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Review

Cytochrome *c* biogenesis System I: An intricate process catalyzed by a maturase supercomplex?☆

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ABSTRACT

Cytochromes *c* are ubiquitous heme proteins that are found in most living organisms and are essential for various energy production pathways as well as other cellular processes. Their biosynthesis relies on a complex post-translational process, called cytochrome *c* biogenesis, responsible for the formation of stereo-specific thioether bonds between the vinyl groups of heme *b* (protoporphyrin IX-Fe) and the thiol groups of apocytochromes *c* heme-binding site (C_1XXC_2H) cysteine residues. In some organisms this process involves up to nine (CcmABCDEFGHI) membrane proteins working together to achieve heme ligation, designated the Cytochrome *c* maturation (Ccm)-System I. Here, we review recent findings related to the Ccm-System I found in bacteria, archaea and plant mitochondria, with an emphasis on protein interactions between the Ccm components and their substrates (apocytochrome *c* and heme). We discuss the possibility that the Ccm proteins may form a multi subunit supercomplex (dubbed "Ccm machine"), and based on the currently available data, we present an updated version of a mechanistic model for Ccm. This article is part of a Special Issue entitled: 18th European Bioenergetic Conference.

1. Introduction

Cytochromes (cyts) are ubiquitous hemoproteins that are key components of energy transduction pathways, and essential for cellular processes spanning from chemical energy (ATP) production to programmed cell death induction (apoptosis) [1–3]. All cyts *c* invariably contain at least one protoporphyrin IX-Fe (heme *b*) cofactor, which is stereo-specifically ligated to the polypeptide by two (rarely one) thioether bonds. These bonds are formed between the vinyl-2 and vinyl-4 of heme tetrapyrrole ring and the thiol groups of Cys_1 and Cys_2 located at a conserved heme-binding motif (C_1XXC_2H) within the apocytochromes (apocyts) *c* [4]. The His residue of this motif together with another Met or His residue coordinate axially the heme-iron [5] (Fig. 1). Variations at the conserved C_1XXC_2H heme-binding motif occur in mitochondrial cyts *c* (AXXCH) and c_1 (FXXCH) of the phylum Euglenozoa that contain a single Cys at the heme-binding site [6], and in bacterial nitrite reductase NrfA (C_1XXC_2K) where His is replaced with a Lys residue [7]. In addition, the number and nature of the amino acid residues between the Cys_1 and Cys_2 residues of the heme-binding motif may also vary, with $C_1(X)_3C_2H$ and $C_{1(}X)_4C_2H$ found in tetraheme cyts c_3 [8,9] and $C_1(X)_{15}C_2H$ in the octaheme MccA [10].

Cyts *c* exhibit different three dimensional (3D) structures, redox properties, and functions (Fig. 1), and can be grouped into four classes based on their major characteristics [11]. Class I cyts *c* is the largest group that includes the small, soluble cyts *c* with a generally globular fold. They usually contain a single amino (N-) terminal heme-binding motif and a Met residue acting as sixth ligand located at their carboxyl (C-) termini (*e.g.* mitochondrial cyt *c* or *Rhodobacter capsulatus* cyt c_2). Class II cyts *c* includes the high spin cyt *c*′ with a C-terminally located heme-binding motif and a four helical bundle fold. Class III comprises the low redox potential (E_m) multi heme cyts *c* with bis-His coordination, and finally cyts *c* that contain additional non-heme cofactors (*e.g.*, flavins) are grouped in class IV (Fig. 1).

2. Diversity of cytochrome *c* biogenesis systems

Cytochrome *c* biogenesis is an intricate process present in virtually all organisms, and ensures the covalent ligation of heme to an apocyt *c* (Fig. 1). It relies on major cellular functions such as protein translocation followed by post-translational modification, extra-cytoplasmic protein folding and degradation, redox homeostasis, metal cofactor acquisition and insertion into target proteins. Several biogenesis machineries (*e.g.*, Systems I to IV) sharing common characteristics were identified [12–17]. First, all apocyts *c* are synthesized in the cytoplasm and translocated *via* the Sec pathway [18,19] across a lipid bilayer into a cellular compartment where they mature and function. This compartment is always on the positive (*p*) side of an energy transducing membrane (*e.g.*, bacterial periplasmic space) with the exception of the cyt b_6f complex cyt c_i (also called c_x or c_n), which is formed on the negative (*n*) side of the thylakoid membranes [17]. Second, biosynthesis and transport of

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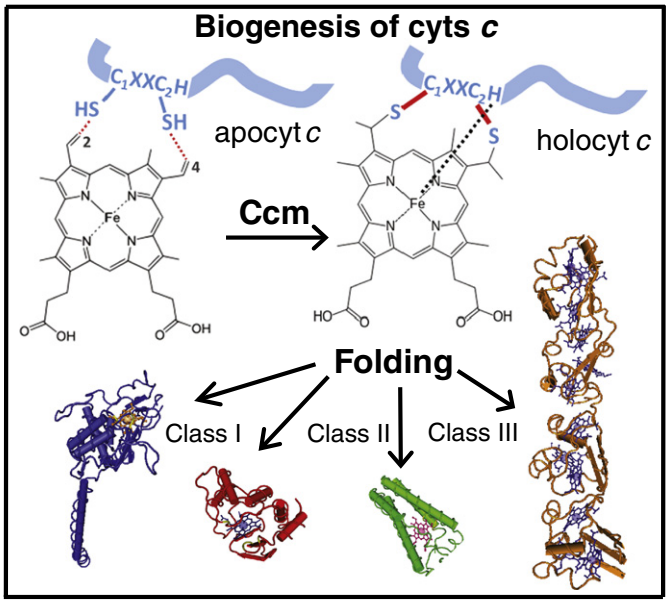


Fig. 1. Heme *b* is stereo-specifically ligated to apocyts *c*. Two thioether bonds are formed between the vinyl groups at positions 2 and 4 of the heme tetrapyrrole ring and Cys_1 and Cys_2 thiol groups at a conserved heme-binding motif (C_1XXC_2H) of apocyts *c*. The His residue of this motif together with another His or Met residues act as axial ligands of the heme-iron (Fe). Following the cyt *c* maturation (Ccm) process, holocyts *c* are folded into their native conformations and become functional. Cyts *c* perform key cellular functions and are very diverse in terms of their 3D structures, heme contents, redox properties, and can be grouped into different classes (*e.g.*, I, II, III).

heme and translocation of apocyts *c* occur per distinct and independent processes, which are coordinated spatially and temporally to minimize cytotoxic effects of heme and proteolytic degradation of apocyts *c* [20,21]. Third, both the heme-iron atom and the apocyt *c* heme-binding motif Cys thiol groups need to be reduced for thioether bond formation [12,22]. Fourth, dedicated chaperones and enzymes are required for ligation of heme to the apocyts *c* in a stereo-specific configuration. Finally, after their biogenesis mature cyts *c* are assembled into their respective cyt *c* complexes.

The most complex process, the Cytochrome *c* maturation (Ccm)-System I, is found in α- and γ-proteobacteria, in archaea, and in mitochondria of plants and red algae. Ccm-System I of *Escherichia coli*, *Rhodobacter capsulatus* and *Arabidopsis thaliana* are best studied, and involve up to nine specific membrane-bound proteins, described below. Ccm-System I is able to ligate heme *b* to a variety of substrates, including cyts *c* that are matured normally by other systems [23], polypeptides that contain multiple hemes [24], and short peptides (*e.g.*, ten amino acid residues long) [25]. Mutagenesis studies revealed that the presence of a C_1XXC_2H heme-binding site is required for Ccm-mediated efficient heme attachment [26–28].

Cytochrome *c* biogenesis-System II, also designated Ccs (Cyt *c* synthesis), is used by the β-, δ- and ε-proteobacteria, Gram-positive bacteria, Aquificales, cyanobacteria, and also algae and plant chloroplasts [15]. Representative examples are found in *Chlamydomonas reinhartii* [29] and *Bacillus subtilis* [30] and are composed of four (sometimes only three) membrane-bound proteins, CcsA (or ResC) and CcsB (or ResB) working together with CcdA and CcsX (ResA). In some organisms, such as *Wolinella succinogenes* CcsB and CcsA are fused into a single polypeptide [31]. CcdA and CcsX are required for thioreduction of apocyt *c* thiol groups, whereas CcsAB complex transports heme *b* to the *p* side of the membrane, recognizes the apocyt *c* substrates and forms the thioether bonds between the apocyt *c* and heme *b*. Substrate specificity of Ccs is not yet fully established, but it may be as broad as the Ccm-System I [32,33].

The simplest form of Cytochrome *c* biogenesis is confined to mitochondria of fungi, metazoans and some protozoa, and is designated System III or CCHL (Cyt *c* heme lyase). In fungi, there are two heme lyases specific for the cyts c_1 (CC_1HL) [34] and *c* (CCHL) [35] while in mammals only one heme lyase (HCCS, as in holocyt *c* synthetase) matures both these cyts [36,37]. Additionally, in yeast a third component (Cyc2p) with NAD(P)H-dependent heme reductase activity was identified [16,38]. CCHL is a heme containing membrane-associated protein that recognizes a consensus sequence ($K/A_5G_6XXL/I_9F_{10}XXXC_{14}XXC_{17-}H_{18}$) at the N-terminal portion of apocyts *c* substrates for heme *b* ligation [39–42].

Additional biogenesis processes dealing with unusual cyts *c* also exist, but these are less studied. System IV catalyzes heme attachment through a single thioether bond in the unique heme c_i (or c_x) from cyt b_6 of the cyt b_6f complex. It acts on the *n* side of the thylakoid membranes, and includes at least four membrane-bound proteins of unknown roles [17]. The occurrence of an additional biogenesis process (System V) is expected in mitochondria of euglenozoans as these organisms lack any of the above described proteins, despite the fact that both of their cyts *c* and c_1 are remarkably similar to other mitochondrial homologues, except that their heme-binding motif (A/FXXCH) contains only a single Cys residue [16].

The present review focuses on the Ccm-System I as found in α- and γ-proteobacteria and plant mitochondria, with an emphasis on *R. capsulatus*. We describe the recent findings related to the Ccm components that are grouped as functional modules. We also discuss an updated version of a working model for the mechanism of stereo-specific heme ligation and the possible occurrence of a multi subunit Ccm supercomplex (dubbed a "Ccm machine").

3. Ccm-System I: functional organization

Ccm-System I involves up to nine membrane-bound components (CcmABCDEFGHI) in *R. capsulatus* that can be grouped into three modules based on their functions and the interactions with their partners (Fig. 2 and Table 1). These three modules accomplish the (*i*) transport and relay of heme *b* (Module 1), (*ii*) preparation and chaperoning of ligation-competent apocyts *c* (Module 2), and (*iii*) ligation of heme-apocyt *c* (Module 3) to yield holocyts *c*.

3.1. Module 1. CcmABCD and CcmE: heme b translocation and relay to Module 3

CcmABCD are homologous to the ABC (ATP-binding cassette) transporters and are responsible for conveying heme *b* to the periplasm and loading it to the heme-chaperone CcmE. CcmA is a peripheral membrane protein with a typical ATP-binding region containing Walker A (GX_4GKS/T) and B ($R/KX_3GX_3LX_3D$) domains [43]. CcmB and CcmC are integral membrane proteins containing six transmembrane (TM) helices. In addition, CcmB has a conserved FX_2DX_2DGSL motif, and CcmC has a hydrophobic tryptophan rich (WWD) motif flanked by two conserved His residues exposed to the periplasm [44–46]. CcmC belongs to the heme handling protein (HHP) family [47] and is the only protein strictly necessary for loading heme *b* in to apoCcmE to synthesize holoCcmE [48]. Together with CcmC, CcmB is required for membrane localization of CcmA to form a putative ABC-type transporter complex with a stoichiometry of $CcmA_2BC$ [31,49–52]. CcmD is a small polypeptide (~60 amino acid residues) with a single TM helix and a hydrophilic C-terminal domain [53,54] that enhances CcmC activity for holoCcmE synthesis [54,55]. Hence, CcmC and CcmD are referred to as a CcmE-specific heme lyase [48,56]. CcmE is a member of the oligo-binding (OB) family of proteins [57], has a single TM helix, and a large periplasmic β barrel core domain ending with a flexible C-terminus [58–60] (Fig. 3A). It binds covalently and transiently one vinyl group (probably vinyl-2) of heme *b* through its conserved and surface-exposed His residue within the HXXXY domain [61–63]. Mutation of this His residue prevents holoCcmE formation, and blocks cyts *c* production [48,64]. In species where the His residue is

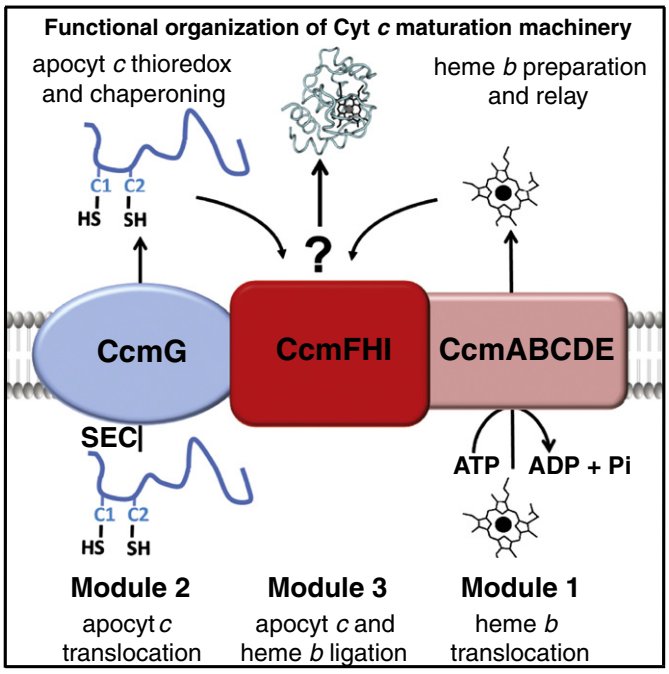


Fig. 2. Functional organization of Ccm. Three membrane-integral functional modules carry out the Ccm process in a coordinated manner. Module 1 (right) transports heme *b* across the membrane while Module 2 (left) translocates and chaperones ligation-competent apocyts *c*. Module 3 (center) traps the Ccm substrates heme *b* and apocyt *c* (provided by the Module 1 and Module 2, respectively) and catalyzes stereo-specifically the thioether bonds between the appropriate vinyl groups of heme and the thiol groups of the heme-binding motifs of apocyts *c*.

naturally replaced by a Cys [27] both the structure of apoCcmE and production of holoCcmE *via* CcmABCD are conserved [60,65] (Fig. 3A and B).

Mutants lacking CcmABCD lack cyts *c* but still produce periplasmic cyts *b* (*i.e.*, noncovalent heme *b* containing cyts) [66], indicating that these components are specific to the Ccm process. As CcmC alone is sufficient for holoCcmE formation [48], CcmAB were proposed [56,67] to transport an unknown molecule (*e.g.*, a reductant) different from heme *b* [55,68]. So far how heme *b* is translocated across the membrane and covalently ligated to CcmE remains unknown. CcmC binds heme *b* non-covalently *via* its two TM His residues [45,69] only in the presence of apoCcmE [46], and lacks any obvious heme delivery pathway unlike its HHP homologues CcmF and CcsAB of the Systems I and II, respectively. Once heme *b* reaches the periplasm by an unknown mechanism, it is thought to interact with the WWD domain of CcmC [68] and the hydrophobic heme-binding region of apoCcmE [63,64]. Heme is ligated covalently to the His residue of CcmE, and the two His residues of CcmC provide the heme-iron axial ligands to form a stable CcmCD–heme–CcmE complex containing oxidized heme-iron (Fe^{3+}) [45,46] (Fig. 3B). The catalytic mechanism behind this unique bond between the N-δ1 of His and the β-carbon of heme *b* vinyl group is not yet established [62]. Imidazole radical (from the His residue) formation with reduced heme-iron (Fe^{2+}), or Michael's addition with oxidized heme-iron (Fe^{3+}), (in both cases involving an unknown redox component) has been proposed [13].

How heme *b* is delivered from holoCcmE to apocyts *c* also remains unclear. In the absence of CcmAB mediated ATP hydrolysis, CcmCD still forms holoCcmE but no cyts *c* production occurs [48,56,65]. Current models suggest that ATP hydrolysis by CcmAB may be required for the "release" of holoCcmE from a stable complex that forms with CcmCD [55]. The His residues of CcmC that act as the heme-iron axial ligands are then replaced by a Tyr residue and another unidentified ligand located at the heme binding domain of CcmE [70] (Fig. 3B). Heme *b* oxidation by an unknown compound is followed by its covalent ligation to CcmE *via* CcmC with the assistance of CcmD. ATP hydrolysis by CcmAB seems needed to induce a "change" in the stable CcmCD–heme–CcmE complex to render holoCcmE proficient for the next steps of Ccm. Recently, a CcmE–heme–apocyt *c* mimic (*i.e.*, cyt *c*-b_{562}, an artificial four helical bundle apocyt *c* engineered by insertion of a heme-binding site in *E. coli* apocyt b_{562}) intermediate was isolated *in vivo*, with one of the heme *b* vinyl groups covalently linked to the His of CcmE and the other to a Cys residue of apocyt *c* derivative [71]. Absence of the conserved His residues of CcmC, or ATP hydrolysis by CcmAB, prevented the formation of this intermediate, which accumulated in the absence of CcmFHG [71]. Additionally, *in vitro* transfer of heme from holoCcmE to an apocyt *c* was previously reported [72], and our recent *in vitro* studies indicate that apoCcmE devoid of heme *b* also interacts with apocyt c_2 provided that it has an intact heme-binding site (preferably containing a disulfide bond) [73]. Clearly, these intermediates are likely to represent step(s) of the Ccm process, but their additional Ccm components and their role(s), if any, remains undefined. Recently, we found that apoCcmE forms a ternary complex with apocyt c_2 and CcmI [73], and using n-dodecyl-β-D-maltoside (DDM) dispersed membrane fractions, we showed that both CcmI and CcmH co-purify with apoCcmE. In addition, holoCcmE was also shown to form a CcmF–heme–CcmE complex in the absence of CcmGH [74].

In *A. thaliana*, CcmABCE proteins are the orthologues of the bacterial CcmABCE components, but CcmD is missing. Functional complementation of an *E. coli* mutant lacking CcmE with its *A. thaliana*

Table 1
Role of the different components of Ccm-System I.

Ccm protein	Structural features	Proposed function
CcmA	Peripheral membrane protein with ATP-binding domain Walker A (GX_4GKS/T) and Walker B motifs ($R/KX_3GX_3LX_3D$)	Part of the CcmABCD complex, a putative ABC-type transporter; required for ATP-dependent "release" of holoCcmE from the CcmCDE complex
CcmB	Six TM helices and a conserved FXXDXXDGSL motif	Part of the CcmABCD complex, a putative ABC-type transporter; required for the "release" of holoCcmE from the CcmCDE complex
CcmC	Six TM helices with a tryptophan-rich (WGXF/Y/WWXWDXRLT) motif and two conserved His residues	Part of the CcmABCD complex, a putative ABC-type transporter; loads heme into apoCcmE and provides axial ligands to the heme-iron; acts as a CcmE-specific heme lyase
CcmD	Small single TM helix protein with no conserved domains	Part of the CcmABCD complex, a putative ABC-type transporter; improves CcmC activity; required for the release of holoCcmE from the CcmCDE complex
CcmE	Single TM helix with a periplasmic domain containing a conserved HXXXY motif	Heme chaperone binding covalently vinyl-2 of heme *via* a conserved His residue; delivers heme to the apocyt *c* substrates
CcmF	Eleven TM helices with a tryptophan-rich (WGGXWFWDP-VEN) motif, and four conserved His residues	Part of the CcmFHI heme ligation complex; interacts with apocyts *c* and holoCcmE, and suggested to reduce heme in holoCcmE for its transfer to apocyts *c*
CcmG	Single TM helix with a periplasmic domain containing a thioredoxin motif (CXXC)	Binds poorly apocyts *c*; involved in thioreduction of apocyts *c* either directly, or indirectly *via* CcmH, or by resolving a CcmH–apocyt *c* mixed-disulfide
CcmH	Single TM helix with a periplasmic domain containing a thioredoxin-like motif (LRCXXC)	Part of the CcmFHI heme ligation complex; interacts with apocyts *c* and suggested to reduce its disulfide bond to enable stereo-specific heme ligation
CcmI	Two TM helices, linked by a cytoplasmic loop with a leucine-zipper-like motif, and a large periplasmic domain containing three TPR repeats	Part of the CcmFHI heme ligation complex; is an apocyt *c* chaperone binding to the C-terminal portion of the apocyt *c* *via* its periplasmic domain

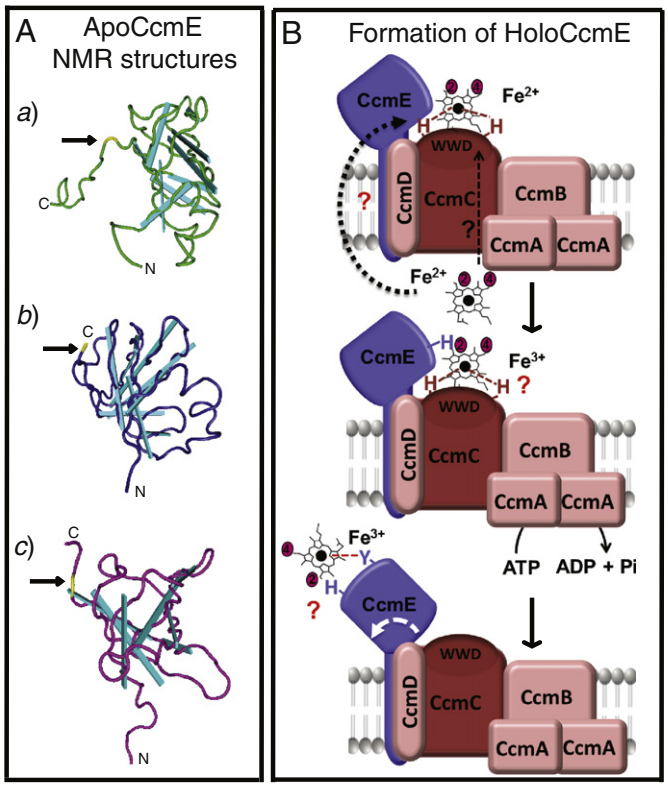


Fig. 3. Module 1 carries out heme *b* translocation and holoCcmE production. A. ApoCcmE structures. Solution NMR structures of the periplasmic C-terminal domains of apoCcmE from different organisms: *a*) *E. coli* (PDB ID: 1SR3) [59], *b*) *Shewanella putrefaciens* (PDB ID: 1J6Q) [58] and *c*) *Desulfovibrio vulgaris* (PDB ID: 2KCT) [60], showing their β-barrel (cyan) OB fold and the flexible C-terminal portions. The conserved His (*a*, *b*) and Cys (*c*) residues that bind covalently heme *b* are represented in yellow, and indicated with an arrow. B. HoloCcmE formation. Specific proteins of Module 1 are depicted as forming a membrane integral complex composed of the ATP-binding CcmA and its partners CcmB, CcmC, CcmD and the heme chaperone CcmE in a putative stoichiometry of $CcmA_2BCDE$. Heme *b* is transported across the membrane by an unknown mechanism and mediates the formation of a CcmCD–heme–CcmE complex. Heme *b* (possibly *via* its vinyl-2 group) becomes covalently bound to the His residue at the conserved heme binding HXXXY domain located at the surface of apoCcmE. Oxidation of heme-iron (Fe^{3+}) and ATP-hydrolysis are postulated to promote a conformational change (indicated by a white arrow), releasing holoCcmE from the stable CcmCD–heme–CcmE, possibly by exchanging the heme-iron axial ligands provided by CcmC with the CcmE Tyr residue. This event renders holoCcmE ready to proceed as a heme *b* donor to the apocyt *c* substrates. Dotted black arrow corresponds to a plausible CcmC-independent heme delivery route, and "?" point out unknown steps of the process.

counterpart, and related protein interaction studies, indicated that the mitochondrial CcmE binds heme *b* *via* a conserved His residue, and that CcmABC forms an ABC-type transporter with ATP hydrolysis activity [49,75,76].

Finally, why the species with a His to Cys variant of CcmE also lack CcmG and CcmH (see below) [77], how the reduced heme-iron (Fe^{2+}) is conveyed to CcmC subsequent to its synthesis by ferrochelatase, and whether ATP hydrolysis by CcmAB is required to transport an unknown compound, remains elusive.

3.2. Module 2. CcmG and CcmH: apocyt c thioredox and relay to Module 3

The thiol-oxidoreductase CcmG together with CcmH maintain the apocyts *c* ligation-competent (*i.e.,* in their reduced state). CcmH being a subunit of the heme ligation complex, CcmFHI, it is also a component of Module 3 (see below). Apocyts *c* with their signal sequences are usually translocated across the cytoplasmic membrane by the general secretory (Sec) pathway prior to their maturation and assembly into their respective complexes. Once on the *p*-side (periplasm in bacteria) the Cys residues of the heme-binding motifs are oxidized *via* the DsbA–DsbB oxidative protein-folding pathway [12,13,78,79]. DsbA is a strong periplasmic thiol-oxidoreductase that acts upon a wide variety of Cys containing substrates with its highly reactive thioredoxin-like (CXXC) domain [80]. Its partner DsbB is an integral membrane protein that contains two thioredoxin-like motifs, and reoxidizes DsbA by shuttling the reducing power to the membrane quinol pool, and eventually to the electron transport chain [81]. This periplasmic disulfide bond formation pathway is important for folding of extracytoplasmic proteins, to provide resistance to proteolysis and to induce conformational changes required for their functions and regulations [82] (Fig. 4A). In the case of the apocyts *c*, any intra-molecular disulfide bond formed at their heme-binding sites needs to be reduced for covalent heme ligation to occur. This reduction is attributed to CcmG, and probably involves CcmH. CcmG receives its reducing equivalents from the cytoplasmic thioredoxin TrxA across the membrane *via* CcdA [83–85]. *R. capsulatus* CcdA is an integral membrane protein with six TM helices containing two redox-active Cys residues located at its first and fourth TM helices [83,84,86,87]. *E. coli* DsbD, which is a homologue of CcdA, is a larger membrane protein containing three distinct domains, each with a pair of Cys [87,88]. CcdA and DsbD are dedicated thiol-oxidoreductases [87], and besides their involvement in cyt *c* biogenesis, they are also involved in virulence gene expression or sporulation in some organisms [89]. CcmG is anchored to the membrane through its unprocessed N-terminal signal sequence, has a *bona fide* thioredoxin fold with a CXXC motif, and is implicated in direct dithiol–disulfide oxidoreduction of apocyts *c* disulfide bonds. Its active site is unusually acidic and has a groove which, in resemblance with its System II homologue ResA [90], might be required for its interaction with CcdA or its substrate(s) [85,91]. Like CcdA, the Cys residues of CcmG are essential for Ccm [92–94]. Mixed disulfide bond formation *in vivo* was detected between CcmG and CcdA, proving their direct interactions during thioreduction [83,85] (Fig. 4A). Addition of exogenous thiol compounds is able to alleviate the Ccm deficiency in CcmG and CcdA mutants, confirming their roles in maintaining reduced the apocyt *c* heme-binding site thiol groups [84,93,95,96]. In the absence of DsbA–DsbB, production of cyts *c* is decreased but not completely abolished, and in some cases, it can be rescued by addition of oxidants [93,96–100]. We found that absence of DsbA or DsbB suppresses Ccm deficiency of *R. capsulatus* CcdA-null [96,98] or CcmG-null mutants [93], indicating that thioreduction *via* CcdA–CcmG is dispensable for Ccm in the absence of thio-oxidation by DsbA–DsbB [93]. Similar findings were also shown recently with *E. coli* [100], reinforcing the proposal that DsbA–DsbB and CcdA–CcmG form a thioredox loop (Fig. 4A). This situation resembles the plant mitochondrial case as in *A. thaliana* neither a DsbA–DsbB dependent oxidative pathway, nor a CcmG orthologue were identified. In addition to thioreduction, *R. capsulatus* CcmG also participates in chaperoning the apocyts *c* as its Cys-less mutant improves cyts *c* production in the absence of DsbA [93].

CcmH has a single TM helix and an unusual three helix-bundle fold distinct from other thioredoxin-like proteins, with a redox active LRCXXCQ motif facing the periplasm [101–103]. Plant CcmH resembles its bacterial counterpart with a membrane anchor and a CXXC motif containing N-terminal domain facing the inter-membrane space. It can interact and reduce an apocyt *c* mimic peptide in spite of its different active site (VRCTECG) [104]. *E. coli* CcmH is a fusion protein with an N-terminal domain corresponding to the redox-active CcmH and a C-terminal portion homologous to CcmI (see below) as found in other organisms, such as *R. capsulatus* and *Pseudomonas aeruginosa* [103,105]. Both Cys residues of *E. coli* CcmH are important for Ccm but not essential under all conditions [105,106]. No thioredox compensation occurs between *R. capsulatus* mutants lacking both DsbA and CcmH, suggesting that the role of CcmH is not restricted to apocyts *c* reduction [93].

Currently, the exact sequence of events that occur during the thioreduction of apocyts *c* is not clear, and different scenarios are plausible (Fig. 4A,B,C). Earlier studies using mutants *in vivo*, and purified

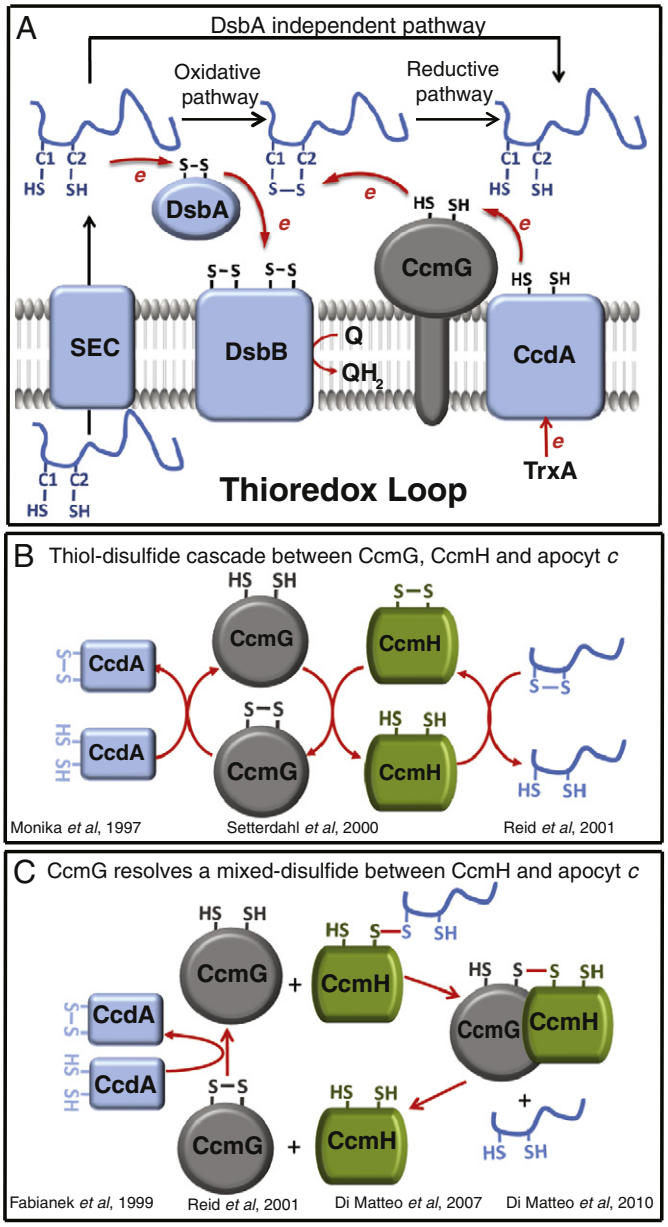


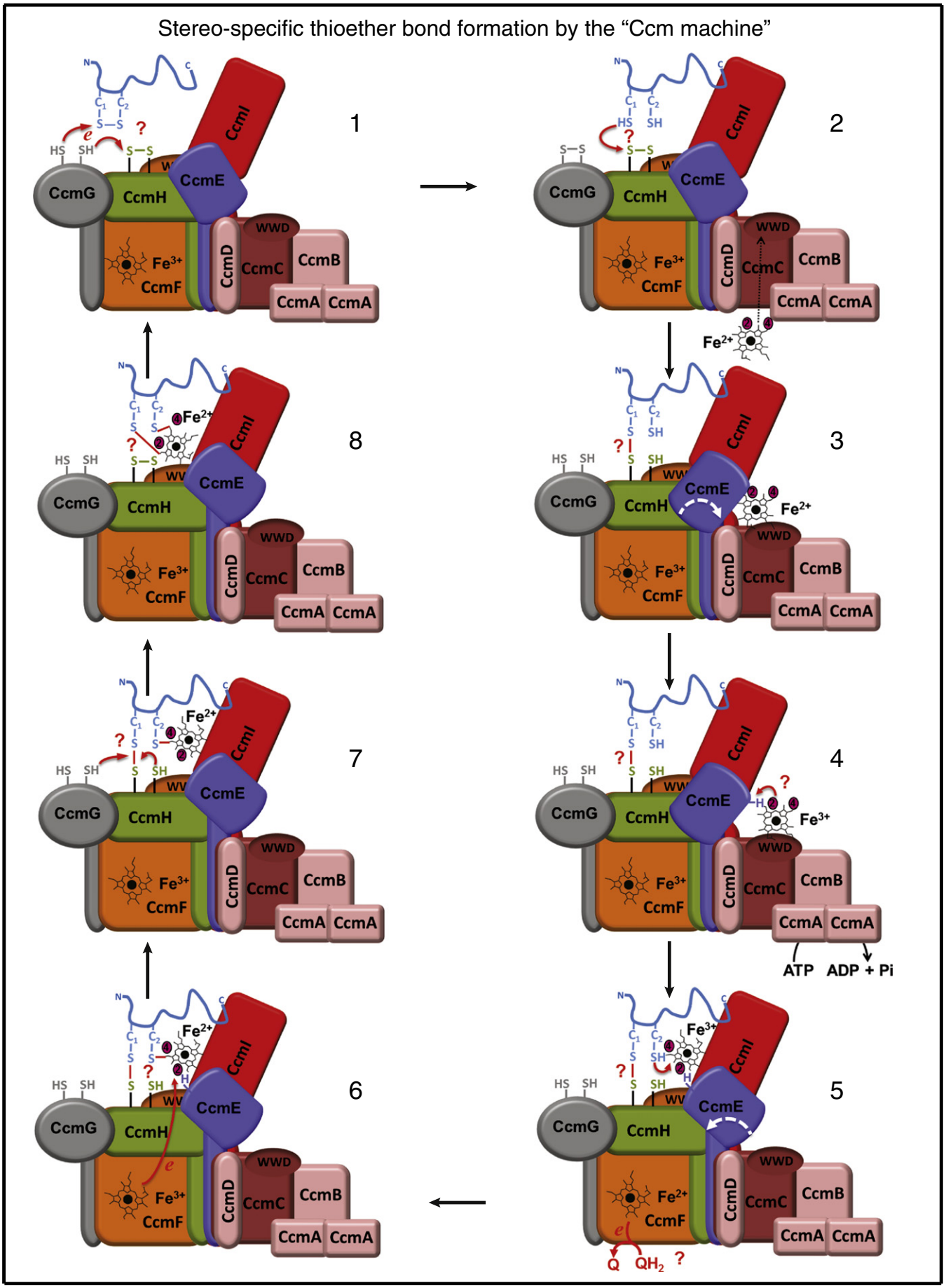
Fig. 4. Thioredox reactions between the CcdA, CcmG, CcmH and apocyts *c* during Ccm. A. DsbA–DsbB and CcdA–CcmG thioredox loop. Components of Module 2 are shown together with the general secretory (Sec) pathway responsible for translocation and cleavage of the signal sequences of apocyts *c*. DsbA and DsbB are thiol–disulfide oxidoreductases thought to oxidize the thiol groups of the heme-binding motif Cys residues (C1 and C2) of apocyts *c*. The disulfide bonds thus formed are then reduced by the CcdA and CcmG components of the Ccm process, forming a thioredox loop of unknown role. In the absence of the oxidative pathway (DsbA or DsbB), CcdA and CcmG are not required as the apocyts *c* stay reduced [93]. This possibility suggests that CcmG (recycled by CcdA) reduces directly the apocyts *c* disulfide bonds, as indicated by the thioredox compensation observed only between the DsbA–DsbB and CcdA–CcmG couples [93]. B. CcmG–CcmH–apocyts *c* thiol–disulfide cascade. The earlier proposed thioreduction pathway involves CcmG, CcmH and apocyt *c*, and is based on combined *in vivo* and *in vitro* studies including the E_m determination of purified CcmG ($E_m = -300$ mV), CcmH ($E_m = -210$ mV) and an 11 amino acid residues peptide mimicking apocyt *c* ($E_m = -170$ mV) [107]. This possibility suggests that CcmH (recycled by CcdA and CcmG) reduces directly the apocyt *c* disulfide bond substrate. C. A mixed-disulfide between CcmH and apocyt *c* resolved by CcmG. This possibility suggests that CcmG reduces a mixed-disulfide formed between CcmH and apocyt *c* (rather than oxidized CcmH or apocyt *c*). It is based on the finding that CcmG is able to reduce a CcmH-TNB mixed disulfide bond, but not an oxidized CcmH [110], and requires that either apocyt *c* or CcmH pairs need to be previously reduced (and the other oxidized) to form the mixed disulfide bond, but how this is accomplished and whether it involves CcmG is unclear.

R. capsulatus and *E. coli* samples *in vitro*, determined the E_m and pKa values of the redox active Cys residues of CcmG, CcmH and apocyts *c*. These studies proposed a linear dithiol–disulfide cascade, with CcmG reducing CcmH, and CcmH reducing the oxidized apocyts *c* substrates [92,103–105,107–109] (Fig. 4B). Alternative models were later proposed based on the different thioredoxin fold and redox properties of CcmH, and on the fact that *P. aeruginosa* CcmG cannot reduce *in vitro* the disulfide of CcmH although it does so a TNB-CcmH mixed disulfide [110]. These findings led to the suggestion that CcmH may form an inter-molecular mixed-disulfide bond with apocyts *c* that is resolved by CcmG, and oxidized CcmG is subsequently reduced by CcdA (or DsbD) [101,105,108,110] (Fig. 4C). In the absence of *in vivo* and *in vitro* evidences showing that CcmG reduces CcmH and/or apocyts *c*, and/or detection of mixed-disulfides between these proteins, the order of the thio-reductive events remains unclear. Establishing the exact sequence of the thioredox reactions between the apocyts *c*, CcmG and CcmH is crucial for understanding the mechanism of heme ligation, especially if CcmH plays a key role in ensuring the stereo-specificity of the thioether bonds [12].

3.3. Module 3. CcmFHI: apocyt c and heme ligation

Heme–apocyt *c* ligation *per se* is attributed to Module 3, which involves in *R. capsulatus* at least the CcmF, CcmH, and CcmI components that form a membrane-integral complex (CcmFHI) [111]. The *E. coli* CcmFH complex is a functional homologue of *R. capsulatus* CcmFHI [112], whereas the plant (*A. thaliana*) mitochondrial CcmF is split into three proteins ($CcmF_{N1}$, $CcmF_{N2}$ and $CcmF_C$). $CcmF_{N1}$ and $CcmF_{N2}$ interact with each other and with CcmH to form a large complex, but a CcmI orthologue seems to be absent [113,114]. CcmI is a bipartite membrane protein that acts as a chaperone for apocyts *c*. Its N-terminal CcmI-1 domain has two TM helices and a cytoplasmic loop with a leucine zipper-like motif, whereas its CcmI-2 domain is a large periplasmic C-terminal extension decorated with three tetratricopeptide repeats (TPR) [115–117]. TPR are ubiquitous protein–protein interaction domains composed of arrays of 34 hydrophobic residues forming two antiparallel α-helices [118]. Early genetic studies indicated that the two domains of CcmI have different functions [115,119–121]. In *R. capsulatus*, CcmI-1 is required for the production of all cyts *c* whereas CcmI-2 is dispensable for the C-terminally membrane-anchored cyt c_1 [115]. Furthermore, overproduction of CcmF and CcmH, or CcmI-1 overcomes partially, while additional overproduction of CcmG, CcmI-2 or apocyt c_2 overcomes fully, the CcmI defects. These findings led to the proposal that CcmI-1 together with CcmF and CcmH are responsible for heme ligation, whereas CcmI-2 and CcmG deliver ligation-competent apocyts *c* to CcmFHI for stereo-specific heme–apocyt *c* ligation [116,122,123]. CcmF is a large integral membrane protein that contains heme *b* [45]. It belongs to the HHP family, like CcmC, and has a periplasmic WWD motif [47]. Of its four conserved His residues, two are ligands of its heme *b* cofactor [124], whereas the remaining two coordinate heme *b* of holoCcmE to form a stable CcmF–heme–CcmE complex [45,74] at least in the absence of CcmGH. It appears that CcmF binds holoCcmE better than apoCcmE [74], and it can be reduced by quinol (QH_2) *in vitro*. CcmF is proposed to reduce the oxidized heme-iron (Fe^{3+}) in holoCcmE, which is a requisite for thioether bond formation. Topological models based on amino acid similarities with other proteins known to interact with quinones identified a putative quinone-binding site in a periplasmic loop of CcmF [45,74]. However, mutations of this quinol binding residues do not abolish cyt *c* production [65], leaving unknown how the oxidized heme-iron (Fe^{3+}) of holoCcmE is reduced.

Recently, we showed that CcmI binds directly to the C-terminal helix of class I apocyt c_2, but not holocyt c_2. This interaction is mainly *via* the large periplasmic domain of CcmI (CcmI-2), in agreement with earlier genetic data, which inferred that CcmI is an apocyt *c* chaperone [117]. Similar observations were also reported for *P. aeruginosa* CcmI and


Stereo-specific thioether bond formation by the "Ccm machine"
1
2
3
4
5
6
7
8
CcmI
CcmE
CcmG
CcmH
CcmF
CcmD
CcmC
CcmB
CcmA
WWD
Fe3+
Fe2+
ATP
ADP + Pi
Q
QH2

apocyt c_{551} [125], as well as the CcmI orthologue NrfG and the pentaheme apocyt *c* NrfA in *E. coli* [126]. So far only *A. thaliana* $CcmF_{N2}$ was reported to interact with both apocyts *c* and c_1 in a yeast two-hybrid assay [113]. Interestingly, using DDM-dispersed *R. capsulatus* membranes, we found that not only CcmI [117] but also CcmH and CcmF can co-purify with apocyt c_2 (Verissimo and Daldal, *unpublished*), in contrast with *E. coli* CcmF that seems unable to interact with apocyts *c* [112]. We observed that co-purification of CcmF with apocyt c_2 is impaired in the absence of its Cys residues or C-terminal helix, and enhanced by addition of purified CcmI (Verissimo and Daldal, *unpublished*). These findings suggest that CcmI-apocyt c_2 interactions might facilitate binding of apocyt c_2 to CcmF, probably indirectly. In addition, co-purification assays done *in vitro* using CcmFHG-enriched solubilized membrane fractions supplemented with purified CcmI revealed that CcmG also co-purifies with CcmI. Thus, *R. capsulatus* CcmFHI heme ligation complex might also contain CcmG (Verissimo and Daldal, *unpublished*). These findings are in agreement with earlier genetic data, which inferred that CcmG and CcmI-2 are functionally related [116], possibly chaperoning apocyts *c* [93]. Direct interactions between CcmG and the heme ligation component CcmH are readily conceivable as these proteins work together during thioreduction of apocyts *c*. Overall findings therefore suggest that *R. capsulatus* heme ligation complex CcmFHI also contains CcmE and CcmG.

4. Do the Ccm components form a membrane-integral supercomplex?

The *E. coli* Ccm components form a single operon, whose products were initially thought to co-localize in the inner membrane, forming a large "maturase" complex [127]. However, in other organisms, these components are dispersed into two (*e.g.*, *ccmABCDG* and *ccmIEFH* in *Bradyrhizobium japonicum* [128]) or more (*e.g.*, *ccmABCDG*, *ccmFH*, *ccmE* and *ccmI* in *R. capsulatus* [84,115,129]) genomic loci. In *A. thaliana*, *ccmBC*, $ccmF_{N1}$, $ccmF_{N2}$, and $ccmF_C$ genes are present in different loci on the mitochondrial genome [130], whereas *ccmA*, *ccmE* and *ccmH* are nuclear-encoded [49,76,104]. Regardless of the genomic distributions, various complexes containing Ccm components were reported. Different genetic strategies were employed to ease the accumulation and isolation of these complexes often from DDM solubilized membrane fractions. However, the nonionic detergent DDM, which is widely used for successful purification of membrane complexes, is known to disrupt membrane supercomplexes (*e.g.,* mitochondrial case) [131]. Large supercomplexes formed of many proteins weakly interacting with each other are difficult to isolate, and require specific lipid dispersion conditions with mild detergents (*e.g.*, digitonin or amphipoles) to keep them intact [131]. Whether the Ccm components form altogether a highly stable membrane supercomplex (dubbed a "Ccm machine") is unknown. Earlier observations in both bacteria and plant mitochondria suggested the occurrence of Ccm components in large membrane entities of unknown compositions. Co-localization of *R. capsulatus* heme ligation components CcmFHI in fractions corresponding to ~800 kDa molecular weight, separated by size exclusion chromatography was reported [111]. Large complexes containing CcmF and CcmH in *A. thaliana* (~500 kDa) [104], and CcmF in wheat (~700 kDa) mitochondria [132] were also described. Yet, earlier experiments indicated that apoCcmE co-immunoprecipitated with CcmF [112], and CcmC co-immunoprecipitated with apoCcmE and CcmD, but not with CcmF in the presence or absence of CcmE [133]. These data precluded the possibility of a CcmCD–CcmE–CcmF complex in *E. coli* membranes. However, the experiments used a complete *ccm*-deletion background, expressing only the tested Ccm components, and the reciprocal co-immunoprecipitation assays were inconclusive. We recently found that *R. capsulatus* apoCcmE interacts directly with CcmI and CcmH, but not with CcmF *in vitro* [73]. In agreement with these findings, an *E. coli* CcmF-heme-CcmE complex was recently isolated from a CcmHG-null background in the presence of heme. It was also shown that, unlike holoCcmE, apoCcmE bounds CcmF poorly [74]. Additional recent findings that documented specific interactions between the Ccm components of different functional modules (*e.g.*, apoCcmE binding CcmI) led us to consider the occurrence in *R. capsulatus* of a CcmABCD–CcmE–CcmFHI–CcmG supercomplex (a Ccm machine) that may catalyze the entire Ccm process. If such a machine exists, then the role of CcmE as a key heme-shuttle between the heme handling (CcmABCD) and the heme ligation (CcmFHI) complexes is particularly attractive as this would render unnecessary the earlier postulated diffusional movement of CcmE between the Ccm complexes. The presence of CcmD, a single transmembrane domain (STMD) topology subunit, known to be important for heme ligation, may mediate tight associations between CcmABCDE and CcmFHI. STMD topology subunits are usually involved in the assembly, stabilization and regulation of large membrane supercomplexes [134]. The occurrence of a large Ccm machine may facilitate concerted availability of the heme and apocyt *c* substrates to enhance the efficiency of maturation of *c*-type cyts. This issue might become even more important in the case of multiheme cyts, where several hemes have to be ligated properly. In this scenario, CcmI may keep the apocyts trapped in the Ccm machine while the remaining components act sequentially to ensure stereo-specific heme ligation and correct axial coordination of the heme iron.

Earlier we developed a hypothetical mechanistic model describing the Ccm process [12]. Here, we updated and further refined this working model in the light of the recent developments discussed above, and the salient aspects of this hypothetical Ccm machine are depicted in Fig. 5. Accordingly, in the absence of heme, apoCcmE is postulated to be associated more closely with CcmI–CcmH than CcmCD (step 1). Upon the availability of an apocyt *c*, CcmI (which is together with CcmFH) binds its C-terminus and apoCcmE recognizes its heme-binding site. Once apocyt *c* is trapped by CcmI(FH), a mixed disulfide bond is formed between its heme-binding Cys_1 and oxidized CcmH (step 2). The identity of the Cys residue that reacts with CcmH (*i.e.*, the availability of a free heme-binding Cys_2) is critical for stereo-specific heme ligation. Once heme *b* is available, a stable non-covalent apoCcmE-heme-CcmCD complex is formed, with CcmC providing a heme platform *via* its WWD domain and the heme-iron axial His ligands (step 3, white arrow) (see also Fig. 3B). Oxidation of heme-iron (Fe^{3+}) occurs together with covalent ligation of (probably) vinyl-2 of heme to the conserved His residue of apoCcmE to produce holoCcmE. Following the CcmAB mediated ATP hydrolysis, a conformational change occurs and CcmC releases holoCcmE (coordinating heme *b via* a Tyr residue at this stage, not shown) for interaction with the heme ligation components (step 4). Next, similar to CcmC, CcmF provides a heme scaffold with its WWD domain and conserved His residues, interacting through the heme with holoCcmE (step 5, white arrow). Upon reduction of the heme-iron (Fe^{2+}), (probably *via* the heme cofactor of CcmF) the heme *b* vinyl-4 that is available interacts with the reduced Cys_2 thiol of apocyt *c* to form the first thioether bond (step 6). Next, the CcdA-reduced

Fig. 5. A possible mechanism for stereo-specific thioether bond formation by the Ccm machine. This hypothetical model depicts all of the Ccm components as a multi subunit supercomplex, facilitating substrate accessibility (heme *b* and apocyt *c*) to the heme ligation site (1). Upon formation of the mixed disulfide bond between apocyt *c* Cys_1 and CcmH (one of them reduced previously by CcmG) (2), the thiol of Cys_2 is free to react with heme *b* to form the first thioether bond (3). Following heme *b* transfer across the membrane, holoCcmE produced by CcmCD carries heme *b* in a unique covalent bond between a His residue and heme vinyl-2 (4). HoloCcmE conveys heme *b* to CcmF upon ATP hydrolysis by CcmAB, and the free vinyl-4 of heme *b* ligates covalently to Cys_2 thiol of the heme-binding (C_1XXC_2H) motif of apocyt *c* site (5). CcmF coordinates axially heme *b* of holoCcmE (not shown), and probably reduces heme-iron *via* QH_2 oxidation, to free its vinyl-2 group (6). Upon resolution of the mixed disulfide bond between apocyt *c* and CcmH by CcmG (7), the free vinyl-2 of heme *b* reacts with apocyt *c* Cys_1 to form the second thioether bond (8) and completes the cycle. Heme *b* incorporated into the apocyts *c* drives its folding into a mature and active holocyt *c* (not shown). See the text for a detailed description of this hypothetical model.

CcmG (or possibly the free Cys of CcmH) resolves the mixed disulfide between CcmH and apocyt *c* Cys_1, rendering it ready to interact with heme *b* (step 7). Upon cleavage of the covalently attached heme from the His of CcmE by an unknown mechanism, the second thioether bond between vinyl-2 and apocyt Cys_1 is formed (step 8). Clearly, many of these steps and their chronological orders are hypothetical, and many alternative possibilities are plausible at present. Yet, we note that the recent *in vivo* trapped CcmCD–heme–CcmE [45], CcmE–heme–apocyt *c* [71] and CcmF–heme–CcmE [74] intermediates, as well as the *in vitro* observed CcmI–apocyt c_2–apoCcmE [73], CcmHI–apoCcmE [73] complexes are consistent with this working model. It is hoped that elucidation of the nature and sequence of the thioredox events involving CcmG, CcmH and apocyts *c* will further define the mechanism of function of the "Ccm machine".

In summary, this review describes the components and the known intermediates of Ccm-System I, and explores the occurrence of a structural multi subunit supercomplex (Ccm machine) formed of functional modules. The mechanism behind the unusual covalent bond formed between heme *b* and CcmE-His residue, the interactions between various Ccm components, their structures, the chronological sequence of the different intermediates and their interactions with the apocyts *c* and heme substrates remain unclear. Again, the order of events that occur during the thioredox reactions leading to the universally conserved stereo-specific heme ligation, and the occurrence and composition of a Ccm supercomplex are among the many issues of critical importance that await answers in future studies for a complete understanding of Ccm.

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