During Cytochrome c Maturation CcmI chaperones Class I Apocytochromes until the Formation of their b-type Cytochrome Intermediates*  

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*Running Title: Specificity of CcmI for different classes of apocytochromes  

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Background: Cytochrome c maturation (Ccm) forms thioether bonds between heme b and c-type apocytochromes.  

Results: CcmI chaperone exhibits for the class I c-type apocytochromes very high binding affinity, which decreases drastically in the presence of heme.  

Conclusion: CcmI holds tightly the c-type apocytochromes until heme coordination yields their b-type intermediates during Ccm.  

Significance: Interactions between the c-type apocytochromes and chaperones are critical for the Ccm process.  

SUMMARY  
The c-type cytochromes are electron transfer proteins involved in energy transduction. They have heme-binding (CXXCH) site(s) that ligate covalently heme b via thioether bonds, and are classified into different classes based on their protein folds, locations and properties of their cofactors. Rhodobacter capsulatus produces various c-type cytochromes using the Cytochrome c maturation (Ccm)-System I, formed of the CcmABCDEFGH proteins. CcmI is a component of the heme ligation complex CcmFHI, interacts with the heme-handling protein CcmE, and chaperones apocytochrome c2 by binding its C-terminal helix. Whether CcmI also chaperones other c-type apocytochromes, and the effects of heme on these interactions, were unknown hitherto. Here, we purified different classes of soluble and membrane-bound c-type apocytochromes (class I c2 and c1 and class II c'), and investigated their interactions with CcmI and apoCcmE. We report that, in the absence of heme, CcmI and apoCcmE recognized different classes of c-type apocytochromes with different affinities (nM to µM KD values). When present, heme induced conformational changes in class I apocytochromes (e.g., c2), and decreased significantly their high affinity for CcmI. Knowing that CcmI does not interact with mature cytochrome c2, and that heme converts apocytochrome c2...
into its $b$-type derivative, these findings indicate that CcmI holds tightly the class I apocytochromes (e.g., $c_2$) until their non-covalent heme containing $b$-type cytochrome-like intermediates are formed. We propose that these intermediates are subsequently converted into mature cytochromes following the covalent ligation of heme via the remaining components of the Ccm complex.

The $c$-type cytochromes are ubiquitous electron transfer proteins involved in energy transduction in almost all living cells, and they also play critical roles in other cellular pathways (e.g., apoptosis in eukaryotes) (1-3). These proteins always contain at least one conserved heme-binding site ($C_1XXC_2H$) where heme $b$ (protoporphyrin IX-Fe) is covalently ligated. The stereo-specificity of the thioether bonds formed between the vinyl-2 and vinyl-4 of heme and the thiols of Cys$_1$ and Cys$_2$ of a $c$-type apocytochrome, respectively, is universally conserved (4). The His residue of the heme binding site, together with another Met or His residue provide axial ligation to heme iron (5). In spite of these common features, the $c$-type cytochromes are diverse in terms of their sizes, three-dimensional (3D) structures, heme contents and physicochemical properties. Earlier, Ambler grouped the $c$-type cytochromes into four broad classes (6). Class I is a large group that includes small, globular and soluble $c$-type cytochromes. They usually contain a single amino (N)-terminal heme-binding site with a Met residue as the sixth ligand located near their carboxyl (C)-termini (e.g. mitochondrial cytochrome $c$). They are divided into subfamilies according to their structures, functions and properties of their cytochrome domains (1,7). Class II $c$-type cytochromes includes the high spin cytochrome $c'$ with a C-terminally located heme-binding motif and a four helical bundle fold. Class III $c$-type cytochromes comprises the low redox potential ($E_m$) multi-heme proteins with generally bis-His coordination, and the $c$-type cytochromes with additional non-heme cofactors (e.g., flavins) such as flavocytochrome $c_3$ are grouped in class IV.

*Rhodobacter capsulatus* produces a variety of $c$-type cytochromes under different growth conditions. These include the class I C-terminally membrane-bound cytochrome $c_1$ subunit of the cytochrome $bc_1$ complex (8), the N-terminally membrane-attached cytochromes $c_p$ and $c_o$ subunits of the $cbb_3$-type oxygen reductase (9,10) as well as the soluble cytochrome $c_2$ and the N-terminally membrane-attached cytochrome $c_3$, as electron carriers (11,12). The class II soluble high spin cytochrome $c'$ is involved in NO detoxification (13), and the class III membrane-attached pentaheme $c$-type cytochrome DorC conveys electrons from the Q/QH$_2$ pool to dimethylsulfoxide (DMSO) reducing it to dimethylsulfide (14).

*Rhodobacter capsulatus* and other $\alpha$- and $\gamma$-proteobacteria, archaea, mitochondria of plants and red algae carry out the process of covalent heme ligation to the $c$-type apocytochromes via a membrane complex, designated as Cytochrome $c$ maturation (Ccm)-System I (15-18). The overall process relies on several cellular pathways, including post-translational modification and secretion of $c$-type apocytochromes, folding and degradation of proteins, as well as maintenance of a suitable thioredox environment conducive to cofactor insertion. The Ccm complex involves nine membrane proteins (CcmABCDEFGHI) that are responsible for chaperoning of $c$-type apocytochromes and heme as well as their covalent ligation (15).

CcmI is composed of two different domains, and forms with CcmF and CcmH a multi-subunit protein complex responsible for heme ligation (19-23). The N-terminal CcmI-1 domain is membrane-integral via two transmembrane (TM) helices and has a cytoplasmic
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loop with a leucine zipper-like motif. The large periplasmic C-terminal CcmI-2 domain contains three tetratricopeptide repeats (TPR) (20,24-26). TPR domains are involved in protein-protein interactions, and form two anti-parallel α-helices packed in tandem arrays as a super helical structure with a convex and a concave surface where the target proteins bind (27). Genetic studies indicated that the CcmI-1 domain of R. capsulatus is required for maturation of all c-type cytochromes, whereas some amount of C-terminally membrane-anchored cytochrome c₁ is produced in the absence of CcmI-2 (20). Recently we (25), and others (28,29), showed that CcmI binds as a chaperone the C-terminal helix of apocytochrome c₂ primarily via its TPR-containing CcmI-2 domain.

CcmE is a heme-handling membrane protein with a β-barrel domain and a flexible C-terminal stretch (30-32). It binds covalently vinyl-2 of heme b through a surface exposed His residue at its conserved HXXXY site (33-35). HoloCcmE formation and delivery of heme to c-type apocytochromes rely on a specific ABC-type transporter complex (CcmABCD). Once apoCcmE is heme-loaded, an ATP-hydrolysis dependent conformational change (36) renders it competent to deliver heme to c-type apocytochromes. Recently, we found that apoCcmE interacts with the N-terminal heme-binding region of apocytochrome c₂, and forms a ternary complex together with CcmI in vitro (37). Moreover, in R. capsulatus membrane fractions, apoCcmE also interacts with both CcmI and CcmH (37). In addition, holoCcmE is known to form a complex with CcmF in E. coli (38). Altogether, these findings point out that the heme ligation complex CcmFHI contains CcmE and CcmG, possibly forming a large 'maturase supercomplex' (15).

In this study, we investigated the binding interactions between CcmI, apoCcmE and different c-type apocytochromes that are distinct from apocytochrome c₂ in order to understand how R. capsulatus Ccm-System I matures many structurally dissimilar c-type cytochromes. We also probed for the first time how the availability of heme affects these chaperone ~ apocytochrome interactions. We found that CcmI and apoCcmE bind different c-type apocytochromes with markedly different affinities (K_D values), and that the strength of these interactions do not correlate with the distinct secondary structures. Remarkably, heme modulates significantly these binding interactions, suggesting that CcmI holds tightly the c-type apocytochromes until their intermediate b-type derivatives are formed. We propose that these intermediates are subsequently converted into mature c-type cytochromes upon completion of covalent heme ligation by the remaining components of the Ccm complex.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this work are described in Table 1. E. coli strains were grown aerobically at 37 °C in Luria–Bertani broth medium, supplemented with ampicillin (100 µg/mL). Cultures were induced with 1 mM IPTG (25). R. capsulatus strains were grown chemoheterotrophically (i.e., by respiration) at 35 °C on enriched medium (MPYE) supplemented with tetracycline or spectinomycin at 2.5 or 10 µg per ml, respectively (39).

Molecular genetic techniques. The apocytochromes c₁ and c' and their derivatives were produced as done earlier for apocytochrome c₂ (25). R. capsulatus native cytochrome c₁ (petC) has four Cys residues (Cys34 and Cys37 of the C₁XXC₂H heme-binding site and Cys144 and Cys167 forming a disulfide bond) (39). Mutating the latter two Cys residues of cytochrome c₁ does not affect its maturation, but lowers its E_m and renders it non-functional. An additional mutation, Ala181Thr in the heme environment corrects
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this defect to yield a fully functional cytochrome $c_1$ variant (40). This disulfide-less apocytochrome $c_1$ was chosen to avoid complications that could arise from the extra Cys residues, and was considered “wild type” for maturation purposes. Two different apocytochrome $c_1$ derivatives (pMAM1 and pMAM2) were constructed by PCR amplification using the mutant petC allele on plasmid pPET1-C144A/C167A/A181T (40) as a template, and the primers NdeI-Cyt$c_1$-Fw (inserting a NdeI restriction site at the 5’ end and removing cytochrome $c_1$ signal sequence) and Cyt$c_1$-BamHI-Rv or Cyt$c_1$t39_BamHI-Rv (inserting a BamHI restriction site either at the 3’ end of petC or 117 bp upstream of its stop codon, respectively) (Table 2). The PCR products were cloned into the same restriction sites in pCS1302 (23) to yield N-terminally Strep II-tagged, signal sequence-less apocytochrome $c_1$ derivatives with a Factor Xa cleavage site for tag removal. Plasmid pMAM1 encoded a soluble variant of apocytochrome $c_1$ lacking its C-terminal 39 amino acid long membrane anchor (apocytochrome $c_1$t39), and pMAM2 encoded a full-length apocytochrome $c_1$ (Table 1). Similarly, a signal sequence-less and N-terminally Strep II tagged apocytochrome $c_1$ (RCC02682 corresponding to cycP) was obtained by PCR amplification using R. capsulatus chromosomal DNA as a template and the primers NdeI-$c_1$-Fw and $c_1$-BamHI-Rv containing the NdeI and BamHI restriction sites, yielding plasmid pAV6 after its cloning into pCS1302 (Tables 1 and 2). In addition, mature form of Hydrogenobacter thermophilus cytochrome $c_{552}$ was obtained by PCR amplification using plasmid pCS1208 (Table 1) (Sanders and Daldal, unpublished) as a template, and the primers Htssdel-Fw (inserting an NdeI site immediately downstream of its signal sequence) and CS46 (located 3’ of the BamHI restriction site) (Table 2). The PCR product was cloned into pCS1302 to yield pAV5, producing Ht-apocytochrome $c_{552}$ lacking its signal sequence. Plasmid pAV5C11AC14A containing the double Cys to Ala substitutions at the heme-binding site of Ht-apocytochrome $c_{552}$ was derived from pAV5 using QuickChange site-directed mutagenesis kit (Invitrogen Inc.) and Htc$C_{552}$C13/C16-Fw and Htc$C_{552}$C13/C16-Rv primers (Tables 1 and 2), according to the supplier’s recommendation. All constructs were analyzed by the Serial Cloner 2.1 and confirmed by DNA sequencing.

**Protein Purification.** The proteins His$_{10}$-CcmI, His$_{10}$-CcmI-2, His$_{10}$-apoCcmE and Flag-CcmI were purified by affinity chromatography using Ni-Sepharose High Performance (GE Healthcare, Inc.) and Anti-Flag® M2 Affinity (SIGMA, Inc.) resins, respectively (25,37). Strep-tagged $c$-type apocytochromes were purified as described earlier (25). The Cys-less variant of $H.\ thermophilus$ Ht-apocytochrome $c_{552}$ was incubated overnight with 50 mM Tris-HCl, 50 mM NaCl, 1M imidazole for heme removal. The imidazole displaced the heme axial ligands, leading to precipitation of the heme that was removed by centrifugation at 14,000 g for 15 min. Purified protein samples were checked by SDS-PAGE for their purity (> 95%), concentrated by ultrafiltration, and desalted using PD-10 columns (GE Healthcare, Inc). A synthetic peptide carrying a Strep II-tag and Factor Xa cleavage site, corresponding to cytochrome $c_1$ residues 222-241 was produced by Pierce, Thermo Fisher.

**Protein-protein interactions monitored by co-purification assays.** Direct interactions between His$_{10}$-apoCcmE, Flag-CcmI and different Strep tagged $c$-type apocytochromes were assayed as described earlier (25). Briefly, equimolar amounts of Strep-tagged $c$-type apocytochromes (∼ 1 µM) were mixed with substoichiometric amounts of His$_{10}$-apoCcmE or Flag-CcmI (∼ 0.1 µM) in the assay buffer (50 mM Tris-HCl, 50 mM NaCl, pH 8.0, final volume of 400 µL) and incubated for 2 h at 25 °C with gentle shaking. The mixture was
loaded onto a mini (200 µl volume) Strep-Tactin resin column equilibrated with the same buffer. The column was washed extensively with 2 ml of assay buffer (10 CV), and eluted with the same buffer containing 2.5 mM desthiobiotin. Flow through and elution fractions were precipitated with methanol:acetone (7:2, v/v) overnight at -20 °C, and interacting partners analyzed by SDS-PAGE. Binding assays using the synthetic peptides instead of the c-type apocytochromes used a similar protocol. As appropriate, different amounts of hemin (i.e., heme-chloride) dissolved in DMSO (determined using the extinction coefficient of 179 cm⁻¹ mM⁻¹ at 400 nm in 40% DMSO (41)) (Frontier Scientific Inc.) were added to the incubation mixtures.

Protein-protein interactions monitored by biolayer interferometry. Binding kinetics of His₁₀-CcmI and His₁₀-apoCcmE to different Strep-tagged c-type apocytochromes was monitored quantitatively in real time by biolayer interferometry (BLI) using Octet RED96 instrument (ForteBio, Inc). Purified c-type apocytochromes (i.e., ligands) were biotinylated using the EZ-Link™ NHS-PEG₄-Biotinylation Kit (Thermo Scientific, Inc) to immobilize them on Streptavidin-coated biosensors (SA-sensors). SA-sensors were loaded with biotinylated c-type apocytochromes (Bt-apocytochromes) by soaking them in a buffer containing ~ 400 nM of desired Bt-apocytochrome, 50 mM Tris-HCl pH 8, 100 mM NaCl, 0.01% DDM and 1% BSA at 30 °C with 1000 rpm shaking. A reference sensor was dipped into a well containing assay buffer lacking the Bt-apocytochrome to assess non-specific binding of the analyte (CcmI or apoCcmE) to the sensor. After washing with the same buffer, unoccupied residual streptavidin sites on the SA-sensors were blocked with biocytin (10 µg/mL), and following another washing step, a baseline signal was recorded. Different Bt-apocytochromes loaded SA-sensors were incubated with increasing concentrations of analyte (i.e., CcmI from 4 nM to 30 µM or apoCcmE from 0.3 to 20 µM) (association step). Subsequent wash of the biosensors with the assay buffer released the analyte (CcmI or apoCcmE) from the immobilized ligand (dissociation step). An assay lacking the analyte was used as a negative control to confirm that the observed shifts were due to the ligand ~ analyte complexes. Collected data were used for the determination of the kinetic parameters. The range of concentrations used depended on the Bt-apocytochrome tested to obtain data under non-saturating binding conditions. Higher concentrations of CcmI or apoCcmE were needed in the case of class II apocytochrome c', which enhanced non-specific binding to the sensors. In order to mitigate this problem, the DDM concentration of the wash buffer was increased to 0.05%.

Kinetics performed in the presence of heme used Flag-CcmI instead of His₁₀-CcmI as analyte to avoid possible binding of heme to the His-tag epitope. In addition, the standard assay buffer that contained BSA (known to bind heme, e.g. (42)) was substituted with 50 mM Tris-HCl pH 8, 150 mM NaCl and 0.01% Tween-20. Under these conditions, hemin was used at concentrations ranging from 0.1 to 6.4 µM to monitor its binding to apocytochrome c₂. In order to investigate the effect of heme on CcmI ~ apocytochrome c₂ interactions, we first repeated full kinetic measurements using this buffer and Flag-CcmI at concentrations ranging from 0.7 to 180 nM. Then, the assay buffer was supplemented with 2 µM hemin to yield a new baseline (accounting for binding of heme to apocytochrome c₂). Increased Flag-CcmI concentrations (from 0.07 to 2.4 µM) were used to account for decreased apparent association responses. The k_on and k_off rates of binding and the K_D values for each interacting pair were determined by fitting the experimental data to 1:1 homogenous or to 2:1 heterogeneous kinetic models describing bimolecular interactions, according to the
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manufacturer’s literature (Forte Bio, Inc) (43). The quality of the fit between the experimental and calculated data was evaluated according to the following parameters: error values for $k_{on}$ and $k_{off}$ (at least an order of magnitude lower than the $k$ values), residual values (< 10% of the maximum response of the fitting curve), $R^2$ (> 0.95) and $X^2$ (< 3) (43).

**SDS-PAGE and Immunoblot Analyses.** SDS-PAGE under reducing conditions (5% β-mercaptoethanol) was performed using 15% gels according to (44), and covalently bound heme containing proteins were detected using tetramethylbenzidine (TMBZ) as in (45). For apocytochrome c’ immunodetection, gel-resolved proteins were electroblotted onto Immobilon-PVDF membranes (Millipore Inc.) and probed with rabbit polyclonal antibodies specific for *R. capsulatus* cytochrome c’ (a kind gift of Dr. R. Prince). Horseradish peroxidase conjugated anti-rabbit IgG antibodies (GE Healthcare Inc.) were used as secondary antibodies, and detection was performed using the SuperSignal West Pico Chemiluminescent Substrate® from Thermo Scientific, Inc.

**Circular Dichroism Spectroscopy.** The far-UV circular dichroism (CD) spectra (195-240 nm) were recorded with a Model 202 spectropolarimeter (AVIV® Instruments, Inc) using a 2 mm path-length cuvette (Hellma, Inc.), as done earlier (25). The CD spectra of proteins (15 µM) in 20 mM sodium phosphate buffer pH 7.5 were recorded using a 3 nm bandwidth, 2 nm step size, and a time constant of 10 s. CD spectrum of the buffer was subtracted from the spectra of the proteins, and the absorbance values were converted into the mean residue ellipticity $[\theta]_\lambda$ (deg cm$^2$ dmol$^{-1}$), as above. For the effect of heme on apocytochrome c$_2$ ~ CcmI interactions, protein mixtures (molar ratio of apocytochrome c$_2$ to CcmI of 2 to 1) were incubated for two hours at room temperature, without or with hemin (at two to eight fold molar excess of apocytochrome c$_2$), and their CD spectra compared with the sum of the spectra of individual proteins obtained under the same conditions, after subtraction of the spectral contributions of the corresponding buffers.

**Reconstitution of b-type cytochrome intermediates.** Stoichiometric amount of hemin dissolved in DMSO was added slowly, from a stock solution of 1 mM, to 10 µM c-type apocytochrome in 50 mM Tris-HCl, 150 mM NaCl, pH 8. The sample was stirred for 5-10 min between each addition to reach equilibrium, and visible spectra between 380-650 nm were taken to monitor binding of hemin to the c-type apocytochromes. Unbound hemin was removed by size exclusion chromatography (PD-10 column, GE Healthcare Inc), and after concentration, visible spectra of the newly formed b-type cytochromes were recorded as prepared (air oxidized) and after dithionite reduction. The
Relative amounts of reconstituted b-type cytochrome derivatives of *R. capsulatus* c-type apocytochromes were determined taking as 100% the amount of *Ht*-apocytochrome b-c552 reconstituted under the same conditions.

**Chemicals.** All chemicals and solvents were of high purity and HPLC spectral grades and purchased from commercial sources.

**RESULTS**

**Overproduction and purification of CcmI, apoCcmE and c-type apocytochromes.** We showed previously that *R. capsulatus* CcmI binds tightly to the C-terminal helix, whereas apoCcmE interacts with the N-terminal heme-binding region of apocytochrome c2 (25,37). As this bacterium produces various c-type cytochromes, in this work we inquired whether these interactions were exclusive to apocytochrome c2 or more general including other c-type apocytochromes. Considering that maturation of cytochrome c' in *R. capsulatus* has not been examined earlier, we first analyzed soluble extracts of *R. capsulatus* mutants lacking CcmI or CcmE (MT-SRP1 or MD2, respectively) using SDS-PAGE/TMBZ staining and immune-detection with cytochrome c2 antibodies. These mutants lacked cytochrome c' (*Fig. 1*), confirming that CcmI and CcmE of Ccm-System I were required for its maturation. Based on these findings, we chose in addition to apocytochrome c2 the class I membrane-anchored cytochrome c1 whose maturation is independent of the CcmI-2 domain of CcmI, and the class II soluble cytochrome c' which has a non-globular 3D structure (*Fig 2A*).

We overproduced in *E. coli* cytoplasm, and purified Strep-tagged versions of the c-type apocytochromes and their derivatives, as done earlier (25) (*Fig. 2B*). The Strep-apocytochrome c' (MW of 15 kDa) was produced at ~1-2 mg/L of culture, and was prone to degradation, like the Strep-apocytochrome c2 (13.5 kDa) (*Fig. 2C*, lanes 1 and 2). The full-length (Strep-apocytochrome c1, 30 kDa), its C-terminal membrane-anchor (39 amino acid residues) truncated (Strep-apocytochrome c1t39, 27 kDa) soluble version and also a Cys-less derivative of Strep-apocytochrome c1t39 were produced at large amounts (> 10 mg/L culture) (*Fig. 2C*). All Strep-Tactin affinity chromatography purified c-type apocytochromes were devoid of heme, as confirmed by TMBZ staining and visible spectroscopy (data not shown). The truncated Strep-apocytochrome c1t39 formed readily intermolecular disulfide bonds to yield homodimers (~55 kDa) even under reducing conditions. We also purified the Flag-CcmI (50 kDa), His10-CcmI (50 kDa), His10-CcmI-2 (42 kDa) and His-apoCcmE (18 kDa) proteins (37). The purity (>95%) of all proteins was confirmed by SDS-PAGE (*Fig. 2C and D*) and immune detection with anti-CcmI and anti-CcmE antibodies (data not shown), and considered suitable for *in vitro* binding assays.

CcmI discriminates different classes of c-type apocytochromes. Chaperone activity of CcmI against the different classes of c-type apocytochromes was probed first using co-purification assays, under the conditions previously established for apocytochrome c2 (25). SDS-PAGE analyses of elution fractions showed that different amounts of CcmI co-purified with different c-type apocytochromes (*Fig. 3A*). Semi-quantitative image analyses of Coomassie stained gels estimated that CcmI co-purified with apocytochrome c1 at about 70 % (lane 4) of the amount of CcmI retained by apocytochrome c2 (lane 2). This decrease in CcmI retention was more obvious with the soluble apocytochrome c1t39 variant (lane 5). Remarkably, no detectable amount of CcmI co-purified with apocytochrome c' (lane 3). We concluded that CcmI associated more readily with the class I than the class II c-type apocytochromes under the conditions used.

Using a full-length apocytochrome c1 and
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its membrane-anchorless variant, apocytochrome c1t39, we next probed the role of the different domains of CcmI in vitro. Co-purification assays conducted using intact CcmI, or only its CcmI-2 domain, with full-length or truncated apocytochrome c1 derivatives showed that the full-length apocytochrome c1 interacted better with CcmI than the truncated apocytochrome c1t39. Also, the amount of CcmI-2 that co-purified with either derivative of apocytochrome c1 was higher than CcmI (Fig. 3B). These findings suggested that neither the membrane-anchor of apocytochrome c1, nor the CcmI-1 domain (i.e., the first TM helix and the adjacent leucine zipper containing cytoplasmic loop absent in CcmI-2 derivative used) of CcmI, is essential for these interactions in vitro. Lastly, using a synthetic peptide (NH2-WSHPQFEKIEGR TVDQMAQVDSAFLMW AAEPK-COOH) corresponding to the C-terminal helix of apocytochrome c1t39 (i.e., the C-terminal helix that interacts with the N-terminal heme-binding helix) we tested whether CcmI recognizes this helical sequence of apocytochrome c1, as observed earlier with that of apocytochrome c2 (25). Incubation of increasing amounts (10 and 20 µg) of this peptide with purified CcmI led to concentration-dependent co-purification of CcmI-peptide complex (Fig. 3C). The amount of CcmI co-purified was lower than that observed with the same amount of the apocytochrome c2 peptide used previously (25), paralleling the findings of the binding assays using apocytochromes c1 and c2 (Fig. 3A). The data indicated that in all class I c-type apocytochromes the C-terminal helix, which is orthogonal to the N-terminal heme-binding site containing helix, is sufficient to promote binding to CcmI.

ApoCcmE recognizes differently class I and class II c-type apocytochromes. In this work, we extended the apoCcmE ~ apocytochrome c2 binding studies carried out earlier (37) to other c-type apocytochromes, and found that apoCcmE, like CcmI, binds apocytochrome c1 but not apocytochrome c’ (Fig. 4A). Semi-quantitative image analyses of Coomassie stained gels revealed that the amounts of apoCcmE co-purifying with apocytochrome c1 decreased to 60% of that seen with apocytochrome c2 (lanes 1 and 3), while no detectable interaction was seen with apocytochrome c’ (lane 2). Moreover, comparison of the truncated apocytochrome c1t39 with its Cys-less derivative (Cys to Ser substitutions at the heme-binding site) indicated that the occurrence of a disulfide bond at the heme-binding site had no effect on apoCcmE ~ apocytochrome c1t39 interactions (Fig. 4B), unlike apocytochrome c2 (37).

Binding kinetics of various c-type apocytochromes to CcmI and apoCcmE. Using BLI, binding affinities of CcmI and apoCcmE to c-type apocytochromes were quantified by real time binding assays. The association (k_on), dissociation (k_off) rates and the binding affinity (K_D) constants of appropriate protein couples were determined as described in Materials and Methods. Negative controls lacking the analytes confirmed that the observed interference shifts originated from the ligand-analyte complexes. Using Octet Data Analysis software (Forte Bio Inc) experimental data (association and dissociation curves) were fit to a homogeneous 1:1 bimolecular protein-protein interaction model, and binding parameters (k_on, k_off and K_D) of the ligand-analyte couple were determined (Table 3).

With the CcmI ~ Bt-apocytochrome c2 complexes, association curves exhibited rapid increases until reaching equilibrium, and dissociation curves followed slow decay kinetics (monitored for longer time periods for better data collection) (Fig. 3D). This behavior reflected fast binding of CcmI to apocytochrome c2 to form a stable complex. Similar experiments conducted with other c-
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type apocytochromes indicated that CcmI interacted strongly with the class I apocytochromes c₁ and c₁t39 (Table 3, ~ nM Kᵯ values). In contrast, although CcmI associated rapidly with apocytochrome c’, it dissociated rapidly, indicating that it bound weakly to this class II c-type apocytochrome (Table 3, ~ µM Kᵯ values). Thus, binding kinetics confirmed and quantified the findings of the co-purification assays (Fig. 3A). In all cases, the kᵯ on rates were comparable, indicating that the c-type apocytochromes bound rapidly to CcmI, but the kᵯ off rates were faster for unstable (i.e., apocytochrome c’) and slower for stable (i.e., apocytochromes c₂, c₁ and c₁t39) binary complexes.

Next, the binding of apoCcmE to c-type apocytochromes was examined using a similar approach (Fig. 4C). Kinetic data showed that apoCcmE associated with apocytochrome c₂ or c₁t39 at rates slower than those seen with CcmI, and dissociated at faster rates, yielding ~ µM Kᵯ values (Table 3). Furthermore, even though apoCcmE bound apocytochrome c’ at rates similar to apocytochromes c₂ or c₁t39, it dissociated rapidly, showing ~ 100 fold higher Kᵯ values. Overall data established that both CcmI and apoCcmE recognized the class I c-type apocytochromes with higher affinities than the class II counterparts.

Probing the secondary structures of various c-type apocytochromes using CD spectroscopy. The secondary structures (globular class I cytochromes c₂ and c₁ versus four helical bundle class II cytochrome c’) of different c-type apocytochromes were examined by CD spectroscopy in the far-UV region (Fig. 5A). CD spectra of the apocytochromes c₂ and c’ exhibited a negative peak around 203 nm and low ellipticity below 215 nm, showing their random coil conformations, respectively. In contrast, both apocytochrome c₁ and apocytochrome c₁t39 exhibited CD spectra more characteristic of α-helical proteins, with two negative peaks at 208 nm and 223 nm. Interestingly though, the two class I apocytochromes c₂ and c₁, which have similar globular folds in their mature forms, exhibited different secondary structures in their apocytochrome forms (48,49). In addition, the R. capsulatus class II apocytochrome c’ also differed from its E. coli homologue cyt b₅₆₂, which forms a molten globule in the absence of heme (50), even though both holocytochromes have four helical bundle structures. Finally, the CD spectra of CcmI and CcmI-2 showed that they had the characteristics of α-helical proteins (Fig. 5B).

Release of apocytochrome c₂ from CcmI is facilitated by the presence of hemin. The observed tight binding of CcmI to class I c-type apocytochromes was remarkable, leading us to probe whether or not heme affected these interactions. This was addressed by choosing cytochrome c₂ as a prototype for class I c-type apocytochrome. We reasoned that if heme induces the formation of a b-type derivative of apocytochrome c₂, with a molten globule-like structure (reminiscent of that of mature cytochrome c₂) then this intermediate might not bind tightly CcmI, similar to what we observed earlier with a native form (25). In order to test this hypothesis, we first investigated the kinetics of heme binding to Bt-apocytochrome c₂ using BLI. The data showed that heme bound to, and dissociated from, apocytochrome c₂ rapidly (Fig. 6A). A 1:1 bimolecular kinetic model indicated that the apocytochrome c₂ ~ heme complex had ~ µM affinity constant (Kᵯ) and was not very stable (Table 3). A similar low affinity has been reported for the horse heart apocytochrome c ~ heme complex under oxidizing conditions (46). Next, we examined the effects of heme on the CD spectra of apocytochrome c₂ and CcmI. Upon addition of heme, the far-UV CD spectrum of apocytochrome c₂ changed drastically, with increased helical content, paralleling increased amount of heme (Fig. 6B). On the other hand,
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For the effect of heme on CcmI ~ apocytochrome c₂ interactions, we first tested co-purification of CcmI with apocytochrome c₂ in the presence of hemin (Fig. 7A). Addition of stoichiometric amounts of hemin (∼ 1 µM) decreased the amount of CcmI that co-purified with apocytochrome c₂ by ∼ 25% (Fig. 7A). Next, binding kinetics of CcmI to apocytochrome c₂ were monitored by BLI in the absence of hemin, but omitting BSA and replacing His₁₀-CcmI with Flag-CcmI to minimize spurious heme interference. As before, we also observed ∼ nM Kᵰ values for CcmI ~ apocytochrome c₂ interactions for these derivatives (Table 3). However, when the assays were repeated in the presence of 2 µM of hemin (∼ five fold molar excess of Bt-apocytochrome c₂), the association and dissociation kinetics could not fit reliably to a 1:1 homogeneous model, suggesting the presence of a nonhomogeneous ligand population. In cases where “active” and “inactive” forms of ligands towards the analyte are expected, the use of a 2:1 heterogeneous model becomes appropriate. Indeed, upon addition of heme, a fraction of apocytochrome c₂ yields a non-covalent heme containing b-type cytochrome derivative (see below). When the kinetic data were fitted to a 2:1 heterogeneous model, two different Kᵰ values (with acceptable X² and R² values) for CcmI ~ apocytochrome c₂ interactions were deduced (Table 3). These values were 15 - 20 times higher than the Kᵰ seen in the absence of hemin, clearly indicating that the CcmI ~ apocytochrome c₂ interactions weakened.

Earlier, CD spectroscopy showed that CcmI and apocytochrome c₂ changed their conformations upon binding to each other in the absence of hemin (25). This approach was used to further document the effect of heme on CcmI ~ apocytochrome c₂ interactions. The secondary structure changes were monitored after incubating CcmI and apocytochrome c₂ in the presence (molar excess) or absence of hemin, and the CD spectra obtained were compared with the sums of the spectra of the individual proteins recorded under the same experimental conditions (Fig. 7B). These comparisons showed that the conformational changes induced by apocytochrome c₂: CcmI interactions decreased markedly in the presence of hemin. This finding further supported that a fraction of apocytochrome c₂ changed its secondary structure upon binding heme, and weakened its interactions with CcmI, lowering the CD detected spectral changes (Fig. 7C).

The c-type apocytochromes can form b-type cytochrome variants in the presence of hemin. Optical spectroscopy was used to monitor non-covalent binding of heme to apocytochrome c₂ and to other selected c-type apocytochromes to assess if indeed they form b-type cytochrome variants. As a control for formation of a b-type cytochrome variant from a c-type apocytochrome, a Cys-less derivative of H. thermophilus cytochrome c₅₅₂ was used. When expressed in E. coli cytoplasm, native H. thermophilus cytochrome c₅₅₂ contains covalently ligated heme (51) even in the absence of the Ccm-System I, and under aerobic growth conditions. Similarly, the Cys-less derivative of H. thermophilus cytochrome c₅₅₂ produces a non-covalent heme containing b-type cytochrome (called cytochrome b-c₅₅₂) (52). Overnight incubation of purified cytochrome b-c₅₅₂ in the presence of 1 M imidazole displaces its heme to yield apocytochrome b-c₅₅₂ (52) (data not shown). We purified these variants of H. thermophilus cytochrome c₅₅₂, and SDS-PAGE/TMBZ analyses confirmed the absence of covalently bound heme in both cytochrome b-c₅₅₂ and its corresponding apocytochrome b-c₅₅₂ (data not shown). CD spectra of these proteins resembled those of typical α-helical proteins with the amounts of secondary structures...
being increased from apocytochrome $c_{552}$, to cytochrome $b_{c552}$ to cytochrome $c_{552}$ (data not shown). Binding of heme enhanced secondary structure formation, even though cytochrome $b_{c552}$ already had some secondary structure in the absence of heme, as compared with the apocytochromes $c_2$ or $c'$ (Fig. 5A). Thus, *H. thermophilus* cytochrome $c_{552}$ and its derivatives provided valid controls for the formation of $b$-type cytochrome from the $c$-type apocytochromes.

Addition of stoichiometric amount of hemin to *R. capsulatus* $c$-type apocytochromes resulted in changes in their visible spectra over time. After 30 min of incubation with hemin, the apocytochromes $c_2$ and $c_1$, but not apocytochrome $c'$, exhibited spectral features that are typical of $b$-type cytochromes, with Soret and $\alpha$-bands at 422 and 557 nm in apocytochrome $c_2$, and 425 and 559 nm in apocytochrome $c_1$, respectively (Fig. 8). Under the conditions where full (100%) incorporation (as confirmed by comparison with similar amount of cytochrome $b_{c552}$ purified from *E. coli*) of heme to apocytochrome $c_{552}$ occurred to yield cytochrome $b_{c552}$, ~20% of available heme was reconstituted into apocytochrome $c_2$, ~12% into apocytochrome $c_1$, and no detectable amount into apocytochrome $c'$ (assuming similar extinction coefficients for all $b$-type cytochrome derivatives). These findings showed that a fraction of apocytochrome $c_2$ was converted to its $b$-type cytochrome derivative in the presence of heme. Moreover, the apocytochromes $c_2$ and $c_1$ behaved in opposing ways with respect to the amounts of ellipticity they exhibited and the $b$-type cytochrome variants they yielded. Thus no direct correlation was seen between the helical content of a $c$-apocytochrome and its ability to bind heme to yield a $b$-type cytochrome variant *in vitro*.

**DISCUSSION**

Previously, we showed that the chaperone protein CcmI binds to the C-terminal helix of apocytochrome $c_2$, whereas the heme-handling protein CcmE recognizes its heme-binding site (25,37). In this work we addressed the next question, which is whether or not CcmI also chaperones other soluble and membrane-bound $c$-type apocytochromes in addition to apocytochrome $c_2$. To this end, we chose the class I membrane-anchored apocytochrome $c_1$ because the TPR motifs containing portion (CcmI-2) of CcmI is not essential for its maturation (20). We also investigated the soluble class II cytochrome $c'$, which has a different (four helical bundle versus globular) structure than the class I members. Production in *E. coli* of cytochrome $c'$ from some species requires co-expression of the Ccm genes (53,54), whereas from others (*e.g.*, *H. thermoluteolus* cytochrome $c'$) do not (54). At the onset of this work, whether cytochrome $c'$ maturation in *R. capsulatus* relied on Ccm-System I was unknown (20). Our data showed that cytochrome $c'$ maturation in *R. capsulatus* requires at least CcmE and CcmI, and established it as a substrate for Ccm-System I. Co-purification and real time binding (BLI) assays demonstrated that both CcmI and apoCcmE distinguished different classes of $c$-type apocytochromes, and that heme affected strongly these interactions.

**Binding of CcmI and apoCcmE to class I and class II $c$-type apocytochromes in the absence of hemin.** Real time binding studies indicated for the first time that *R. capsulatus* class I $c$-type apocytochromes ($c_2$, $c_1$ and its anchor-less derivative $c_139$) had very high (~nM $K_D$), while the class II apocytochrome $c'$ had much lower (~$\mu$M $K_D$) binding affinities for CcmI (Table 3) (Fig. 9). Protein-protein interaction studies between the $c$-type apocytochromes and their chaperones are scarce. The only other experiment using the soluble portion of *Pseudomonas aeruginosa* CcmI and the class I apocytochrome $c_{551}$ (or a dansylated peptide
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corresponding to its C-terminal end) reported a low equilibrium binding K_D (~ >100 µM) (29). As a different experimental approach is used in the latter studies, direct data comparison is difficult, and why different K_D values were obtained remains unclear.

A major structural determinant of apocytochrome \( c_2 \) for binding CcmI is its most C-terminal helix, the equivalent of which is conserved in most class I \( c \)-type cytochromes (55). This helix packs orthogonally against the heme-binding site containing N-terminal helix in mature \( c \)-type cytochromes. The presence of a hydrophobic molecule (e.g., a porphyrin ring) induces conversion of a random coiled \( c \)-apocytochrome to a molten globular structure, as a folding intermediate (56), and promotes interactions between these N- and C-terminal helices. In cytochrome \( c_1 \), unlike the other \( R. \) capsulatus \( c \)-type cytochromes (i.e., \( c_2, c_y, c_o \) and \( c_p \)), this “C-terminal helix” precedes its most C-terminal membrane-anchoring helix, but becomes the most C-terminal helix in the truncated apocytochrome \( c_1t39 \). Availability of these two variants allowed us to probe the role of the anchoring and the “C-terminal helix” of cytochrome \( c_1 \) on binding CcmI. Earlier genetic studies indicated that CcmI-2 portion of CcmI is not necessary for cytochrome \( c_1 \) production, inferring that enough interactions occur between the Ccm-System I and membrane-anchored apocytochrome \( c_1 \) during its maturation (20). In vitro data obtained here further complemented these findings and showed that roughly similar K_D values were observed for binding of native or truncated forms of apocytochrome \( c_1 \) to CcmI (Table 3). Moreover, co-purification assays showed that CcmI-2 binds both apocytochromes \( c_1 \) derivatives. We deduced that the structural determinant(s) recognized by CcmI-2 must be present in the anchor-less apocytochrome \( c_1t39 \). Indeed, similar to apocytochrome \( c_2 \), a peptide corresponding to the “C-terminal helix” preceding the membrane-anchor of cytochrome \( c_1 \) is readily recognized by CcmI (Fig. 3C). Overall data therefore showed that apocytochrome \( c_1 \) interacted via its “C-terminal helix” with CcmI-2, and inferred that it might interact via its membrane-anchor with CcmI-1 domains of CcmI. It remains to be seen if the second TM helix of CcmI-1, which is present in the CcmI-2 variant used here, is also involved in CcmI ~ apocytochrome \( c_1 \) interactions.

How the class I \( c \)-type apocytochromes interact with the TPR motifs is not well known. The TPR-containing domain of \( E. \) coli NrfG (a functional homologue of \( R. \) capsulatus CcmI and \( E. \) coli CcmH), which is specific for maturation of unusual \( c \)-type cytochromes (with a heme-binding sequence of C\(_1\)XXC\(_2\)K like the \( E. \) coli NrfA (28)) exhibits a K_D of ~ 10 µM towards a peptide mimicking the C-terminus of NrfA. In this case, the TPR binding groove apparently recognizes a helix followed by a loop composed of six C-terminal residues (28). However, the TPR proteins are highly versatile with respect to the amino acid compositions of their TPR motifs that modulate their ligand affinity. The 3D structures of these proteins bound to their ligands show an intricate network of contacts, including electrostatic, hydrophobic and Van der Waals interactions (27). For example, such a structure between a TPR protein and the Hsp70 or Hsp90 proteins define a conserved peptide (Met-Glu-Glu-Val-Asp) at the C-termini of these proteins, binding to the concave groove of the TPR protein (57). Yet, other TPR proteins present alternative interaction modes, including a binding site not located in the TPR groove, but composed of the loops connecting the TPR domains (58). Clearly, much remains to be learned about the interactions of the \( c \)-type apocytochromes with their TPR motifs containing chaperones.

This study also showed that, like CcmI, the heme-handling protein apoCcmE interacts not only with apocytochrome \( c_2 \) (37), but also...
with other c-type apocytochromes. These interactions are weaker (µM versus nM $K_D$ values) than those seen with CcmI, but still highly discriminatory between the different classes of c-type apocytochromes with about a hundred fold lower binding affinities for the class II apocytochrome c'. They are also unaffected by the redox state of the Cys residues at the heme-binding sites of apocytochromes.

**Binding of heme to c-type apocytochromes in the absence of the Ccm components.** Heme triggers folding of unstructured c-type apocytochromes, increasing their secondary structures (46). Several c-type apocytochromes, like the mitochondrial apocytochrome c (46,59), *Paracoccus denitrificans* apocytochrome c$_{550}$ (59) and *R. capsulatus* apocytochrome c$_2$, are random coils that can form compact molten globular structures upon binding heme. However, they often yield inefficiently b-type cytochrome derivatives. Conversely, some other c-type apocytochromes from thermophilic organisms like *Hydrogenobacter thermophilus* cytochrome c$_{552}$ (51), *Aquifex aeolicus* cytochrome c$_{555}$ (60), *Thermus thermophilus* cytochrome c$_{552}$ (61), and *Hydrogenophilus thermoluteolus* cytochrome c' (62) exhibit secondary structures with high helical contents in the absence of heme (52,62,63), and yield readily b-type cytochrome derivatives upon heme availability in vitro (64). Even in exceptional cases, thermophilic molten globular c-type apocytochromes yield b-type cytochrome intermediates that are conducive to spontaneous covalent heme ligation (51). Hence, they are poor models for cytochrome c maturation studies due to their structures evolved to bind and ligate heme independently of the Ccm-System I.

CD spectral data indicated that no direct correlation exists between the increased helical contents of c-type apocytochromes and their ability to yield b-type cytochrome derivatives upon binding of heme. For example, the class I apocytochrome c$_1$ exhibited high degree of secondary structure but was less efficient than apocytochrome c$_2$ for binding heme (Fig. 8). On the other hand, *R. capsulatus* apocytochrome c' had no secondary structure discernable by CD spectroscopy, like its homologue from mesophilic *Allochromatium vinosum* (62), and was unable to bind heme and yield a b-type cytochrome derivative. Conversely, the apocytochrome b-c$_{562}$ derivative of *E. coli* cytochrome b$_{562}$ (structural homologue of cytochrome c') exhibits a fold that matches closely its final conformation, and incorporates efficiently stoichiometric amount of hemin, independently of the Ccm machinery. So far, the only known mesophilic c-apocytochrome that binds heme efficiently to form a b-type cytochrome derivative is the horse cytochrome c (46,59), although it is matured naturally by the cytochrome c biogenesis-System III (65). Although a few c-type apocytochromes, due to their structure and intrinsic stability can bind heme to yield either b-type cytochromes derivatives, or mature c-type cytochromes in the absence of the Ccm components, apparently, the “designed” four helical bundles, which bind heme efficiently, still need the Ccm machinery to produce their c-cytochrome derivatives (66). Thus, the high helical content of a c-type apocytochrome in the absence of hemin is not predictive of a form conducive to efficient heme binding. In addition to the interactions between the heme iron and its axial ligands, as well as those between the porphyrin ring and the polypeptide, the amino acid sequences of c-type apocytochromes might also contribute to providing a suitable environment for trapping heme. If this structural information is virtually absent in a c-type apocytochrome then it relies on the Ccm processes.

**Effect of hemin on the interactions of apocytochrome c$_2$ with CcmI.** A remarkable
finding of this study is the very high binding affinity (~ nM) of CcmI for class I c-type apocytochromes (Fig. 9). Clearly, this high affinity is advantageous for efficient capture of the c-type apocytochromes by the Ccm complex following their translocation across the cytoplasmic membrane. However, it also raises an intriguing issue, which is their subsequent release upon maturation. Our earlier observation that CcmI does not bind holocytochrome c₂ suggested that heme, and heme mediated folding, might affect these interactions. Indeed, addition of hemin to apocytochrome c₂ promoted the formation of its b-type cytochrome-like derivative (Fig. 9). Co-purification assays in the presence of hemin indicated that the amount of CcmI that co-purified with apocytochrome c₂ decreased. Interestingly, the decrease (~ 25%) in the amount of CcmI that was retained by apocytochrome c₂ coincided roughly with the amount (~ 20%) of heme incorporated into apocytochrome c₂ to yeild its b-type derivative in vitro. This coincidence suggested that the b-type cytochrome derivative formed upon addition of hemin might interact poorly with CcmI, which was also supported by the decreased conformational changes seen by the CD spectra of CcmI ~ apocytochrome c₂ complexes in the presence of hemin (Fig. 7). In agreement with these findings, quantitative binding assays done in the presence of hemin documented higher $K_D$ values for binding of apocytochrome c₂ to CcmI (Table 3). It should be noted that thorough analyses of tripartite interactions between hemin, apocytochrome c₂ and CcmI are complicated due to the heterogeneity induced by binding of hemin to only a fraction of apocytochrome c₂, to yield a b-type cytochrome-like derivative in the absence of any Ccm component in vitro. Nonetheless, data analyses based on a 2:1 heterogeneous kinetic model supported a decrease in the affinity of CcmI for apocytochrome c₂ (and possibly for other class I c-type apocytochromes) in the presence of hemin, possibly via promoting the formation of a partially folded apocytochrome c₂ with coordinated heme moiety.

In summary, detailed analyses of the binding process of CcmI to apocytochrome c₂ summarized in Fig. 9, support the notion that the release of a c-type apocytochrome from its chaperone during the Ccm process could occur upon the availability of heme (probably via CcmE). If so, then a b-type cytochrome derivative forms as an intermediate, which subsequently undergoes covalent heme ligation by the remaining components of the Ccm complex.
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Specification of CcmI for different classes of apocytochromes

Anderson, J. L., Armstrong, C. T., Kodali, G., Lichtenstein, B. R., Watkins, D. W., Mancini, J. A., Boyle, A. L., Farid, T. A., Crump, M. P., Moser, C. C., and Dutton, P. L. (2014) Constructing a man-made c-type cytochrome maquette: electron transfer, oxygen transport and conversion to a photoactive light harvesting maquette. Chem. Sci. 5, 507-514

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FOOTNOTES
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2The abbreviations used are: Ccm, Cytochrome c maturation; MPYE, Mineral-peptone-yeast-extract medium; Res, Respiratory; Ps, Photosynthetic; Nadi stain, α-naphthol + N,N-dimethyl-p-phenylenediamine yielding indophenol blue in the presence of O2 to reveal cytochrome c oxidase activities of bacterial colonies; DDM, n-dodecyl-β-D-maltoside; IPTG, isopropyl β-D-1-thiogalactopyranoside; BLI, Biolayer interferometry; CD, Circular Dichroism; TM, transmembrane; TPR, tetratricopeptide repeat; TMBZ, tetramethylbenzidine; DMSO, dimethylsulfoxide.

FIGURE LEGENDS

Figure 1. CcmI and CcmE are required for maturation of class II cytochrome c’. A. SDS-PAGE/TMBZ heme staining using 100 µg of total soluble protein extracts from appropriate R. capsulatus strains: 2 µg of purified cytochrome c’ as a positive control for heme staining (lane 1), wild type MT1131 (lane 2), CcmI-null strain MTSRP1 (lane 3), CcmE-null strain MD2 (lane 4) and cytochrome c2-null strain MT-G4/S4 (lane 5). B. SDS-PAGE/immunoblot using anti-cytochrome c’ polyclonal antibodies using 100 µg of total soluble protein extracts from appropriate R. capsulatus strains: 2 µg of purified cytochrome c’ as a positive control (lane 1), wild type MT1131 (lane 2), cytochrome c2-null strain MTG4/S4 (lane 3), CcmI-null strain MTSRP1 (lane 4) and CcmE-null strain MD2 (lane 5).

Figure 2. Purification of various R. capsulatus c-type apocytochromes, CcmI and apoCcmE. A. 3D structures of class I cytochrome c2 (cyt c2, PDB:1C2R), cytochrome c1 (cyt c1, PDB:1ZRT) and class II cytochrome c’ (cyt c’, PDB:1RCP). B. Schematic representations of c-apocytochrome constructs with N-terminal Strep-Tag followed by a Factor Xa cleavage site. The heme-binding site C1XXC2H is located close to the N- and C-termini in class I and class II c-type apocytochromes, respectively. The 6th axial ligand M in class I c-type apocytochromes is located...
Specificity of CcmI for different classes of apocytochromes

at the C-terminus, while in class II apocytochrome c’ heme has no 6th axial ligand. CcmI, formed of the CcmI-1 and CcmI-2 (with its TPR motifs) domains, has an N-terminal Flag-tag followed by a Factor Xa cleavage site. ApoCcmE (with its HXXXY heme binding motif) has an N-terminal His10-tag fused at its membrane anchor. C. Coomassie Blue stained SDS-PAGE containing 3 µg of Strep Tactin Sepharose purified Strep-apocytochrome c2 (lane 1), Strep-apocytochrome c’ (lane 2), Strep-apocytochrome c1t39 (lane 3), Cys-less derivative of Strep-apocytochrome c1t39* (lane 4) and Strep-apocytochrome c1 (lane 5); t39 refers to the truncation of 39 C-terminal amino acid residues encompassing the membrane anchor of cytochrome c1. D. Coomassie Blue stained SDS-PAGE containing 3 µg of Anti-Flag Sepharose purified CcmI (lane 1) and Ni-Sepharose purified His10-CcmI-2 (lane 2) and apoCcmE (lane 3).

Figure 3. CcmI recognizes differently class I and class II c-type apocytochromes. A. Co-purification of Flag-CcmI with different c-type apocytochromes. Flag-CcmI does not bind to the Strep Tactin resin (lane 1); Co-purification of CcmI with apocytochrome c2 (lane 2), apocytochrome c’ (lane 3), apocytochrome c1 (lane 4) and apocytochrome c1t39 (lane 5). The amounts of CcmI that co-purified with apocytochrome c2 was taken as 100% for semi-quantitative estimation using Image J software, and compared with those seen with other c-type apocytochromes. Although the samples were reduced with β-mercaptoethanol, homodimers of apocytochrome c1t39 (marked •, above CcmI) were observed when its heme-binding site Cys residues were intact. B. Co-purification of Flag-CcmI and its derivative His10-CcmI-2, with apocytochrome c1 and its truncated derivative apocytochrome c1t39. The amount of CcmI that co-purified with apocytochrome c1 (lane 3) was taken as 100% and those seen with the other c-type apocytochromes were determined as in Fig. 1. C. Co-purification of Flag-CcmI with different amounts of a peptide corresponding to the “C-terminal helix” (see the text) following the membrane anchor of apocytochrome c1. Flag-CcmI does not bind alone to the Strep Tactin resin (lane 1). CcmI co-purified with 10 (lane 2) or 20 (lane 3) µg of this cytochrome c1 peptide and compared with 20 µg of the corresponding peptide from cytochrome c2 (i.e., its C-terminal helix) as control. D. Real time protein ~ protein interactions between biotinylated Strep-apocytochrome c2 (Strep-Bt-apocytochrome c2) immobilized on a streptavidin SA biosensor (ligand) and His10-CcmI (analyte). Aligned sensorgram traces showing baseline (B) followed by association (A) and dissociation (D) steps obtained using 400 nM Bt-apocytochrome c2 and varying concentrations of Flag-CcmI. Raw data were fitted with high accuracy to a homogeneous 1:1 bimolecular interaction model and the kinetic parameters determined (Table 3).

Figure 4. ApoCcmE recognizes differently class I and class II c-type apocytochromes. A. Co-purification of His10-apoCcmE with stoichiometric amount of different c-type apocytochromes. The amounts of apoCcmE co-purified with apocytochrome c2 (lane 1) was taken as 100%, and compared with those seen with apocytochromes c’ and c1 (lanes 2 and 3, respectively). B. Co-purification of His10-apoCcmE with apocytochrome c1t39 and with its Cys-less derivative apocytochrome c1t39Cys/Ser*. The amounts of ApoCcmE co-purified with apocytochrome c1t39 (lane 1) and its Cys-less derivative (Cys/Ser*) were determined as in Fig. 1. C. Real time protein ~ protein interactions between Strep-Bt-apocytochrome c2 immobilized on a streptavidin SA biosensor (ligand) and His10-apoCcmE (analyte). Aligned sensorgram traces showing baseline (B) followed by association (A) and dissociation (D) steps obtained using 400 nM Bt-apocytochrome c2 and varying concentrations of apoCcmE. Raw data were fitted with high accuracy to a homogeneous 1:1 bimolecular interaction model and the kinetic parameters determined (Table 3).
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determined (Table 3).

Figure 5. CD spectra of various \(c\)-type apocytochromes and their interacting partners CcmI and CcmI-2 in the absence of hemin. A. Far-UV CD spectra between 195 and 240 nm of various \(c\)-type apocytochromes (15 \(\mu\)M) were recorded in the absence of hemin. The apocytochromes \(c_2\) and \(c'\) exhibited CD spectra typical of disordered coils while the apocytochrome \(c_1\) and its truncated derivative apocytochrome \(c_{1t39}\) showed characteristics of typical \(\alpha\)-helical proteins. B. Far-UV CD spectra between 195 and 240 nm of His\(_{10}\)-CcmI and His\(_{10}\)-CcmI-2 (1.5 \(\mu\)M) in the absence of hemin indicated their high \(\alpha\)-helical contents. Both the apocytochrome \(c_{1t39}\) and CcmI-2 are one helix shorter than apocytochrome \(c_1\) and CcmI, respectively, and the amplitudes of their ellipticity are comparatively lower.

Figure 6. Binding of hemin to apocytochrome \(c_2\). A. Real time interactions between Strep-Bt-apocytochrome \(c_2\) immobilized on a streptavidin SA biosensor (ligand) and hemin (analyte). Aligned sensorgram traces showing baseline (B) followed by association (A) and dissociation (D) steps obtained using 400 nM of Bt-apocytochrome \(c_2\) and varying concentrations (0 to 3.2 \(\mu\)M) of hemin. Raw data were fitted with high accuracy to a homogeneous 1:1 bimolecular interaction model, and the kinetic parameters determined (Table 3). B. Far-UV CD spectra between 195 and 240 nm of Strep-apocytochrome \(c_2\) (2.5 \(\mu\)M) recorded in the absence, or presence of 5 and 20 \(\mu\)M hemin. The arrows indicate the displacements of the spectra, reflecting increased \(\alpha\)-helical content of apocytochrome \(c_2\) in the presence of hemin. C. Far-UV CD spectra between 195 and 240 nm of Flag-CcmI (1.25 \(\mu\)M) in the absence or presence of 5 and 20 \(\mu\)M hemin. Note that hemin has no major effect on the \(\alpha\)-helical content of CcmI.

Figure 7. Effect of heme on CcmI \(\sim\) apocytochrome \(c_2\) interactions. A. Co-purification of Flag-CcmI with Strep-apocytochrome \(c_2\) in the absence (lane 1), or presence, of 1 (lane 2), 2 (lane 3), 3 (lane 4) and 4 (lane 5) \(\mu\)M hemin. The amounts of the interacting partners were as in Materials and Methods, and semi quantitative comparisons (as in Fig. 1) showed that CcmI \(\sim\) apocytochrome \(c_2\) interactions weakened, but not completely abolished, in the presence of hemin. B. Effect of heme on the CD spectra of CcmI \(\sim\) apocytochrome \(c_2\) mixture in the absence or presence of hemin (20 \(\mu\)M). Spectra were taken after 2 hours of incubation to ensure that all spectral changes were complete. “exp” and “sum” refer to the experimental and calculated spectra, respectively. “Sum” spectra were obtained by summing the spectra of each protein alone in the absence or presence of hemin (Fig. 5B and 5C). C. Difference spectra between the experimental (exp) spectra of CcmI \(\sim\) apocytochrome \(c_2\) minus the calculated (sum) spectra shown in panel B, in the absence or presence of hemin (20 \(\mu\)M). Decreased conformational changes were seen upon binding of apocytochrome \(c_2\) to CcmI in the presence of hemin, suggesting decreased binding interactions.

Figure 8. In vitro reconstitution of hemin to \(R.\) capsulatus \(c\)-type apocytochromes in the absence of Ccm-System I components. Reduced minus oxidized optical difference spectra between 380 and 650 nm of 6.5 \(\mu\)M \(b\)-type cytochrome derivatives formed after stoichiometric addition of hemin to 10 \(\mu\)M of \(H.\) thermophilus apocytochrome \(b-c_{552}\) (purple) was used as a “heme reconstitution” control. Similar reconstitution experiments were repeated using the same amounts of \(R.\) capsulatus apocytochrome \(c_2\) (black), apocytochrome \(c_1\) (blue) and apocytochrome \(c'\) (red) with stoichiometric amounts of hemin, and obtained spectra compared

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Specificity of CcmI for different classes of apocytochromes taking 100% that of *H. thermophiles* apocytochrome b-c₅₅₂. Inset spectra depict the β- and α-bands of the b-type cytochrome derivatives of *R. capsulatus* apocytochromes c₂, c₁t39 and c’.

**Figure 9. CcmI ~ apocytochrome c₂ interactions in the absence and presence of hemin during the Ccm process.** Panel A shows the Ccm machinery and its two substrates, the apocytochrome c₂ and heme, forming holocytochrome c₂. Panel B depicts the interactions between CcmI and apocytochrome c₂ in the absence and presence of hemin (thought to be provided by CcmE). This hypothetical scheme takes into account all available data to show that the CcmI-2 portion of CcmI binds tightly the “C-terminal helix” of apocytochrome c₂, bringing its heme-binding site near CcmE (left drawing); upon the availability of heme via CcmE, apocytochrome c₂ binds heme non covalently to form a b-type cytochrome intermediate that interacts less tightly with CcmI-2 (middle two drawings). Subsequent formation of covalent thioether bonds between heme and apocytochrome c₂, via currently unknown steps catalyzed by the remaining components of the Ccm-System I, yields holocytochrome c₂ (right drawing).
Table 1. Strains and plasmids used in this work.

| Strains/Plasmids | Relevant Characteristics | References |
|------------------|--------------------------|------------|
| **Bacteria**     |                          |            |
| *R. capsulatus*   | wild type *ctrD121*: Rif<sup>+</sup>; Res<sup>+</sup> Nadi<sup>+</sup> Ps<sup>+</sup> |            |
| MT1131           |                          |            |
| MT-G4/S4         | Δ(*cycA::kan*); Res<sup>+</sup> Nadi<sup>+</sup> Ps<sup>+</sup> m | [58]       |
| MTSRP1           | Δ(*ccmI::kan*); Res<sup>+</sup> Nadi<sup>-</sup> Ps<sup>-</sup> | [19]       |
| MD2              | Δ(*ccmE::spec*); Res<sup>+</sup> Nadi<sup>-</sup> Ps<sup>-</sup> | [44]       |
| **Escherichia coli** |                      |            |
| HB101            | F<sup>-</sup> Δ(*gpt-proA*)62 araC14 leuB6(Am) glnV44(AS) galK2(Oc) lacY1 Δ(*merC-mrr*) rplL20*(Str<sup>+</sup>) xylA5 mtl-1 thi-1 | Stratagene |
| **Plasmids**     |                          |            |
| pMAM2            | pCS1302 derivative, contains full length *R. capsulatus petC*<sup>*</sup>, cloned into the Ndel/BamHI sites without its signal sequence but with a Strep-tag II fused at its 5'<sup>′</sup> end, Amp<sup>+</sup>. | This work |
| pMAM1            | pCS1302 derivative, containing a truncated *R. capsulatus petC*<sup>*</sup> gene, cloned Ndel/BamHI, with a Strep-tag II fused at its 5'<sup>′</sup>-end, without a signal sequence and misses the last 39 C-terminal residues, Amp<sup>+</sup>. | This work |
| pMAM1ΔCys        | pMAM1 derivative with Cys34 and Cys37 mutated to Ser, Amp<sup>+</sup>. | This work |
| pAV6             | pCS1302 derivative, contains mature *R. capsulatus cycP*, cloned into the Ndel/BamHI sites without its native signal sequence, and with a Strep-tag II fused at its 5'<sup>′</sup> end, Amp<sup>+</sup>. |            |
| pAV5             | pCS1302 derivative, contains mature *H. thermophilus* cyt<sub>c<sub>552</sub></sub> gene from pCS1208, cloned into the Ndel/BamHI, without its signal sequence but with a Strep-tag II fused at its 5'<sup>′</sup> end, Amp<sup>+</sup>. | This work |
| pAV5C13SC16S     | pAV5 derivative with Cys13 and Cys16 mutated to Ser, Amp<sup>+</sup>. | This work |
| pCS1302          | pET-3a derivative with a Strep-tag II sequence fused to GFP, rendering GFP replaceable by cloning any gene of interest in-frame into Ndel and BamHI sites, Amp<sup>+</sup>. |            |
| pCS1208          | pBSK derivative, contains full length *H. thermophilus* cyt<sub>c<sub>552</sub></sub> gene carrying both 5’end Ndel and 3’end BamHI sites, cloned into the EcoRV site, Amp<sup>+</sup>. | This work |
| pPET1-C144A/ C167A/A181T | pPET1 derivative contains petABC operon (encoding *R. capsulatus* cyt<sub>bc<sub>1</sub></sub>) with petC<sup>*</sup> carrying the Cys144Ala, Cys167Ala and Ala181Thr mutations, Amp<sup>+</sup>. | (40)       |

Res and Ps refer to respiratory and photosynthetic growth, respectively. Nadi refers to cytochrome c oxidase-dependent catalysis of α-naphtol to indophenol blue. *R. capsulatus* MT1131 is referred to as wild-type with respect to its c-type cytochrome profile and growth.
Table 2. Nucleotide sequences of the oligonucleotide primers used in this study.

| Designation       | Constructs   | Nucleotide sequence (5’ to 3’)                                                                 |
|-------------------|--------------|-----------------------------------------------------------------------------------------------|
| NdeI_Cytc1-Fw     | pMAM1, pMAM2 | GCCTTTGCGAACCTCCCATATGCCGGATCACGCCTTCAGC                                                     |
| Cytc1t39 BamHI-Rv | PMAM1        | CCCATCTGCTTGCAGCGATCCAGTTACGGTTCCGGCGGCC                                                 |
| Cytc1 BamHI-Rv    | pMAM2        | CAGCTGTCCGGATCCTCTTTAGCGCTTTGCGCC                                                       |
| Cytc1t39 C34SC37S-Fw pMAM1Cys/Ser | CTACAAACGAAGTCAGTCAGCTCGGCCAGCCAGCCAGGCATGAG                                                  |
| Cytc1t39 C34SC37S-Rv |             | CTTCATGCGGTGGCTGGCGACCAGCTTTGCTTTGAG                                                       |
| NdeI-c’-Fw        | pAV6         | GGCTCGGCGCGCATATGGCTGATACC                                                                |
| c’-BamHI-Rv       | pAV5         | CCCCGCAGCCGGATCCTTACTTCTTCTTG                                                           |
| Htssdel-Fw        | pAV5         | GGCAATACATATGGCAATAGACAGCTTGCAAGAG                                                        |
| CS46              |              | GTAGTTATTCGCTACGCC                                                                   |
| Htc552C13/C16-Fw  | pAV5C13SC16S | GCTTGGCAAGCAAAAGGGCGCTATGGCTGGCCACGATCTGAAAGCTAAG                                       |
| Htc552C13/C16-Rv  |              | CTTAGCTTTCAGATCGTGGCAAGCCATAGCAGGCCTTTTGGGCAAGCC                                      |
Table 3. Kinetic analysis of protein: protein interactions between CcmI or apoCcmE and different c-type apocyts and in the absence or presence of heme.

| Protein:protein interactions | $k_{on}$ (1/Ms) ($\times 10^4$) | $k_{off}$ (1/s) ($\times 10^{-5}$) | $K_D$ (nM) | $X^2$ | $R^2$ | Assay Buffer |
|-----------------------------|-------------------------------|---------------------------------|------------|-------|-------|--------------|
| **Bimolecular model 1:1**   |                               |                                 |            |       |       |              |
| Apocyt c$_2$: CcmI          | 10.3 ± 3.30                   | 2.6 ± 0.15                      | 0.3 ± 0.08 | 0.30  | 1.00  | A            |
| Apocyt c$_{t39}$: CcmI      | 6.0 ± 1.40                    | 13.4 ± 1.4                      | 2.3 ± 0.3  | 0.12  | 0.99  | A            |
| Apocyt c$_1$: CcmI          | 7.1                           | 5.1                             | 0.7        | 0.23  | 1.00  | A            |
| Apocyt c': CcmI             | 3.1 ± 0.20                    | 118500±1500                     | 38200± 3100| 0.47  | 0.99  | A            |
| Apocyt c$_2$: apoCcmE       | 0.3                           | 65.6                            | 210        | 0.11  | 0.99  | A            |
| Apocyt c$_{t39}$: apoCcmE   | 0.2                           | 40.1                            | 221        | 0.15  | 0.99  | A            |
| Apocyt c’: apoCcmE          | 1.3                           | 868000                          | 67200      | 0.15  | 0.98  | A            |
| Apocyt c$_2$: heme          | 1.26 ± 0.05                   | 1325 ± 15                       | 1050 ± 100 | 0.41  | 0.97  | B            |
| Apocyt c$_2$: CcmI          | 12.0 ± 0.9                    | 14.0 ± 1.2                      | 1.14 ± 0.03| 0.46  | 0.99  | B            |
| **Heterogeneous model 2:1** |                               |                                 |            |       |       |              |
| $k_{on1}/ k_{on2}$          | 1.6 ± 0.02/ 0.2 ± 0.05        | 16.7 ± 2.5/ 21.0 ± 0.2          | 1.10       | 0.99  |       | B            |

A: Assay buffer: 50 mM Tris-HCl, pH 8, 100 mM NaCl, 0.01% DDM and 1% BSA.
B: Assay buffer: 50 mM Tris-HCl, pH 8, 150 mM NaCl and 0.01% Tween-20.
Specificity of CcmI for different classes of apocytochromes

Verissimo et al., Figure 1

A

B

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Specificity of CcmI for different classes of apocytochromes

Verissimo et al., Figure 2
Specificity of Ccml for different classes of apocytochromes

Verissimo et al., Figure 3

[Image: A, B, C, D]
Specificity of CcmI for different classes of apocytochromes

Verissimo et al., Figure 4

A

|       | 100 | ~1 | 60 |
|-------|-----|----|----|
| apocytc₁ |     |    |    |
| apoCcmE  |     |    |    |
| apocytc'  |     |    |    |
| apocytc₂  |     |    |    |

Strep Tactin resin

B

|       | 100 | 120 |
|-------|-----|-----|
| apocytc₁₁₃₉ |     |     |
| apoCcmE  |     |     |

Strep Tactin resin

C

Sensorgram

B

A

D

[apoCcmE] µM

20

10

5

2.5

1.25

0

200 300 400 500 600

Time (sec)

Binding (nm)
Specificity of CcmI for different classes of apocytochromes

Verissimo et al., Figure 5

![Diagram A: Graph showing CD data for different apocytochromes.](image)

![Diagram B: Graph showing CD data for CcmI and CcmI-2.](image)
Specificity of CcmI for different classes of apocytochromes

Verissimo et al., Figure 6

[Diagram A: Sensorgram showing binding over time with different heme concentrations.]

[Diagram B: Spectrophotometric analysis showing absorption at various wavelengths with different apocytochromes and heme concentrations.]

[Diagram C: Circular dichroism spectrum with different CcmI samples and heme concentrations.]
Specificity of CcmI for different classes of apocytochromes

Verissimo et al., Figure 8

[Graph showing absorption spectra for different classes of apocytochromes, with peaks at specific wavelengths.]
Specificity of CcmI for different classes of apocytochromes

Verissimo et al., Figure 9

A

Heme

+ apocyto c₂

B

Ccm machinery

CcmE captures apocyto c₂

CcmE is loaded with heme and delivers it apocyto c₂

apocyto c₂ binds heme non-covalently

formation of thioether bonds

Tight CcmI: apocyto c₂ complex

Loose CcmI: apocyto c₂ complex
During Cytochrome c Maturation CcmI chaperones Class I Apocytochromes until the Formation of their b-type Cytochrome Intermediates
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