The Cytochrome c Maturation Components CcmF, CcmH and CcmI Form a Membrane-integral Multisubunit Heme Ligation Complex

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Running title: Protein-protein interactions between CcmF, CcmH and CcmI

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Summary

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. Absence of any of these components abolishes the ability of cells to form cytochromes c, leading in the case of Rhodobacter capsulatus to the loss of photosynthetic proficiency and respiratory cytochrome oxidase activity. Based on earlier molecular genetic studies, we inferred that R. capsulatus CcmF, CcmH and CcmI interact with each other to perform heme-apocytochrome c ligation. Here, using functional epitope-tagged derivatives of these components coproduced in appropriate mutant strains, we determined protein-protein interactions between them in detergent-dispersed membranes. Reciprocal affinity purification as well as tandem size exclusion and affinity chromatography analyses provided the first biochemical evidence that CcmF, CcmH and CcmI associate stably 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.

Introduction

Cytochromes (cyts) c 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-Xxx-Yyy-Cys2-His (C1XXC2H) motifs within the apocyts (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 apocyts 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 (Turkarslan et al., in
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 apocyts c thio-reduction process (Turkarslan et al, in press).

CcmH is comprised of a N-terminal signal peptide, an extracytoplasmic loop with a redox-reactive CXXC motif and a C-terminal transmembrane domain (10,11). It has an unusual thioredoxin-like structure (12), and interacts physically with CcmF in E. coli (13). Based on genetic studies using R. 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 apocyts c (13,16). However, direct association of CcmF with apocyts c has not yet been shown experimentally in bacteria (13). CcmI is thought to chaperone the apocyts 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 repeats (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 chromatographies, 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 CcmFHI-containing heme ligation complex lacking CcmG for the heme-apocyts 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) (pYZ4) and ccmH::spe (pYZ12) alleles (23) were introduced by interposon mutagenesis (21) into the CcmH-null mutant MT-SRP1 Δ(ccmF::kan) (17) using the gene transfer agent (GTA) (24) to yield the R. capsulatus CcmH-null CcmI-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 “QuickChange™ 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 TGG GAG CAC CCG CAG TTC GAA AAA GGC GTC GAG ACC GCC CAT-3’) and CcmF-StrepN-Rev (5’-GAA ATC GCC GTT CTC GAC GCC TTT TTT GAA CTG CGG GTG GCT CCA GCT GAT CAT GCC GGG TTC-3’) to create pYZ5. The 2.95 kb XbaI and KpnI restricted fragment of pYZ5 carrying Strep::ccmF, ccmH and the

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G488A promoter-up mutation overexpressing the ccmF-ccmH gene cluster (23) was then cloned into the respective sites of pRK415 to yield pYZ6. Plasmids pST6, pST7 and pST8 were constructed as described in Table 1 (Turkarslan et al., in press). The plasmids pCS1581 (ccmH::Strep ccmH::FLAG) and pCS1582 (Strep::ccmF-ccmH ccmI::FLAG) were constructed by cloning the 1.7 kb KpnI fragment of pCS1564 (19) containing ccmI::FLAG expressed via its own promoter into the unique KpnI site of pST6 and pYZ6, respectively. To construct pCS1718, the R. capsulatus cycAmat, corresponding to the matured form of cyt c2 protein encoded by cycA, was PCR-amplified using pHM14 (27) as a template and RecycAmat/Ndel-Fwd (5' - CAT ATG GGC GAC GCC GCG AAG GGC GA-3') and RecycAmat/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 thePCR-amplification from pHM14, and the DNA fragment corresponding to cycAmat 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 cycAmat 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 e-amino-capric 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 (21). Chromatophores were homogenized in TNE1 buffer at a protein concentration of 8 mg/ml, solubilized by addition of n-Dodecyl β-D-maltoside (DDM; Sigma-Aldrich, St. Louis, MO) 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 40 000 x g to collect solubilized membrane proteins in the supernatants for further use.

Various protein chromatographies- 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 TNED1 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 eluats 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 weight ranges across the fractions collected. As needed, appropriate high molecular weight fractions were pooled for further analyses.

For tag-affinity chromatographies, the TNED1 buffer of the solubilized membrane proteins was exchanged with TNED2 (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA and 0.05% (w/v) DDM), not to 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, both St. Louis, MO).

For tandem size exclusion and affinity chromatographies, high molecular weight fractions separated by the size exclusion column were pooled, the TNED1 buffer was exchanged with TNED2, 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 TNED2 buffer. Following sample loading, the respective columns were washed twice with 5 ml of TNED2 buffer, and Strep-tagged (i.e.,
CcmF and CcmH) or FLAG-tagged (i. e., CcmI) proteins were eluted using 5 × matrix volumes (E1 to E5 fractions, 1 ml each) with TNED2 buffer containing 5 mM DTB or 0.1 mg/ml FLAG peptide, respectively. For each Ccm component, samples corresponding to 100 µg 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 to E5) were precipitated, and analyzed via SDS-PAGE and immunodetection. Protein concentrations were determined using the Bicinchoninic Acid kit from Sigma-Aldrich (St. Louis, MO), and SDS-PAGE and immunoblot analyses were performed as described below.

Production of CcmF antisera- Antisera generated towards 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 (16)) was amplified. The forward primer sequence was 5’-GGG CCC ATG GAG GAT ATC CGC GTG GCG AAG, 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 KPFN represents the final residues within the CcmF fragment (ending at residue 609). NcoI and HindIII sites (underlined) were engineered into the forward and reverse 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 a N-terminally hexahistidine-tagged polypeptide of about 20 kDa. Rabbits were immunized either directly with nickel affinity chromatography purified CcmF-P6 fragment, or after its separation via SDS-polyacrylamide gel and subsequent electrophoresis. The antisera towards the polypeptide (called CcmFP6D) obtained from the latter procedure yielded the best results, and were used in (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 20000 × 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% [v/v] bromophenol blue) by incubation at 42 °C for 45 min prior to loading. SDS-PAGE was performed according to (29) using 15% (T) polyacrylamide gels. Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA) at 1 mA/cm² for 2 h using a Trans-Blot SD semi-dry transfer cell (BioRad, Hercules, CA, USA). Membranes were then washed twice for 5 min at RT 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 RT, washed twice for 5 min at RT 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, St. Louis, MO) (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-Aldrich, St. Louis, MO) (1:2000) or stabilized goat anti-rabbit IgG horse raddish peroxidase conjugates (GE Healthcare Bio-Sciences, Piscataway, NJ) (each 1:10000) for 1h at room temperature. Antibody-antigen complexes were visualized by chromogenic detection using the BCIP/NBT liquid substrate (Sigma-Aldrich, St. Louis, MO) or by enhanced chemiluminescence (ECL) detection using the SuperSignal west pico chemiluminescent substrate (Pierce, Rockford, IL).
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 in 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 cys c profiles, producing the membrane-bound cys c$_p$, c$_1$, c$_y$ and c$_o$ profiles, and the soluble cyt c$_2$ (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 x pYZ6, Table 1). Desthiobiotin (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 x 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 x pNJ2, Table 1). In fractions eluted with FLAG peptide, we detected CcmI-FLAG (M$_r$ of ~55 kDa) (19) and CcmH (M$_r$ of ~17 kDa), but neither CcmF (M$_r$ of ~58 kDa) nor CcmG (M$_r$ of ~18 kDa) (Fig. 2A), suggesting that CcmI-FLAG and CcmH also associated with each other.

Just like for CcmF and CcmH, additional reciprocal copurification experiments were needed to further support the interactions between CcmI and CcmH. Since no CcmI-specific antibodies were available, constructs expressing 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 CcmF-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 CcmI-null
CcmH-null double mutant, complemented with coexpressed CcmI-FLAG and CcmH-Strep (MD15 x pCS1581, Table 1), indicated that the DTB eluted fractions contained, in addition to CcmH-Strep (Mₘ of ~20 kDa), both 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.

Lastly, to further probe whether CcmF and CcmI interacted directly, affinity purification of the Strept-CcmF derivative was repeated using membranes from the CcmF-null CcmI-null double mutant complemented with coexpressed Strept-CcmF and CcmI-FLAG derivatives (MD13 x pCS1582, Table 1). DTB eluted fractions containing Strept-CcmF (Mₘ of ~58 kDa) 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 Strept-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 chromatographies, 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-FLAG (MT-SRP1.r1 x pNJ2, Table 1) 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 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 thioredox states of CcmH. Calibration of the size exclusion chromatography column with proteins of known molecular weights (Experimental Procedures) indicated that CcmF, CcmH and 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-CcmF and CcmH-Strep components. Reciprocal affinity purification as well as size exclusion fractionation followed by affinity chromatography yielded for the first time strong supporting evidence that CcmF, CcmH and CcmI associate with each other to form a multisubunit membrane protein complex. Pairwise copurification results suggested that CcmF and CcmI interact poorly or indirectly with each other despite their strong and presumably direct association with CcmH (Fig. 5). In addition, in the early experiments we noted that the flow through fractions contained CcmH not associated with CcmF and vice versa, CcmF not associated with CcmH (Fig 1). These observed substoichiometries suggest that either free pools of these components are also present in membranes, or that the solubilization conditions used here are not optimal to fully preserve the intactness of the CcmFHI containing complex. Thus, the experiments reported here do not address the degree of purity or stoichiometry of the components.

Clearly, the Ccm complex identified here does not contain CcmG, but whether it includes CcmE or other components awaits the availability of specific antibodies. CcmG was present in readily detectable amounts in solubilized membranes of all utilized R. capsulatus strains, and its absence of interaction with CcmH was surprising because CcmG reduces oxidized CcmH in vitro (11). However, no mixed disulfides between CcmG and CcmH have so far been trapped in vivo. Furthermore, the compensatory thio-redox interactions between DsbA and CcdA or CcmG apparently do not include CcmH (Turkarslan et al., in press). It therefore seems that CcmG associates either too transiently or indirectly with CcmF, CcmH or CcmI under our experimental conditions. Interestingly, our preliminary data indicate that addition of E. coli-produced and purified apocytc2 (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 (Sanders et al., unpublished data), in agreement with our current thoughts about how the apocytc 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 coimmuno-precipitation 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 weight 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 weight complexes (~700 kDa 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 apocytc (32). In bacteria, coimmuno-precipitation data indicated that E. coli CcmF interacts with CcmE, but not with apocytc (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 apocytc is unknown, precluding the conclusion that it is composed of solely three subunits (Fig. 5). Indeed, future identification of the high molecular weight derivatives of CcmH and CcmI seen among the size exclusion chromatography fractions 51-60 will be informative.
In summary, establishment of a CcmFHI-containing complex will hopefully enable us to further define the molecular events occurring during heme-apocyt c ligation in organisms using Ccm-system I for cyt c production.
References


**Acknowledgments**

This work was supported by grants from DOE 91ER20052 and NIH GM38237 to F. D., and R. G. K is supported by NIH GM47909. The authors thank Barry Goldman for work on CcmF polypeptide production, and Meenal Deshmukh and Yan Zheng for constructing the *R. capsulatus* mutants MD13 and MD15 and plasmids pYZ5 and pYZ6, respectively.

**Figure Legends**

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) x MD12 (CcmF-null) (**panel A**) or pST6 (CcmH-Strep) x MD14 (CcmH-null) (**panel B**) were loaded onto a StrepTactin® Sepharose affinity chromatography column, washed, and eluted with 5 mM DTB as described in Materials and Methods. Aliquots from different steps of purification (CM: chromatophore membranes, SM: solubilized membranes, FT: flow-through after the third passage, W: column wash, and E2 to E5: DTB elution fractions 2-5) were analyzed by SDS-PAGE and immunoblots (100 µg proteins in lanes 2-4, 25 µg proteins in lane 5, and 5 µg proteins in lanes 6-9). Polyclonal antibodies against CcmF, CcmH and CcmG are as indicated on the right, and molecular weight markers (in kDa) are shown on the left of each panel. In each case, lane 1 contained 100 µg proteins of CM from appropriate mutant strains (*Table 1*) as negative controls for the respective immunoblots, as depicted on the top left of each panel.

Figure 2. Copurification of CcmH with CcmI-FLAG and coexpression of *ccmI:*FLAG with *ccmH::Strep or Strep::*ccmF. **Panel A**: Copurification experiment was conducted as in Fig. 1 except that chromatophore membranes from *R. capsulatus* strain pNJ2 (CcmI-FLAG) x MT-SRP1.r1 (CcmI-null overproducing CcmH and CcmF), ANTI-FLAG® Agarose column for affinity chromatography, and FLAG peptide for elution were used as described in Materials and Methods. Aliquots from different steps of purification were analyzed by SDS-PAGE and immunoblots. Polyclonal antibodies against CcmF, CcmH and CcmG are as indicated on the right, and molecular weight markers (in kDa) are shown on the left. In each case, lane 1 contained 100 µg proteins of chromatophore membranes (CM) from appropriate mutant strains (*Table 1*) used as negative controls for the respective immunoblots, as depicted on the top left. **Panel B** illustrates the plasmids pCS1581 coproducing CcmH-Strep and CcmI-FLAG, and pCS1582 coproducing Strep-CcmF, CcmH and CcmI-FLAG. The corresponding genes and their epitope sequence fusions are represented as rectangles, and the promoters (P) of *ccmI*, *cycA* and up-regulated *ccmFH* indicated with arrows. See the Materials and Methods and *Table 1* for the construction of these plasmids.

Figure 3. Copurification of CcmI-FLAG and CcmF with CcmH-Strep, and of CcmI-FLAG and CcmH with Strep-CcmF. Copurification experiments were conducted as in Fig. 1 except that
chromatophore membranes from <i>R. capsulatus</i> strains pCS1581 (CcmH-Step and CcmI-FLAG) x MD15 (CcmH-null CcmI-null double mutant) (panel A), and pCS1582 (Strep-CcmF, CcmH and CcmI-FLAG) x MD13 (CcmF-null CcmI-null double mutant) (panel B) were used. StrepTactin<sup>®</sup> Sepharose affinity chromatography was conducted, and aliquots from different steps of purification were analyzed by SDS-PAGE and immunoblots as in Fig. 1. Polyclonal antibodies against the FLAG epitope (fused to CcmI), CcmH, CcmF and CcmG are indicated on the right of each panel, and molecular weight markers (in kDa) are shown on the left. In each case, lane 1 contained 100 µg proteins of chromatophore membranes (CM) from an appropriate mutant strain (Table 1) as a negative control for the respective immunoblot analysis, as depicted on top left of each panel.

**Figure 4. Size exclusion chromatography of solubilized membranes and copurification of CcmF and CcmH with CcmI-FLAG by affinity chromatography.** Panel A: DDM-solubilized membrane proteins from <i>R. capsulatus</i> strain pNJ2 (CcmI-FLAG) x MT-SRP1.r1 (CcmI-null overproducing CcmH and CcmF) were fractionated via FPLC on a precalibrated Sephacryl S-400 HR column (Materials and Methods), as indicated on top of panel A. The size exclusion column was eluted with TNED2 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 (A<sub>280</sub> in milli absorbance units or mAU) (panel A, upper section). Proteins from every third elution fraction (from 30 to 78) were precipitated, subjected to SDS-PAGE, and stained with Commassie (panel A, middle section). Immunoblot analyses were carried out using polyclonal antibodies against CcmF, CcmH, and the FLAG epitope (fused to CcmI) (panel A, lower section). In each case, the molecular weight markers (in kDa) are indicated on the right and the specific antibodies as probes are shown on the right. Panel B: Elution fractions 30 to 49 containing high molecular weight materials were pooled, concentrated and utilized for the purification of FLAG-tagged CcmI as described in Materials and Methods. Samples from different purification steps (P: concentrated fraction pool 30-49, FT: flow-through after the ANTI-FLAG<sup>®</sup> Agarose column, W: column wash, E2-E5: FLAG peptide eluted fractions 2 to 5) were analyzed by SDS-PAGE (100 µg protein in lanes 3 and 4, 25 µg protein in lane 5 and 5 µg protein in lanes 6-9) and immunoblots using polyclonal antibodies as indicated on the right. Lanes 1 and 2 correspond to 100 µg protein of chromatophore membranes (CM) from a mutant strain used as a negative control for the respective immunoblot analysis (lane 1) or from <i>R. capsulatus</i> strain pNJ2 (pccmI::FLAG) x MT-SRP1.r1 (∆ccmI ccmFH<sup>Δ</sup>) (lane 2), as depicted on the top, left side. Molecular weight markers (in kDa) are shown on the left.

**Figure 5. A minimalistic heme-apocyt<sub>c</sub> ligation process**

The known ten (CcmABCDEFGHI and CcdA) components required for the Ccm-system I could be reduced to a set of five components as a minimalistic heme-apocyt<sub>c</sub> ligation apparatus per se, because in the absence of a thio-redox pathway (i. e., DsbA, CcdA) cyt<sub>c</sub> production still occurs and CcmABCD is involved in loading heme to the holoCcmE. Thus, the apocyt<sub>c</sub> thioreductase/holdase CcmG, 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.
Fig. 1. (Sanders et al.)
Fig. 2. (Sanders et al.)

A

$\Delta$ceml cemFH up x pceml::FLAG

<table>
<thead>
<tr>
<th>CM</th>
<th>$\Delta$ceml</th>
<th>cemFH up</th>
<th>CM</th>
<th>SM</th>
<th>FT</th>
<th>W</th>
<th>E2</th>
<th>E3</th>
<th>E4</th>
<th>E5</th>
</tr>
</thead>
</table>

- CemI-FLAG

22

17

6

A

$\Delta$ceml

CemH

60

42

CemF

22

17

B

$p_{ycC}:cemH$:Strep

$p_{ceml}:ceml::FLAG$

$p_{cemFH}::ccmF$

$p_{ceml}:ceml::FLAG$

1 2 3 4 5 6 7 8 9

CemG

Downloaded from http://www.jbc.org/ by guest on July 11, 2017
Fig. 3. (Sanders et al.)
Fig. 4. (Sanders et al.)
Fig. 5. (Sanders et al.)
Table 1: Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Relevant phenotype</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
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</tr>
<tr>
<td><em>E. coli</em></td>
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<tr>
<td>HB101</td>
<td>F^Δ(gpt-proA)62 araC14 leuB6(Am) glnV44(AS) galK2(Oc) lacY1 Δ(mcrC-mrr) rpsL20(Str') xylA5 mtl-1 thi-1</td>
<td>Str'</td>
<td>(25)</td>
</tr>
<tr>
<td>R. capsulatus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT1131^a</td>
<td>crtD121 Rif'</td>
<td>Wild type</td>
<td>Res'/Nadi', Ps', cyts c'</td>
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<tr>
<td>MD12</td>
<td>ccmF::spe (ccmH')</td>
<td>Res'/Nadi', Ps', cyts c'</td>
<td>(23)</td>
</tr>
<tr>
<td>MD14</td>
<td>(ccmF') ccmH::spe</td>
<td>Res'/Nadi', Ps', cyts c'</td>
<td>(23)</td>
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<td>MT-SRP1</td>
<td>Δ(ccmI::kan)</td>
<td>Res'/Nadi', Ps', cyts c'</td>
<td>(17)</td>
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<tr>
<td>MT-SRP1.r1</td>
<td>Δ(ccmI::kan) G488A in promoter of ccmFH</td>
<td>Res'/Nadi', Ps', cyts c' on MedA</td>
<td>(23)</td>
</tr>
<tr>
<td>MD13</td>
<td>Δ(ccmI::kan) ccmF::spe (ccmH')</td>
<td>Res'/Nadi', Ps', cyts c'</td>
<td>This work</td>
</tr>
<tr>
<td>MD15</td>
<td>Δ(ccmI::kan) (ccmF') ccmH::spe</td>
<td>Res'/Nadi', Ps', cyts c'</td>
<td>This work</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pBluescriptIIISK+</td>
<td>(also called pBSK)</td>
<td>Amp'</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Broad host-range vector, gene expression supported by <em>E. coli</em> lacZ promoter</td>
<td>Kan', helper</td>
<td>(36)</td>
</tr>
<tr>
<td>pRK415</td>
<td>Broad host-range expression vector with <em>R. capsulatus</em> cycA (cyt c2) promoter</td>
<td>Tet'</td>
<td>(37)</td>
</tr>
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<td>pCHB500</td>
<td>2.82 kb fragment carrying ccmFH overexpressed via a promoter-up mutation in pBSK</td>
<td>Amp'</td>
<td>(23)</td>
</tr>
<tr>
<td>pYZ1</td>
<td>pYZ10 derivative with a non-polar spe cassette in ccmF (ccmF::spe ccmH') in pRK415</td>
<td>Tet', Spe', CcmH'</td>
<td>(23)</td>
</tr>
<tr>
<td>pYZ4</td>
<td>pYZ10 derivative, but with a polar spe cassette in ccmH (ccmF' ccmH'::spe)</td>
<td>Tet', Spe', CcmF'</td>
<td>(23)</td>
</tr>
<tr>
<td>pYZ12</td>
<td>ccmI::FLAG expressed from its own promoter in pRK415</td>
<td>Tet', CcmI-FLAG'</td>
<td>(19)</td>
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<tr>
<td>pNJ2</td>
<td>ccmI::FLAG including its own promoter sequence (1.69 kb fragment) in pBSK</td>
<td>Tet', Cyt c2'</td>
<td>(27)</td>
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<tr>
<td>pCS1564</td>
<td>1.25 kb BamHI–HindIII fragment containing <em>R. capsulatus</em> cycA in pRK415</td>
<td>Amp'</td>
<td>(19)</td>
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<tr>
<td>pHM14</td>
<td>pET-3a derivative (Novagen, Madison, WI) with T7 promoter region replaced by a DNA fragment encoding LacI and the</td>
<td>Amp', P sac, LacI'</td>
<td>(28)</td>
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<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Selection markers</td>
<td>Notes</td>
</tr>
<tr>
<td>-----------</td>
<td>------------------------------------------------------------------------------</td>
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<tr>
<td>pST6</td>
<td><em>tac</em> promoter region <em>ccmH::Strep</em> from pST8 cloned into the XbaI-KpnI sites of pCHB500</td>
<td>Tet', CcmH-Strep⁺</td>
<td>Turkarslan <em>et al.</em>, <em>in press</em></td>
</tr>
<tr>
<td>pST7</td>
<td>0.5 kb fragment of pYZ1 carrying <em>ccmH</em> cloned into the XbaI-KpnI sites of pBSK</td>
<td>Amp'</td>
<td>Turkarslan <em>et al.</em>, <em>in press</em></td>
</tr>
<tr>
<td>pST8</td>
<td>0.5 kb fragment carrying a Strep-tag sequence added at the 3'-end <em>ccmH</em> from pST7, cloned into the XbaI and KpnI sites of pBSK</td>
<td>Amp'</td>
<td>Turkarslan <em>et al.</em>, <em>in press</em></td>
</tr>
<tr>
<td>pYZ5</td>
<td>Strep-tag sequence added by site directed mutagenesis to the 5' end of <em>ccmF</em> in pYZ1</td>
<td>Amp', Strep-CcmF⁺</td>
<td>This work</td>
</tr>
<tr>
<td>pYZ6</td>
<td>XbaI-KpnI fragment of pYZ5 carrying Strep::ccmF-ccmH (expressed via a promoter-up mutation) in pRK415</td>
<td>Tet', Strep-CcmF⁺, CcmH⁺</td>
<td>This work</td>
</tr>
<tr>
<td>pCS1581</td>
<td><em>ccmI::FLAG</em> including its own promoter sequence from pCS1564 cloned KpnI into pST6</td>
<td>Tet', CcmH-Strep⁺, CcmI-FLAG⁺</td>
<td>This work</td>
</tr>
<tr>
<td>pCS1582</td>
<td><em>ccmI::FLAG</em> including its own promoter sequence from pCS1564 cloned KpnI into pYZ6</td>
<td>Tet', Strep-CcmF⁺, CcmH⁺, CcmI-FLAG⁺</td>
<td>This work</td>
</tr>
<tr>
<td>pCS1302</td>
<td>pCS905 derivative, Strep-tag II sequence (IBA, St. Louis, MO) fused <em>GFP</em>, <em>GFP</em> replacable by cloning any gene of interest in frame into Ndel and BamHI sites</td>
<td>Amp', P*&lt;sup&gt;tac&lt;/sup&gt;, LacI⁺, Strep-GFP⁺</td>
<td>This work</td>
</tr>
<tr>
<td>pCS1718</td>
<td>PCR amplification of a <em>cycA</em> fragment from pHM14 (encoding the matured form of cyt *&lt;sub&gt;c&lt;/sub&gt;₂, introducing Ndel and BamHI sites), phosphorylation and cloning into EcoRV-restricted pBSK</td>
<td>Amp'</td>
<td>This work</td>
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<tr>
<td>pCS1726</td>
<td><em>cycA</em>&lt;sup&gt;mat&lt;/sup&gt; from pCS1718 cloned Ndel-BamHI into pCS1302.</td>
<td>Amp', P*&lt;sup&gt;tac&lt;/sup&gt;, LacI⁺, Strep-cyt *&lt;sub&gt;c&lt;/sub&gt;₂⁺</td>
<td>This work</td>
</tr>
</tbody>
</table>

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

*All plasmids listed contain genes or gene derivatives originated from *R.* capsulatus, unless indicated otherwise.*
The cytochrome c maturation components CcmF, CcmH and CcmI form a membrane-integral multisubunit heme ligation complex
Carsten Sanders, Serdar Turkarslan, Dong-Woo Lee, Ozlem Onder, Robert G. Kranz and Fevzi Daldal

J. Biol. Chem. published online August 27, 2008

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