Genetic Techniques**—Molecular genetic techniques were performed using standard procedures (25). All constructs were confirmed by DNA sequencing. Sequence analyses and comparisons were conducted using MacVector (Accelrys, San Diego, CA) and BLAST software packages (26). Constructions of various epitope-tagged Ccm derivatives were as follows. A Strep-tag sequence was added in-frame to the 5′-end of *ccmF* in pYZ1 (23) via the QuikChange™ site-directed mutagenesis kit according to the manufacturer’s instructions (Stratagene, La Jolla, CA) using the primers CcmF-StrepN-Fwd (5′-GGA GGA CCC CGC ATG ATC AGC TGG AGC CAC CCG CAG TAT GGA AAA GGC GTG ACC GCC CAT-3′) and CcmF-StrepN-Rev (5′-GAA ATC GCC GGT CTC GAC GGC TTT TTC GAA CTG CGG GTT GCC GCT GAT CAT GGC GGG TCC-3′) into the unique HindIII site of pCS1302, a derivative of pHM14 (27) as a template and RccycAmat/NdeI-Fwd (5′-GGA TCC TAT TTC ACG ACC GAG GCC AG-3′) and RccycAmat/Ndel-Fwd (5′-GGA TCC TAT TTC ACG ACC GAG GCC AG-3′) as primers. The generated 0.37-kb fragment was phosphorylated and cloned into EcoRV-restricted pBSK. Plasmid pCS1718 was then digested with Ndel and BamHI sites, which were introduced during the PCR amplification from pHM14, and the DNA fragment corresponding to *cycA* mat cloned into the same sites of pCS1302, a derivative of pCS905 (28), to yield pCS1726. This plasmid contains an in-frame Strep-tag sequence fused at the 5′-end of *cycA* mat expressed from a Ptac-lac promoter-operator system in *E. coli*.

**Detergent-solubilized Membrane Protein Preparation**—*R. capsulatus* cells grown by respiration were resuspended in TNE1 buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 25 mM EDTA, 0.1 mM ε-amino-caproic acid, and 0.1 mM Pefabloc SC) at a ratio of 5 ml per g cell wet weight, and intracytoplasmic membrane vesicles (chromatophores) prepared using a French pressure cell as described in Ref. 21. Chromatophores were homogenized in TNE1 buffer at a protein concentration of 8 mg/ml, solubilized by addition of n-dodecyl β-maltoside (DDM; Sigma-Aldrich) at a protein:detergent ratio of 1:1 (0.8% w/v DDM) from a 20% (w/v) stock solution under continuous stirring for 1 h at 4°C, and then centrifuged for 2 h at 4°C and 120,000 × g to collect solubilized membrane proteins in the supernatants for further use.

**Various Protein Chromatography**—For size exclusion chromatography, solubilized membrane proteins were loaded onto a Sephacryl S-400 HR column (GE Healthcare Biosciences, Piscataway, NJ) pre-equilibrated with five column volumes of TNE1 buffer (TNE1 plus 0.01% (w/v) DDM), which was also used as elution buffer. The flow rate was adjusted to 0.8 ml/min, the absorption of the eluates monitored at 280 nm, and 2.4 ml per fraction were collected. For each Ccm component monitored, proteins present in 400-μl aliquots of desired fractions were precipitated and subjected to SDS-PAGE and immunodetection. The size exclusion column was calibrated using blue dextran (2000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa) to estimate the molecular mass ranges across the fractions collected. As needed, appropriate high molecular mass fractions were pooled for further analyses.

For tag-affinity chromatography, the TNE1 buffer of the solubilized membrane proteins was exchanged with TNE2 buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.05% (w/v) DDM), to not damage the tag-affinity matrices with high EDTA amounts, using a PD-10 column (GE Healthcare Biosciences, Piscataway, NJ). Protein concentrations were adjusted with the same buffer to a final concentration of 2 mg/ml, and loaded by three successive passages to appropriate affinity columns (StrepTactin®-Sepharose from IBA or ANTI-FLAG®-agarose from Sigma-Aldrich).

For tandem size exclusion and affinity chromatography, high molecular weight fractions separated by the size exclusion column were pooled, the TNE1 buffer was exchanged with TNE2, and proteins were concentrated to 1 mg/ml protein using 10-kDa cut-off Centriplus YM-10 centrifugal filter units (Millipore, Billerica, MA). Affinity columns contained 1-ml matrix volume and were pre-equilibrated with 20 ml of TNE2 buffer. Following sample loading, the respective columns were
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TABLE 1
Bacterial strains and plasmids used in this study

| Strain or plasmid | Description | Relevant phenotype | Source or Ref. |
|-------------------|-------------|--------------------|---------------|
| **Strains**        |             |                    |               |
| E. coli           |             |                    |               |
| HB101             | F: Δ(gpt-proA)Δ62 araC14 leuB6(Am) glnV44(AS) galK2(Oc) lacY1 Δ(mcr-c-mrr) rpsL20(Strep') xylA5 mtl-1 thi-1 | Str<sup>*</sup> | (25) |
| **R. capsulatus** |             |                    |               |
| MT1131<sup>+</sup> |             | Wild type Res<sup>+</sup>/Nadi<sup>+</sup>, Ps<sup>+</sup>, cyts c<sup>+</sup> | (35) |
| MT1131<sup>+</sup> |             | Res<sup>+</sup>/Nadi<sup>+</sup>, Ps<sup>+</sup>, cyts c<sup>+</sup> | (23) |
| MT1131<sup>+</sup> |             | Res<sup>+</sup>/Nadi<sup>+</sup>, Ps<sup>-</sup>, cyts c<sup>-</sup> | (17) |
| MT1131<sup>+</sup> |             | Res<sup>+</sup>/Nadi<sup>+</sup>, Ps<sup>-</sup>, cyts c<sup>-</sup> | (23) |
| MD12              |             | Res<sup>+</sup>/Nadi<sup>+</sup>, Ps<sup>-</sup>, cyts c<sup>-</sup> | This work |
| MD14              |             | Res<sup>+</sup>/Nadi<sup>+</sup>, Ps<sup>-</sup>, cyts c<sup>-</sup> | This work |
| MT-SRP1           |             | Res<sup>+</sup>/Nadi<sup>+</sup>, Ps<sup>-</sup>, cyts c<sup>-</sup> | This work |
| MT-SRP1<sub>r1</sub> | Δ(ccm(kan)) G48A in promoter of ccmFH | Res<sup>+</sup>/Nadi<sup>+</sup>, Ps<sup>-</sup>, cyts c<sup>-</sup> | This work |
| MD13              | Δ(ccm(kan)) cmc: spe (ccmH<sup>+</sup>) | Res<sup>+</sup>/Nadi<sup>+</sup>, Ps<sup>-</sup>, cyts c<sup>-</sup> | This work |
| MD15              | Δ(ccm(kan)) cmc: spe (ccmF<sup>+</sup>) | Res<sup>+</sup>/Nadi<sup>+</sup>, Ps<sup>-</sup>, cyts c<sup>-</sup> | This work |
| **Plasmids**      |             |                    |               |
| plbuescriptIISK+ + |             |                    |               |
| prK2013           | Broad host-range vector, gene expression supported by E. coli lacZ promoter | Amp<sup>+</sup>, Kan<sup>+</sup>, helper | Stratagene (36) |
| prK415            | Broad host-range expression vector with R. capsulatus cycA (cyt c<sup>-</sup>) promoter | Tet<sup>+</sup> | (37) |
| pCH500            | 2.82 kb fragment carrying ccmFH overexpressed via a promoter-up mutation in pBSK | Amp<sup>+</sup> | (23) |
| pYZ1              | pYZ10 derivative with a non-polar spe cassette in ccmF (ccmF: spe cmc: spe cmcH<sup>+</sup>) in pRK415 | Amp<sup>+</sup>, Spe<sup>+</sup>, CcmH<sup>+</sup> | (23) |
| pYZ12             | pYZ10 derivative, but with a polar spe cassette in ccmH | Tet<sup>+</sup>, Spe<sup>+</sup>, CcmH<sup>+</sup> | (23) |
| pN2               | ccmF:FLAG expressed from its own promoter in pRK415 | Tet<sup>+</sup>, CcmF:FLAG<sup>+</sup> | (19) |
| pCS1564           | ccmF:FLAG including its own promoter sequence (1.69 kb fragment) in pBSK | Amp<sup>+</sup> | (19) |
| pHM14             | 1.25 kb BamHI−HindIII fragment containing R. capsulatus cycA in pRK415 | Tet<sup>+</sup>, Cyt c<sup>-</sup> | (27) |
| pCS905            | pET-3a derivative (Novagen, Madison, WI) with T7 promoter region replaced by a DNA fragment encoding lacI and the tac promoter region | Amp<sup>+</sup>, P<sub>lac</sub> LacI<sup>+</sup> | (28) |
| pST6              | ccmH:Strep from pST8 cloned into the XbaI-KpnI sites of pCH500 | Tet<sup>+</sup>, CcmH-Str<sup>+</sup> | (39) |
| pST7              | 0.5 kb fragment of pYZ1 carrying ccmH cloned into the XbaI-KpnI sites of pBSK | Amp<sup>+</sup> | (39) |
| pST8              | 0.5 kb fragment carrying a Streptag sequence added at the 3′-end of ccmH from pST7, cloned into the XbaI and KpnI sites of pBSK | Amp<sup>+</sup> | (39) |
| pYZ5              | Streptag sequence added by site directed mutagenesis to the 5′-end of ccmF in pYZ1 | Amp<sup>+</sup>, Strept-CcmF<sup>+</sup> | This work |
| pYZ6              | XbaI-KpnI fragment of pYZ5 carrying Strep: ccmF: ccmH (expressed via a promoter-up mutation) in pRK415 | Tet<sup>+</sup>, Strep-CcmF<sup>+</sup>, CcmH<sup>+</sup> | This work |
| pCS1581           | ccmF:FLAG including its own promoter sequence from pCS1564 | Tet<sup>+</sup>, CcmH-Str<sup>+</sup>, CcmF:FLAG<sup>+</sup> | This work |
| pCS1582           | ccmF:FLAG including its own promoter sequence from pCS1564 | Tet<sup>+</sup>, Strep-CcmF<sup>+</sup>, CcmH<sup>+</sup>, CcmF:FLAG<sup>+</sup> | This work |
| pCS1302           | pCS905 derivative, Strep-tag II sequence (IBA, St. Louis, MO) fused GFP, GFP replaceable by cloning any gene of interest in frame into Ndel and BamHI sites | Amp<sup>+</sup>, P<sub>lac</sub> LacI<sup>+</sup>, Strep-GFP<sup>+</sup> | This work |
| pCS1718           | PCR amplification of a cyaA fragment from pHM14 (encoding the matured form of cyt c<sub>550</sub> introducing Ndel and BamHI sites), phosphorylation and cloning into EcoRI-restricted pBSK | Amp<sup>+</sup> | This work |
| pCS1726           | Amp<sup>+</sup>, P<sub>lac</sub> LacI<sup>+</sup>, Strep-cyt c<sub>550</sub> | This work |

*All R. capsulatus mutant strains listed are derivatives of strain MT1131, which is referred to as a wild-type strain with respect to its cyt c profile and growth properties.

*All plasmids listed contain genes or gene derivatives originated from R. capsulatus, unless indicated otherwise.

washed twice with 5 ml of TNED2 buffer, and Strept-tagged (i.e. CcmF and CcmH) or FLAG-tagged (i.e. CcmI) proteins were eluted using 5× matrix volumes (E1-E5 fractions, 1 ml each) with TNED2 buffer containing 5 mM desthiobiotin (DTB) or 0.1 mg/ml FLAG peptide, respectively. For each Ccm component, samples corresponding to 100 μg of total proteins of non-solubilized or DDM dispersed chromatophore membranes, 25 μg of flow-through or column wash solutions, and 5 μg of elution fractions (E2-E5) were precipitated, and analyzed via SDS-PAGE and immunoblot analyses were performed as described below.

Production of CcmF Antisera—Antisera generated toward predicted small (~10 amino acids) soluble antigenic CcmF peptides did not detect CcmF in crude extracts by immunoblotting, even when CcmF was overproduced. Therefore, longer polypeptides (~several tens of amino acids) from soluble domains were generated and purified for antisera production. The following was determined to give the best results in immunoblots and was used in these studies. The R. capsulatus ccmF gene was used as template for synthesis of a PCR product that encoded the sixth periplasmic domain of R. capsulatus CcmF (P6, as designated in Ref. 16) was amplified. The forward primer sequence was 5′-GGG CCC ATG GAG GAT ATC CGC GTG CGC AAC, beginning at the amino acid residue 512 of CcmF with the sequence EDIRV (the ATG in the primer encodes the initiating methionine). The reverse primer sequence was 5′-ATC CCA AAG CTT GTT CGC GAA AGG CTT GAC, whereby the amino acid sequence KPNF represents the final residues within the CcmF fragment (ending at residue 609). Ncol and HindIII sites (underlined) were engineered into the
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FIGURE 1. Copurification of CcmH with Strep-CcmF and copurification of CcmF with CcmH-Strep. Solubilized chromatophore membranes from R. capsulatus strains pYZ6 (Strep-CcmF) × MD12 (CcmF-null) (A) or PST6 (CcmH-Strep) × MD14 (CcmH-null) (B) were loaded onto a StrepTactin®-Sepharose affinity chromatography column, washed, and eluted with 5 mM DTB as described under “Experimental Procedures.” Aliquots from different steps of purification (lanes 2–5), column wash, flow-through (lanes 6–9), DTB elution fractions 2–5) were analyzed by SDS-PAGE and immunoblots (100 μg of protein in lanes 2–4. 25 μg of protein in lane 5, and 5 μg of protein in lanes 6–9). Polyclonal antibodies against CcmF, CcmH, and CcmG are as indicated on the right, and molecular mass markers (in kDa) are as shown on the left of each panel. In each case, lane 1 contained 100 μg of protein of CM from appropriate mutant strains (Table 1) as negative controls for the respective immunoblots, as depicted on the top left of each panel.

forward and revers primers for cloning purposes, respectively. This NcoI and HindIII restricted ccmF fragment cloned into the cytoplasmic expression vector pRSETB (Invitrogen, Carlsbad, CA) produced high yields of an N-terminally hexahistidine tagged polypeptide of about 20 kDa. Rabbits were immunized either directly with via nickel affinity chromatography purified CcmF-P6 fragment, or after its separation via SDS-polyacrylamide gel and subsequent electro-elution. The antisera toward the polypeptide (called CcmFP6D) obtained from the latter procedure yielded the best results, and were used in Ref. 23, and in this study.

SDS-PAGE and Immunoblot Analyses—Proteins were precipitated at −20 °C with 90% (v/v) ice-cold acetone overnight and centrifuged for 30 min at 20,000 × g and 4 °C. The obtained pellets were air-dried and then re-solubilized in SDS loading buffer (62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 0.1 M dithiothreitol, 25% (v/v) glycerol, and 0.01% (w/v) bromphenol blue) by incubation at 42 °C for 45 min prior to loading. SDS-PAGE was performed according to Ref. 29 using 15% (T) polyacrylamide gels. Gel-separated proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA) at 1 mA/cm² for 2 h using a Trans-Blot SD semi-dry transfer cell (Bio-Rad). Membranes were then washed twice for 5 min at room temperature with TBS buffer (25 mM Tris/HCl, pH 7.5, and 150 mM NaCl), saturated with TTBS buffer (TBS + 0.05% (v/v) Triton X-100 and 0.05% (v/v) Tween 20) containing 5% (w/v) nonfat dry milk for 1 h at room temperature, washed twice for 5 min at room temperature with TTBS buffer, and subsequently probed with rabbit antisera against CcmG (1:5000), CcmH (1:5000), and CcmF (1:1000), or rabbit ANTI-FLAG® polyclonal antibodies (Sigma) (1:2000) for 16 h at 4 °C. Thereafter, membranes were washed three times for 5 min with TTBS buffer and reprobed with either monoclonal anti-rabbit (immunoglobulins clone RG-16) alkaline phosphatase conjugate (Sigma-Al drich) (1:2000) or stabilized goat anti-rabbit IgG horseradish peroxidase conjugates (GE Healthcare Bio-Sciences, Piscataway, NJ) (each 1:10,000) for 1 h at room temperature. Antibody-antigen complexes were visualized by chromogenic detection using the BCIP/NBT liquid substrate (Sigma-Aldrich) or by enhanced chemiluminescence (ECL) detection using the SuperSignal West Pico chemiluminescent substrate (Pierce).

Chemicals—All chemicals were of reagent grade and obtained from commercial sources.

RESULTS

Functional Epitope-tagged Derivatives of CcmF, CcmH, and CcmI—For protein-protein interaction studies between CcmF, CcmH, and CcmI, plasmids containing epitope sequence-tagged gene derivatives (Strep::ccmF, CcmH::Strep, and ccmI::FLAG) and appropriate mutant strains were generated as described under “Experimental Procedures” and Table 1. Production of an N-terminally Strep-tagged derivative of CcmF(Strep-CcmF, expressed together with CcmH via a promoter-up mutation variant of the ccmFH cluster) complemented both CcmF-null (MD12) and CcmH-null (MD14) mutants for Ccm, and hence Ps, proficiency. Similarly, a C-terminally Strep-tagged CcmH (CcmH-Strep) and a FLAG-tagged CcmI (CcmI-Flag) derivatives also complemented the CcmH-null (MD14) and CcmI-null (MT-SRP1) mutants for Ps growth, respectively (Table 1) (19, 23). Using TMBZ–SDS/PAGE, examination of chromatophore membranes and soluble fractions confirmed that all complemented strains exhibited wild-type cyt c profiles, producing the membrane-bound cyts cpr, c1, c2, and c0 (30), and the soluble cyt c2 (21) (data not shown).

Epitope-tagged Ccm components were probed for protein-protein interactions by affinity chromatography using chromatophore membranes prepared from semi-aerobically grown cells and subsequently solubilized with DDM. Ccm components thus purified or copurified were monitored in flow-through, wash, and ligand-eluted fractions by immunodetection using specific antibodies against R. capsulatus CcmH (11), CcmF (“Experimental Procedures”) and CcmG (11) or the FLAG epitope fused to CcmI. Additional coimmunoprecipitation and cross-linking experiments were also conducted to verify the affinity purification results (data not shown).

CcmF and CcmH Interact with Each Other, but Not with CcmG—In E. coli, CcmF and CcmH are known to coimmunoprecipitate (13). To test whether similar interactions also occur in R. capsulatus, affinity purification of Strep-CcmF using StrepTactin®-Sepharose was carried out with an appropriately complemented CcmF-null mutant (MD12 × pYZ6, Table 1).
DTB-eluted fractions contained Strep-CcmF and CcmH, but not CcmG (Fig. 1A), suggesting that at least a fraction of CcmH copurified with Strep-CcmF, and hence associated with each other. Reciprocal affinity purification was next performed to confirm this finding. Membranes from a CcmH-null mutant complemented with a CcmH-Strep derivative (MD14 × pST6) were used, and the data showed that at least a fraction of CcmF, but again not CcmG, copurified with CcmH-Strep (~3 kDa larger than native CcmH) under the same experimental conditions (Fig. 1B). These physical interactions therefore indicated that CcmF and CcmH form a stable complex, devoid of CcmG.

CcmI Also Associates with CcmH, but Not with CcmG—Our earlier genetic studies indicated that CcmF and CcmH also interacted with CcmI (14). Thus, we purified CcmI-FLAG using ANTI-FLAG®-Agarose from DDM-solubilized membranes of a complemented CcmI-null mutant (MT-SRP1.r1 × pNJ2, Table 1). In fractions eluted with FLAG peptide, we detected CcmI-FLAG (M, of ~55,000) (19) and CcmH (M, of ~17,000), but neither CcmF (M, of ~58,000) nor CcmG (M, of ~18,000) (Fig. 2A), suggesting that CcmI-FLAG and CcmH also associated with each other.

Similar to CcmF and CcmH, additional reciprocal copurification experiments were needed to further support the interactions between CcmI and CcmH. Because no CcmI-specific antibodies were available, constructs coexpressing CcmI-FLAG with CcmH-Strep (pCS1581) or with Strep-CcmF (pCS1582) were generated (Fig. 2B) (“Experimental Procedures”). As expected, these plasmids complemented for Ps growth the single CcmI-null (MD12), CcmH-null (MD14), and CcmI-null (MT-SRP1) as well as the CcmF-null CcmI-null (MD13) and CcmH-null CcmI-null (MD15) double mutants of \( R. \) capsulatus (Table 1, and data not shown).

Interactions between CcmI and CcmH Appears to Be Stronger Than Those between CcmI and CcmF—Affinity purification of the CcmH-Strep derivative from membranes of a CcmH-null CcmI-null double mutant, complemented with coexpressed CcmI-FLAG and CcmH-Strep (MD15 × pCS1581, Table 1), indicated that the DTB-eluted fractions contained, in addition to CcmI-FLAG and CcmF but not CcmG (Fig. 3A). This result confirmed the close interactions of CcmI-FLAG and CcmF with CcmH-Strep, as seen earlier (Figs. 1 and 2A). Moreover, it also suggested that CcmF, CcmH, and CcmI are part of a stable multisubunit membrane-integral complex.

Finally, to further probe whether CcmF and CcmI interacted directly, affinity purification of the Strep-CcmF derivative was repeated using membranes from the CcmF-null/CcmI-null double mutant complemented with coexpressed Strep-CcmF and CcmI-FLAG derivatives (MD13 × pCS1582, Table 1). DTB-eluted fractions containing Strep-CcmF (M, of ~58,000) had copious amounts of CcmH as shown above (Fig. 1), but included only trace amounts of CcmI-FLAG, and no CcmG (Fig. 3B). This observation suggested that, under the conditions used, the Strep-CcmF and CcmI-FLAG derivatives interacted either weakly with each other, or indirectly via CcmH, which was further assessed as described below.

CcmF, CcmH, and CcmI Form a Multisubunit Membrane-Integral Complex—A different purification approach, which consisted of tandem size exclusion and affinity chromatography, was next used to establish that CcmF, CcmH, and CcmI are part of a multisubunit membrane-integral protein complex. DDM-dispersed proteins from a strain overproducing CcmF, CcmH, and CcmI were fractionated by FPLC within 8000–20 kDa ranges using a Sephacryl S-400 HR column (180 ml). The CcmF, CcmH, and CcmI-FLAG contents of the elution fractions were determined using SDS-PAGE and immunoblot analyses. Large amounts of
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CcmF, CcmH, and CcmI-FLAG were detected in the fractions 36–48 and 66–78, 33–51 and 54–78, and 36–45 and 54–63, respectively (Fig. 4A). Additional protein bands of unknown identities with molecular weights larger than those of CcmH and CcmI-FLAG but still reacting with CcmH- and CcmI-FLAG-specific antibodies were also observed in fractions 51–60. Some of these bands might reflect the different thio-redox states of CcmH. Calibration of the size exclusion chromatography column with proteins of known molecular masses (“Experimental Procedures”) indicated that CcmI-FLAG colocalized mainly in the elution fractions 30–50, corresponding to molecular masses of protein complexes larger than 400 kDa with detection peaks for all three components around ~800 kDa (Fig. 4A). These fractions were pooled after eight independent FPLC runs (~40 mg total proteins), concentrated, and subjected to ANTI-FLAG®-agarose affinity chromatography to purify CcmI-FLAG. Fractions eluted with the FLAG peptide contained CcmI-FLAG, CcmH, and CcmF, but not CcmG (Fig. 4B). Thus, CcmH and CcmF copurified readily with CcmI-FLAG from the high molecular weight fractions pool, demonstrating that they formed a multisubunit membrane protein complex, which was devoid of CcmG under the conditions used here.

DISCUSSION

Based on our earlier studies using the suppressors of CcmI-null mutants, we proposed that CcmF, CcmH, and CcmI form a multisubunit membrane-integral complex in R. capsulatus (14, 19). Here, we sought direct biochemical evidence to substantiate this hypothesis. At the onset, this task appeared daunting as most of the Ccm components are poorly characterized membrane proteins (2, 3). Purification of membrane-integral protein complexes, especially those lacking optically detectable cofactors, is challenging because it requires empirical definition of adequate conditions for membrane lipid dispersion while keeping the subunit interactions intact. In our case, reliable detection means for individual Ccm components were also restricted (2). First we generated functional epitope-tagged derivatives of CcmF, CcmH, and CcmI, and expressed them individually or in pairs in appropriate mutants to supplement available Ccm antibodies. After establishing that the tagged Ccm derivatives were functional, we initiated purification of various Ccm components. Lack of CcmI-specific antibodies initially restrained our ability to monitor it in some instances, but this difficulty was surmounted upon coproduction of a CcmI-FLAG derivative with Strep-Cm and CcmH-Strep...
Protein-Protein Interactions between CcmF, CcmH, and CcmI

FIGURE 4. Size exclusion chromatography of solubilized membranes and copurification of CcmF and CcmH with CcmI-FLAG by affinity chromatography. A, DDM-solubilized membrane proteins from R. capsulatus strain pNU2 (CcmI-FLAG) × MT-SRP1.r1 (CcmI-null overproducing CcmH and CcmF) were fractionated via FPLC on a precalibrated Sephacryl S-400 HR column (“Experimental Procedures”), as indicated on top of A. The size exclusion column was eluted with TNEDE2 buffer at a flow rate of 0.8 ml/min, and the absorption values of the elution fractions (2.4 ml per fraction) were recorded at 280 nm (A450 nm, in milliabsorbance units or mAU) (A, upper section). Proteins from every third elution fraction (from 30 to 78) were precipitated, subjected to SDS-PAGE, and stained with Coomassie (A, middle section). Immunoblot analyses were carried out using polyclonal antibodies against CcmF, CcmH, and the FLAG epitope (fused to CcmI) (A, lower section). In each case, the molecular mass markers (in kDa) are indicated on the left and the specific antibodies as probes are shown on the right. B, elution fractions 30–49 containing high molecular weight materials were pooled, concentrated, and utilized for the purification of FLAG-tagged CcmI as described under “Experimental Procedures.” Samples from different purification steps (P, concentrated fraction pool 30–49; FT, flow-through after the ANTI-FLAG® Agarose column; W, column wash; E2–E5, FLAG peptide eluted fractions 2–5) were analyzed by SDS-PAGE (100 μg of protein in lanes 3 and 4, 25 μg of protein in lane 5, and 5 μg of protein in lanes 6–9) and immunoblots using polyclonal antibodies as indicated on the right. Lanes 1 and 2 correspond to 100 μg of protein of CM from a mutant strain used as a negative control for the respective immunoblot analysis (lane 1) or from R. capsulatus strain pNU2 (pcmI/I-FLAG) × MT-SRP1.r1 (ΔccmI/I ccmFFHI) (lane 2), as depicted on the top, left side. Molecular mass markers (in kDa) are shown on the left.

FIGURE 5. A minimalistic heme-apocyt c ligation process. The known ten (CcmABCDDEFHI and CcdA) components required for the Ccm-system I could be reduced to a set of five components as a minimalistic heme-apocyt c ligation apparatus per se, because in the absence of a thio-redox pathway (i.e. DsbA, CcdA) cyt c production still occurs, and CcmABCD is involved in loading heme to the holoccmE. Thus, the apocyt c thioreductase/holase CcmE, the heme chaperone CcmE and the heme ligation complex involving CcmFHI are the three major partners of this membrane confined process that is essential for cellular energy production.

(“Experimental Procedures” and Table 1) to detergent-dispersed membranes leads to its colocalization with CcmG in a subcomplex of ~100 kDa and also with the ~800 kDa complex including CcmFHI, in agreement with our current thoughts about how the apocyt c might interact with the Ccm components in R. capsulatus (14, 19, 31). In any event, establishing that the CcmFHI components are parts of a multisubunit complex supports the idea that cyt c maturation process is carried out by a well-defined membrane-integral apparatus (Fig. 5).

Furthermore, using R. capsulatus as a model for Ccm-system I, we report here the first biochemical evidence for physical association of CcmH and CcmI. We found that CcmF and CcmI interact weakly with each other, or possibly indirectly via CcmH, to form a complex, which does not contain CcmG. Previously available communoprecipitation data with E. coli indicated that only CcmF and CcmH (13, 32) (as well as Arabidopsis thaliana CcmH (32)) interact with each other. Remarkably, colocalization of CcmH, CcmF, and CcmI-FLAG peaking in high molecular mass fractions of ~800 kDa during size exclusion chromatography is intriguing. This observation suggested that the Ccm components either form large aggregates escaping solubilization or are inherent parts of a large multisubunit complex with additional proteins. In mitochondria from T. aestivum (33) and A. thaliana (32), high molecular mass complexes (~700 and ~500 kDa, respectively) containing the bacterial CcmF or CcmH homologs have also been detected. Using yeast two-hybrid assays, the A. thaliana CcmH homolog was shown to interact with apocyt c (32). In bacteria, communoprecipitation data indicated that E. coli CcmF interacts with CcmE, but not with apocyt c (13). Similarly, the C-terminal helix and its adjacent loop of the pentaheme cyt c NrfA was found to interact with the CcmI ortholog NrfG via a TPR domain (34). Currently, to what extent or under which conditions, the CcmFHI-containing complex also associates with other Ccm components (such as CcmG and CcmE) or with apocyt c is unknown, precluding the conclusion that it is composed of solely three subunits (Fig. 5). Indeed, future identification of the high

C. Sanders and F. Daldal, unpublished data.
molecular weight derivatives of CcmH and CcmI seen among the size exclusion chromatography fractions 51–60 will be informative. In summary, establishment of a Ccm-FHI-containing complex will hopefully enable us to further define the molecular events occurring during heme-apocytc ligation in organisms using Ccm-system I for cyt c production.

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