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Cytochrome *c* biogenesis: the Ccm system

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Abstract

Cytochromes of *c*-type contain covalently attached hemes that are formed via thioether bonds between the vinyls of heme *b* and cysteines of the C₁XXC₂H motifs of apocytochromes. In various organisms, this post-translational modification relies on membrane-associated specific biogenesis proteins, referred to as cytochrome *c* maturation systems. A highly complex version of these systems, Ccm or System I, is found in Gram-negative bacteria, archaea and plant mitochondria. Here, we describe emerging functional interactions between the Ccm components categorized into three conserved modules, and present a mechanistic view of the molecular basis of ubiquitous vinyl-2~Cys₁ and vinyl-4~Cys₂ heme *b*-apocytochrome *c* thioether bonds in *c*-type cytochromes.

Keywords

cytochrome *c*; heme; cytochrome *c* maturation or biogenesis; Ccm system; Gram-negative bacteria

Diversity of cytochrome *c* maturation processes

Cytochromes (cyts) *c* are hemoproteins with covalently attached iron-protoporphyrin IX (or heme *b*) groups (see Glossary). Primarily, they are electron carriers between enzymes involved in cellular energy transduction processes such as photosynthesis or respiration [1,2]. They also act as enzymes for biosynthesis of cofactors (*e.g.* tryptophan tryptophylquinones) [3] and lipidic signaling molecules (*e.g.* long chain fatty acid-glycines) [4]. Moreover, cyts *c* bind gases such as carbon monoxide, nitric oxide or oxygen and play roles in the oxidation of hydroxylamines and thiosulfates, as well as in other cellular processes [5]. In some eukaryotes, they contribute to signaling events that lead to apoptosis (programmed cell death) [6].

Despite diverse functions, different three-dimensional structures, and heme *b* contents, all *c*-type cyts invariably contain at least one heme *b* cofactor that is attached to the polypeptide by one or two thioether bonds (Figure 1). These bonds are always formed between the vinyls at positions 2 and 4 of the tetrapyrrole ring of heme *b* and the thiols of the amino (N)- and carboxyl (C)-terminal cysteines (Cys₁ and Cys₂), respectively, of a heme binding motif (C₁XXC₂H) within apocyts *c* [7,8]. The number of residues between the cysteines of this

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motif sometimes varies [9], but commonly, the histidine residue together with another amino acid (e.g. methionine or histidine) serve as axial ligands to hexa-coordinate the heme-iron atom (Figure 1). Remarkably, the orientation of the heme *b* group relative to the cyt *c* peptide (i.e. its stereo-specificity) is universally conserved [5].

In prokaryotic or archaeal cells and in eukaryotic organelles, the pathways leading to covalent ligation of heme *b* to an apocyt *c* are complex, but they share common characteristics [7,10]. First, apocyts *c* are synthesized and translocated across a lipid bilayer into a cellular compartment where they mature and function. This compartment is always on the positive (or *p*) side of an energy-transducing membrane, and corresponds to the bacterial extracytoplasmic space, plastid thylakoid lumen, or mitochondrial inter membrane space. Second, biosynthesis and transport of heme *b* and translocation of apocyts *c* occur via distinct and independent mechanisms. Third, thioether bond formation needs the iron atom of heme *b* and the thiols of the apocyt *c* heme binding motif to be available in their reduced states [10,11]. Fourth, heme *b* is always attached to apocyts *c* in a specific orientation via an unknown mechanism, and specialized proteins acting as chaperones and catalysts are always required for *c*-type cyt production. This process can be subdivided into three highly conserved modules [12]. Two of these modules prepare and deliver the substrates (i.e. heme and apoprotein) to a third module that catalyzes the heme *b*-apocyt *c* ligation *per se*, to yield active cyts *c* (Figure 2). Indeed, protein composition of the modules vary broadly among organisms [7,13], but the modular organization of cyt *c* biogenesis appears omnipresent, possibly accommodating common biochemical requirements under varying cellular environments [14].

This review deals exclusively with the cyt *c* maturation pathway found in α - and γ -proteobacteria, *Deinococcus* species, mitochondria of plants and red algae, and archaea called Ccm system (or System I) [7]. Recently, Kranz *et al.* have published a comprehensive review of cyt *c* biogenesis, with emphasis on topologies of Ccm components, heme trafficking and heme-iron redox processes [15]. Here, we categorize the various Ccm components into three common modules, and describe their emerging functional interactions with each other. We also present a mechanistic view for the occurrence of universally conserved stereo-specific vinyl-2~Cys₁ and vinyl-4~Cys₂ heme *b*-apocyt *c* ligations seen in cyts *c*, and underline outstanding questions critical for understanding this important biological process.

Other types of closely related cyt *c* biogenesis processes, such as those referred to as the Ccs system (or System II) seen in some Gram-positive bacteria, cyanobacteria, chloroplasts of plants and algae, and some β -, δ - and ϵ -proteobacteria [16,17], or the CCHL system (or System III) confined to mitochondria of fungi, metazoans and some protozoa [14,18] are not discussed here. Equally, the process of covalent heme attachment to cyt *b* subunit of the cyt *b₆f* complexes (i.e. CCB components) in cyanobacteria and chloroplasts of photosynthetic eukaryotes [19], or additional systems proposed to occur in some Gram-positive bacteria [13,20], or in mitochondria of *Euglenozoa* (e.g. *Trypanosoma* and *Leishmania*) [21] are not discussed here. Excellent reviews concerning these similar cyt *c* biogenesis processes are available [22,23].

Modular organization of the Ccm system

The Ccm system consists of up to ten (CcmABCDEFGHI and CcdA or DsbD) membrane-bound components [10,15] (Table 1). The three operational modules of this system accomplish (i) the transport and relay of heme *b* (Module 1), (ii) preparation and chaperoning of ligation-competent apocyts *c* (Module 2), and (iii) heme *b*-apocyt *c* ligation

per se (Module 3) to form holocyts *c* (Figure 2). The three modules are described below with examples from mainly Gram-negative bacteria.

Module 1: heme translocation and relay processes

Module 1 consists of five Ccm components (CcmABCDE), of which four (CcmABCD) are involved in conveying heme *b* to CcmE to yield a heme-loaded CcmE (holoCcmE) (Figure 3). CcmE is a monotopic membrane protein with a periplasmic heme binding domain, and functions as a transient heme chaperone to supply heme *b* for cyt *c* production [24]. Its periplasmic domain has a compact β -barrel core structure with a flexible C-terminal extension [25,26]. A surface exposed histidine residue (His130 in *Escherichia coli* CcmE) is located in this region within a conserved HXXXY motif [27]. This residue is covalently linked to vinyl-2 of heme *b*, which apparently is oxidized (Figure 4) [15]. Mutational analyses indicated that this histidine residue is critical for the function of CcmE [28,29], although it is not conserved and in most archaeal as well as some bacterial species it is substituted with a cysteine [30]. Remarkably, species harboring a histidine to cysteine variant of CcmE lack the CcmH component of Module 3 (described below). CcmC is a member of the putative heme handling protein (HHP) family [31] with six transmembrane helices, a tryptophan-rich (WWD) motif and two conserved periplasmic histidine residues that are essential for loading heme to CcmE to yield holoCcmE [32]. CcmD is a poorly conserved, small (about 60 amino acids long) monotopic membrane protein that enhances the activity of CcmC [33], and is implicated in the release of holoCcmE from a putative CcmABCD complex [34]. CcmA is a peripheral membrane protein with a nucleotide-binding domain, conserved Walker A and B motifs, and ABC (ATP-binding cassette) transporter signature sequences [35]. In plant mitochondria, the homologue of CcmA was found in a high molecular weight membrane protein complex of unknown composition [37]. CcmB is an integral membrane protein with six transmembrane helices, and together with CcmC, is required for membrane localization of CcmA. Membrane topologies of CcmABCD components are described in detail in Kranz *et al.* [15]. Immunobiochemical data indicate that CcmA, CcmB, CcmC and CcmD interact with each other [36], suggesting that these components form an ABC-type transporter complex under some conditions.

How heme *b* is translocated across membranes, how it is loaded onto CcmE, and what is the role of CcmABCD in this step remains unclear. Mutants lacking the entire set of Ccm components can produce periplasmic *b*-type cyts that have non-covalently bound heme *b* [38], indicating that Ccm-independent heme *b* transport across the cytoplasmic membrane also occurs. Thus, if CcmABCD are involved in heme *b* translocation, as initially proposed [36], then they must be specific to the *c*-type cyts only. In the absence of CcmA and CcmB, CcmC can load heme *b* to CcmE, but transfer of heme *b* from holoCcmE to apocyt *c* is blocked, suggesting that CcmC is a CcmE-specific heme lyase [32,35]. Moreover, recent work indicates that ATP hydrolysis by CcmA is required for the release of holoCcmE from a CcmABCD-containing complex to enable holoCcmE to supply heme *b* for cyts *c* production [34]. However, whether this ATP hydrolysis could also be associated with an ABC-type transporter activity of CcmAB for an unknown compound required for cyt *c* maturation [35] remains undetermined.

Module 2: apocyt *c* thioredox and chaperoning processes

Upon Sec-dependent protein translocation of apocyts *c* across the membrane, the cysteines of the heme binding motifs are thought to be oxidized by the extracytoplasmic DsbA-DsbB oxidative protein-folding pathway to form intramolecular disulfide bonds [10,15], possibly to render the apocyts *c* less degradation-prone (Figure 5) [39]. The *in vivo* occurrence of these disulfide bonds has not been proven experimentally, but if formed, they require reduction prior to covalent heme *b* addition [40]. Among the Ccm components, CcdA (or

DsbD in some species) [41], CcmG and CcmH [42,43] contain thioredoxin-like motifs (CXXC) involved in dithiol-disulfide oxidoreduction reactions to reduce such disulfide bridges. CcdA is a homolog of the central part of *E. coli* DsbD, which has additional periplasmic thioredoxin-like domains at its N- and C-terminal ends [44]. CcmG has a N-terminal unprocessed signal peptide that attaches it to the membrane, and a thioredoxin domain that faces the *p*-side of the membrane. In some archaea, the homolog of CcmG is C-terminally fused to CcdA [45]. Both CcdA and the central part of DsbD have six transmembrane helices, of which the first and fourth ones contain two invariant cysteines. These amino acids are required for transfer of reducing equivalents across the membrane from the cytoplasmic thioredoxin TrxA to the periplasmic CcmG [29,44]. Similarly, the cysteines of CcmG and the apocyt *c* heme binding motifs are required for efficient *c*-type cyt production [42,46]. Mixed disulfide bond formation *in vivo* has been detected between CcmG and DsbD in *E. coli*, and between CcmG and CcdA in *Rhodobacter capsulatus*, indicating that CcmG and CcdA/DsbD interact directly [44,47]. Current models support step-wise, mixed disulfide bond formation between TrxA and DsbD, and subsequently between DsbD and CcmG on the *n*- and *p*- sides of the membrane, respectively [48]. Remarkably, DsbA-null mutants suppress the *c*-type cyt deficiency of CcdA-null [49] or CcmG-null mutants [46], indicating that in the absence of DsbA, both CcdA and CcmG are dispensable for cyt *c* production *per se*. Such compensatory interactions render these reactions unnecessary in the absence of the thio-oxidation reactions, as presumably apocyts *c* remain reduced and competent for heme *b* ligation. Clearly, Module 2 includes DsbA-DsbB and CcdA-CcmG pairs forming a thio-redox loop in bacteria (Figure 5), but CcdA and CcmG are apparently absent in plant mitochondria [14,18].

Earlier experiments conducted *in vitro* with purified soluble variants of Ccm components indicated that reduced CcmG can reduce oxidized CcmH, while an oxidized synthetic apocyt *c* fragment with a heme binding motif can oxidize a reduced CcmH in bacteria [42] or plant mitochondria [50]. These findings were interpreted as indicating that electrons are shuttled from CcmG to CcmH, and then to the apocyts *c* to reduce the disulfide bonds at their heme binding motifs [41-43,51]. However, recent observations questioned the role of CcmH in these reactions. First, no mixed disulfide bonds between CcmG and CcmH or CcmH and apocyt *c* have so far been observed *in vivo*. Second, unlike the CcdA-null or CcmG-null mutants, no thio-redox compensation has been evidenced between the DsbA-null and CcmH-null mutants [46]. Third, the recent structure of CcmH indicates that, although the bulk of the protein faces the *p*-side of the membrane with its C-terminal helix acting as a membrane anchor, it does not have a canonical thioredoxin fold despite its conserved LRCXXCQ motif [52]. These findings suggest that the cysteines of CcmH, which are required for cyt *c* production [41,46] might not convey reducing equivalents directly from CcmG to apocyts *c* to reduce the disulfide bond at the heme binding motifs. Could oxidized apocyt *c* instead of CcmH be the direct partner of CcmG *in vivo*, and subsequently, could reduced apocyt *c* reduce an oxidized CcmH (Figure 6)?

Biochemical and structural studies of the CcmG homolog CcsX/ResA (a System II component) indicated that it forms a cavity close to its active site upon its reduction. This cavity was proposed to act as a clamp for the histidine residue of the apocyt *c* heme binding motif to provide a mechanism for recognition, binding and reduction of apocyt *c* by CcsX/ResA [53,54]. Moreover, a cysteine-less (*i.e.* thioreduction-defective) derivative of CcmG significantly increases holocyt *c* production *in vivo* when expressed in *R. capsulatus* DsbA-null and CcmG-null double mutants [46]. Thus, CcmG might act as both a thioredoxin to reduce the disulfide bonds at the heme binding motifs and also as a chaperone (“holdase”) to convey reduced apocyts *c* to the heme ligation sites (Figures 5 and 6).

Module 3: apocytochrome c and heme b ligation processes

In the Ccm system the process of heme *b*-apocytochrome *c* ligation *per se* has been attributed specifically to CcmF and CcmH in *E. coli* [55], CcmF, CcmH and CcmI in *R. capsulatus* [56], and CcmF_{N1}, CcmF_{N2}, CcmF_C and CcmH [57] components in *Arabidopsis thaliana* mitochondria, and these might form multi-subunit complexes (Figure 2). Note that the *E. coli* CcmH [41] is a fusion protein between CcmH and the C-terminal portion of CcmI found in other organisms [58]. Moreover, in plant mitochondria, the bacterial CcmF homolog is split into multiple proteins (*e.g.* into CcmF_{N1}, CcmF_{N2} and CcmF_C in *A. thaliana*) while a CcmI homolog is apparently absent [18]. Thus, the numbers of subunits that form Module 3 differ among species. CcmI is a bipartite membrane protein thought to be involved in periplasmic apocytochrome *c* chaperoning (Figure 6) [59]. It contains two N-terminal transmembrane helices encompassing a leucine zipper-like motif in its cytoplasmic loop (CcmI-1), and a large periplasmic C-terminal extension (CcmI-2) decorated with tetratricopeptide repeat (TPR)-motifs to facilitate protein-protein interactions [58]. CcmI-1 is required for the biogenesis of all *c*-type cyst, while CcmI-2 is dispensable for the C-terminally membrane-anchored cyt *c*₁ [59]. Recently, the C-terminal portion of the pentaheme cyt *c* NrfA was shown to be associated with the CcmI orthologue NrfG via a TPR domain [60]. Whether this is also the case for the TPR motifs of CcmI-2, possibly interacting with apocytochrome *c* remains to be seen. Remarkably, CcmI-null mutants are suppressed by overproduction of CcmF, CcmH and CcmG via distinct, synergistic events [61]. In *R. capsulatus*, overproduction of CcmF and CcmH, or of CcmI-1, partially overcomes the CcmI defect, while additional overproduction of CcmG, CcmI-2 or apocytochrome *c*₂ overcomes it fully [58,61,62]. Thus, CcmI could act as a bridging component between Module 2 and Module 3, which is also implied by the fusion structure of *E. coli* CcmH. In this scenario, the CcmI-1 domain could perform the heme ligation function of the Ccm system (with CcmF and CcmH) and the CcmI-2 domain the apocytochrome *c* thio-redox (with CcmG) function of the Ccm system [10].

CcmF is a large integral membrane protein, and like CcmC (Module 1), it belongs to the putative heme handling protein (HHP) family (Figure 6) [31]. Kranz *et al.* discussed in detail its topology [15], periplasmic tryptophan-rich (WWD) signature motif, and essential histidine residues for its heme *b* cofactor [55]. Note that *E. coli* CcmF co-immunoprecipitates with holoCcmE or CcmH (both *E. coli* and *A. thaliana* homologs) but not with apocytochrome *c*, and no interaction has been seen between holoCcmE and CcmH [50,55]. Similarly, CcmF, CcmH and CcmI, but not CcmG could be co-purified from solubilized *R. capsulatus* membranes, where they might be part of a high molecular weight complex (~800 kDa) [58]. Equally, the WWD domain-containing plant mitochondrial counterpart of CcmF, CcmF_{N2}, interacts in a yeast two-hybrid approach with CcmF_{N1}, CcmF_C, CcmH, and also with apocytochromes *c* and *c*₁, while CcmH interacts with CcmF_{N1} and apocytochrome *c* in *A. thaliana* [50,57]. In wheat (*Triticum aestivum*) mitochondria CcmF_C has been found in a ~700 kDa complex [63], whereas in *A. thaliana* mitochondria, CcmF_{N1}, CcmF_{N2}, CcmF_C and CcmH have been identified in a ~500 kDa complex [50,57]. Currently, the exact composition of these large molecular weight complexes are undefined, but their co-occurrence with holoCcmE, CcmF, CcmG, CcmH, CcmI and apocytochrome *c* supports the idea that they might be involved in the catalysis of the terminal steps of heme *b*-apocytochrome *c* ligation.

A mechanistic view for heme-apocytochrome c ligation

In all cytochromes *c*, the vinyl-2 and -4 are always attached specifically to the N- and C-terminally located Cys₁ and Cys₂ of apocytochrome *c* heme binding motifs. The mechanism underlying this ubiquitous specificity of heme *b*-apocytochrome *c* ligation is unknown. The possibility that CcmH might not be the direct target of CcmG *in vivo* suggested to us that, together with holoCcmE, CcmH and its essential Cys residues might ensure this specificity (Figure 6) [64]. Considered that Module 1 attaches heme *b* via its vinyl-2 group to holoCcmE (Figure 4),

only the vinyl-4 group is available to form the first thioether bond with a free cysteine at the apocytochrome *c* heme-binding motif (Figure 6a). Thus, we surmise that the oxidized CcmH acts as a specific dithiol-disulfide oxidoreductase for reduced apocytochrome *c* (which might be either free or held by CcmG), trapping the Cys₁ of the C₁XXC₂H motif in an intermolecular disulfide bond (Figure 6b). If so, then the stereospecifically correct thio-ether bond between the vinyl-4 of heme *b* and the C-terminal Cys₂ of apocytochrome *c* becomes obligatory, and could occur subsequent to heme *b* reduction via CcmF (Figure 6c-f) [65] ensuring the stereospecifically correct thioether bonds formation. The sequence of these events is further described in the legend of Figure 6. Although this hypothetical model is far from being established, the atypical thioredoxin structure of the *Pseudomonas aeruginosa* CcmH periplasmic domain with its disulfide bond as described earlier [52] renders this idea worthy of experimental scrutiny.

Conclusions and future perspectives

In recent years, our understanding of how cells carry out the seemingly simple task of forming thioether bonds between heme *b* and apocytochrome *c* to yield cytochrome *c* has progressed enormously. This process looks unnecessarily convoluted at first sight, but a closer inspection indicates that the Ccm components could be grouped into three conserved units, presenting heme *b* (Module 1) and apocytochrome *c* (Module 2) as ligation-competent substrates to a third entity that carries out covalent and specific heme *b*-apocytochrome *c* ligation (Module 3). The apparent complexity of the Ccm system might be a mere reflection of the different life styles that organisms evolved to manage similar chemical necessities in different cellular compartments and physiological redox homeostasis, extending from extreme anaerobiosis to degrees of aerobiosis. With significant progress at hand, the general outlines of the Ccm process are now shaping up, its essential components are being well-defined, and mechanistic issues between them are appearing as timely targets for future investigations to better understand this important process. Organization of the overall Ccm process into simpler functional modules that perform defined tasks might allow more in-depth studies of each unit. Clearly, we need to solve the three-dimensional structures of the components and modules, and define the temporal and spatial interactions between the players. Perhaps the time is now ripe to initiate the development of a well-defined *in vitro* system with catalytic thioether bond formation capabilities using holoCcmE and a proper apocytochrome *c* as substrates. Although this effort might not be facile or rapid, achieving this daunting task surely will allow a unique and much needed opportunity to tackle related mechanistic questions in-depth (Box 1). In the meantime, sustained progress in deciphering the riddles of the Ccm system continues to pave the road towards a basic understanding of efficient production of *c*-type cytochromes that have broad biological significance and important implications for energy-related applications.

Box 1. Outstanding questions

- What are the exact composition, stoichiometry, specific cargo and transport activities of the heme delivery Module 1 that involves CcmA, CcmB, CcmC, CcmD and CcmE?
- Does the loading of heme *b* to CcmE, and its unloading from holoCcmE, require only CcmC-CcmD and CcmF, respectively?
- Do the constituents of each module form stable complexes? Do the modules interact with each other?
- If the iron of heme *b* on holoCcmE is in its oxidized state, then how is it reduced to facilitate thioether bond formation? Is there a need for a heme iron reductase

activity *in vivo*, and could CcmF carry out this reaction as a QH₂: heme oxidoreductase as proposed?

- Which component(s) of Module 2 directly interact with an apocyt *c* during its thioredox shuffling and delivery to heme ligation Module 3?
- Do CcmG and CcmH directly interact with each other? If not, could they interact indirectly via apocyt *c* linking them together?
- Between which components, and in which order, are mixed disulfide bonds formed all the way from the secretion to the delivery of apocyt *c* to Module 3?
- What is the validity of the “stereospecific heme ligation” model, and in particular, does CcmH provide a disulfide bond to form a mixed disulfide with the Cys₁ of the heme binding motif of an apocyt *c*, to ensure stereo-specific heme *b* incorporation?
- Although not essential for heme *b* ligation, does the presence of a disulfide bond in apocyt *c* protect against its periplasmic degradation?

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Glossary

<i>c</i> -type cytochromes (cyts <i>c</i>)	are typically characterized by the covalent attachment of heme <i>b</i> to polypeptide through one or, more generally, two thioether bonds with the Cys residues of a C ₁ XXC ₂ H motif. In many Gram-negative bacteria, the heme is ligated to the polypeptide by a periplasmic process conducted by the cytochrome <i>c</i> maturation (Ccm) proteins.
Cytochrome <i>c</i> maturation (Ccm) system	refers to post-translational and post-export protein modification processes that involve up to ten (CcmABCDEFGHI and CcdA) components in most Gram-negative bacteria.
Heme <i>b</i>	the most abundant metalloprotoporphyrin (iron-protoporphyrin IX) of the prosthetic group of hemoproteins, such as hemoglobins, peroxidases and cytochromes.
Thioether bond	a covalent chemical bond formed by a cysteine thiol side chain of a polypeptide and a vinyl group of heme <i>b</i> . In <i>c</i> -type cytochromes, thioether bonds are always formed between the vinyl at positions 2 and 4 of the tetrapyrrole ring of heme <i>b</i> and the thiols of the N- and C-terminal cysteines (Cys ₁ and Cys ₂), respectively, of a conserved heme-binding motif (C ₁ XXC ₂ H) within apocytochrome <i>c</i> .
Cytochrome <i>c</i> -type heme ligation complex	a set of proteins that carry out covalent ligation of heme <i>b</i> to an apocytochrome <i>c</i> .

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Figure 1. Heme binding domain of a cyt *c*

A three-dimensional structural close-up view of the covalent heme binding domain of *R. capsulatus* cyt *c*₂ (PDB 1C2R) is shown with the peptide backbone (gray) surrounding the heme group (red) and its thioether linkages between the vinyl-2~Cys34 and vinyl-4~Cys37 (yellow). His38 (blue) of the heme binding motif (C₁XXC₂H) acts as the 5th axial ligand of heme iron.

Figure 2. Modular organization of cyt *c* maturation system

In general, cyt *c* maturation is carried out by three coordinately operating membrane integral modules: Module 1 (heme translocation and relay, right panel) deals with the transport of heme *b* across energy transducing membranes and its availability to Module 3 (apocyt *c*–heme *b* ligation, middle panel), to which the apocyt *c* is conveyed by Module 2 (apocyt *c* thioredox and chaperoning, left panel) upon its translocation. Module 1 and Module 2 produce ligation-competent heme *b* and apocyt *c* substrates, respectively, and Module 3 catalyzes the thioether bonds between the appropriate vinyl and cysteine groups of these substrates. The arrows indicate possible simultaneous or sequential paths followed by the substrates to yield active *c*-type cyts. In all organisms, regardless the type of the native maturation system used, some forms of these modules are always present, but the number of components in each module is variable.

Figure 3. Module 1 components of Ccm system

Specific protein components of Module 1 (heme translocation and relay) drawn as a large complex formed of the ATP hydrolyzing CcmA subunits and their membrane integral partners CcmB, CcmC and CcmD loading heme *b* to the heme chaperone CcmE are shown. The conserved WWD motif of CcmC, found in heme handling proteins, is indicated. On the left, HoloCcmE is shown after its dissociation from CcmABCD following heme *b* loading and ATP hydrolysis. The CcmABCDE-independent heme *b* transport pathway of unknown nature is depicted with a dotted arrow and “?”.

Figure 4. Structure of holoCcmE and its bound heme *b*

The three-dimensional structure of CcmE (PDB 1J6Q) is shown in gray, with a heme molecule (in red) modeled as linked covalently via its vinyl-2 to His130 (blue) of CcmE. This model was generated by docking the structure of cyt *c*₂ with its heme (PDB 1C2R) to that of CcmE without heme, followed by minimizing the obtained binary structure and rendering invisible the cyt *c*₂ backbone. Note that the structure of holoCcmE is unknown. This hypothetical structure is simply to illustrate the covalent ligation between heme vinyl-2~His130 and axial heme iron coordination via Tyr134, leaving heme vinyl-4 exposed and available for subsequent ligation to apocyt Cys2.

Figure 5. Module 2 components of Ccm system

Module 2 (apocyt *c* thioredox and chaperoning) is shown together with the Sec translocon (secretion and signal sequence cleavage), the extracytoplasmic oxidative DsbA/DsbB pathway (linked to membrane quinone (Q/QH₂) pool) and the reductive CcdA/CcmG pathway (linked to cytoplasmic thioredoxin [TrxA] and NADH). As indicated by black, thin arrows marked with e⁻ the redox loop oxidizes and then reduces the cysteines at the heme binding motifs (C₁XXC₂H) of apocyt *c* at the expense of conveying cytoplasmic reducing equivalents to membrane Q/QH₂ pool. The DsbA-independent pathway of reduced apocyt *c* is shown with a dotted arrow, and possible enhanced degradation/protection of reduced or oxidized apocyt *c* with a “?”. Note that the exact nature of the interactions between reduced (re) or oxidized (ox) CcmG and oxidized or reduced apocyt *c* is unknown, the “+ ?” is to reflect that the final product of Module 2 is hypothesized to be a reduced disulfide bond containing apocyt *c* associated with CcmG.

Figure 6. Module 3 components of Ccm system and stereospecific ligation of heme *b*-apocyt *c*
 (a) Module 3 (apocyt *c*-heme *b* ligation) contains CcmI with its two domains, proposed to interact with apocyt *c* (provided by Module 2), CcmH proposed to have a disulfide bond, and CcmF known to interact with heme *b* loaded holoCcmE (provided by Module 1). (b) Upon formation of a mixed disulfide bond between the Cys₁ of reduced apocyt *c* and oxidized CcmH, Cys₂ of apocyt *c* is free to react with vinyl-4 of heme *b* to yield the first vinyl-4~Cys₂ thioether linkage, while His of the heme binding motif coordinates the oxidized heme iron (Fe ox) (c). Following reduction of heme iron proposed to occur via CcmF/QH₂ (d), vinyl-2 of heme *b* is released from CcmE, and the CcmH-apocyt *c* mixed disulfide bond is resolved (e), leading to the formation of the second vinyl-2~Cys₁ thioether linkage and regeneration of oxidized CcmH (f). Upon subsequent folding of correctly ligated heme *b*-apocyt *c*, holocyt *c* is produced. For a more detailed description of this hypothetical model, see the text and Sanders *et al.* [64].

Table 1**Ccm system components**

Component	Structural characteristics	Proposed function	Significant variation
CcmA	Peripheral membrane protein with a nucleotide-binding domain, conserved Walker A, B motifs, and ABC signature sequences	Involved in ATP dependent release of holoCcmE from the CcmABCDE complex, and/or is a part of a putative transporter required for releasing heme <i>b</i> from holoCcmE	Unknown
CcmB	Integral membrane protein with six transmembrane (TM) helices and conserved FXXDXXDGSLS motif	Involved in the release of holoCcmE from the CcmABCDE complex, and/or is a part of a putative transporter required for releasing heme <i>b</i> from holoCcmE	Unknown
CcmC	Six TM helices with two conserved histidine and a tryptophan rich motif (WGX[F,Y,W]WXWDXRLT)	Involved in translocation and ligation of heme <i>b</i> to CcmE and in possible release of holoCcmE from the CcmABCDE complex	Unknown
CcmD	Small monotopic membrane protein without any conserved motif	Enhances CcmE specific heme <i>b</i> lyase activity of CcmC, and possible release of holoCcmE from the CcmABCDE complex	Not found in plant mitochondrial Ccm system
CcmE	Single TM helix, single histidine within a conserved HXXXXY motif able to covalently bind heme <i>b</i> via its vinyl-2 group	Heme chaperone of the Ccm system; contributes to stereospecific apocyt <i>c</i> - heme <i>b</i> ligation	Histidine of HXXXXY motif is substituted by cysteine in most archaeal and some bacterial species
CcmF	Eleven TM helices with tryptophan rich motif (WGXWFWDPVEN), and four conserved histidines	Binds holoCcmE and probably performs apocyt <i>c</i> -heme <i>b</i> ligation in the CcmHFI complex	Multiple split homologs in Ccm system of plant mitochondria
CcmG	Single TM helix with a periplasmic thioredoxin (CXXC) motif	Reduction and transfer of apocyt <i>c</i> to the heme <i>b</i> ligation site	Not found in Ccm system of plant mitochondria; fused to CcdA in some archaeal species
CcmH	Single TM helix and a periplasmic thioredoxin-like (LRCXXC) motif	Binds apocyt <i>c</i> and possibly contributes to stereo-specific apocyt <i>c</i> -heme <i>b</i> ligation in the CcmFHI complex	Fused with the C-terminal domain of CcmI in some species like <i>E. coli</i> ; not found in species that contains a CcmE with a modified heme <i>b</i> binding motif
CcmI	Two TM helices, a cytoplasmic leucine zipper-like motif, and three or four TPR motifs in its periplasmic C-terminal portion	Possibly binds apocyt <i>c</i> as a part of the CcmFHI complex	Not found in Ccm system of plant mitochondria
CcdA/Dsbd	Six TM helices and two conserved cysteines; only the central domain of Dsbd is homologous to CcdA	Thioreduction of CcmG	Not found in Ccm system of plant mitochondria; CcdA and CcmG are fused together in some archaeal species