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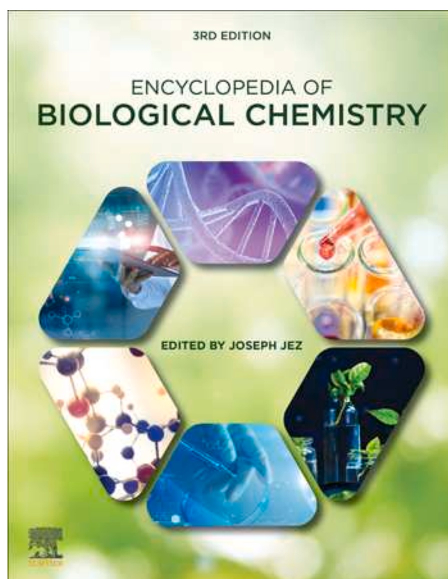


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# Bioenergetics Theory and Components | Cytochrome *c* Assembly

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## Introduction

### Definition of a Cytochrome *c*

Cytochromes, a family of metalloproteins performing one-electron transfer reactions, are found in biological membranes involved in transducing energy (Ma and Ludwig, 2019; Bertini *et al.*, 2006). Initially described in 1884 by Alexander McMunn, cytochromes were first named by David Keilin (*cyto* for cell and *chrome* for pigment, meaning merely cellular pigment) based on their different spectral characteristics upon oxidation and reduction (Bendall, 2016; Ma and Ludwig, 2019). Cytochromes contain heme as a prosthetic group and are further classified in *a*-, *b*-, *c*-, *d*- and *o*-types depending of the type of heme attached (Ma and Ludwig, 2019). Cytochromes of type *c*, also referred to as cytochromes *c*, are the only class of cytochromes with a heme *b* (ferroprotoporphyrin IX or protoheme IX) covalently attached to the protein (Alvarez-Paggi *et al.*, 2017). Covalent linkage of heme to the apocytochrome occurs via formation of two (or rarely one) thioether bonds between the side chain carbons in heme and the cysteine thiols in the polypeptide. The most common cytochromes *c* occur on the positive side (or *p*-side) of energy-transducing membranes (i.e., the bacterial or archaeal periplasm, the thylakoid lumen and mitochondrial intermembrane space), and carry heme covalently attached to a heme binding site (CXXCH) on the apoprotein (**Fig. 1**). Cytochromes *c* on the negative side (*n*-side) of the energy-transducing membrane (i.e., bacterial cytoplasm, chloroplast stroma and mitochondrial matrix) are rare. The only known example is cytochrome $b_6$ (in the $b_6f$ complex) in the chloroplast and cyanobacteria, and cytochrome *b* (in the *bc* complex) in firmicutes. Cytochromes *c* on the *n*-side harbor one heme *b* covalently attached to a single cysteine that is not contained within a motif, in addition to two *b*-hemes, which are non-covalently bound (de Vitry, 2011; Zito and Alric, 2016). This article is only concerned with cytochromes *c* on the *p*-side of the energy-transducing membranes.

### Cytochromes *c* on the *p*-Side of the Energy-Transducing Membrane

Cytochromes *c* on the *p*-side of the membrane are soluble or membrane-bound molecules with the heme binding domain always exposed to the *p*-side. Cytochromes of the *c*-type typically function as electron carriers or enzyme subunits (Ma and Ludwig, 2019; Thöny-Meyer, 1997; Bertini *et al.*, 2006). Bacteria contain numerous cytochromes *c* and some can carry multiple hemes (Salgueiro

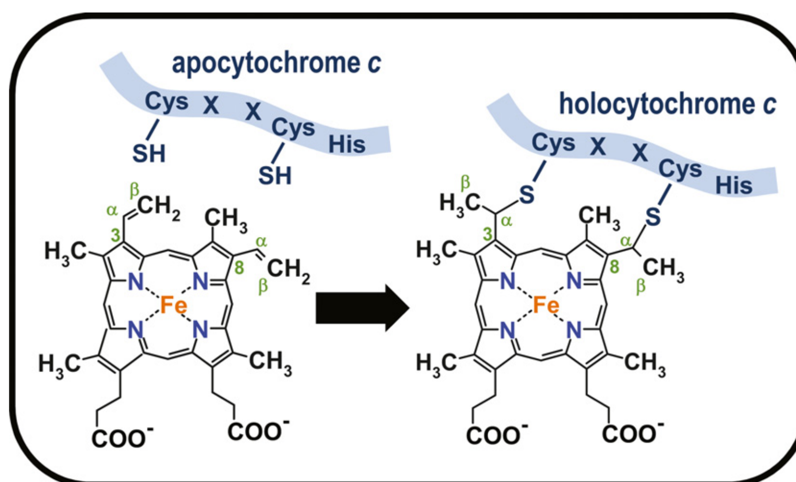


**Fig. 1** *Stereospecific covalent attachment of heme prosthetic group to apocytochrome c.* The free thiols of the cysteinyl groups of the heme binding motif (CXXCH, highlighted in light blue) in apocytochrome *c* form thioether linkages with the vinyl groups of Fe-Protoporphyrin IX or heme (shown in black, iron in orange, nitrogen in dark blue). The first and second cysteines of the heme binding motif forms a link with the α carbons at position C3 (2-vinyl) and position C8 (4-vinyl), respectively on the porphyrin ring. The proximal ligand is the histidine residue of the CXXCH motif and the distal ligand (not shown) is usually a distant methionine or, less frequently, another residue (such as histidine, asparagine or tyrosine). The carbon atoms in the porphyrin rings are numbered according to the rules of the International Union of Pure and Applied Chemistry (IUPAC). The Figure is adapted from Hamel, P., Corvest, V., Giege, P., Bonnard, G., 2009. Biochemical requirements for the maturation of mitochondrial c-type cytochromes. Biochim. Biophys. Acta 1793, 125–138.

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and Dantas, 2016). For example, NrfA is a pentaheme cytochrome *c* in *Escherichia coli* (Bamford *et al.*, 2002) and HmcA is a hexadecaheme cytochrome *c* in *Desulfovibrio vulgaris* (Matias *et al.*, 2002). Cytochromes *c* in archaea are under-investigated but they do not appear to be present in the majority of the archaeal proteomes deduced from genome sequencing analyses (Bertini *et al.*, 2006; Kletzin *et al.*, 2015; Lee *et al.*, 2007). In eukaryotes, cytochromes *c* always contain a single heme and reside in the thylakoid lumen and mitochondrial intermembrane space (IMS) where they function in photophosphorylation and oxidative phosphorylation, respectively (Gabilly and Hamel, 2017; Hamel *et al.*, 2009). While chloroplasts can house two or three *c*-type cytochromes, mitochondria typically harbor only two cytochromes *c* (Gabilly and Hamel, 2017; Hamel *et al.*, 2009; Bertini *et al.*, 2006). The most common heme-binding site (or motif), in *p*-side localized cytochromes *c*, contains two cysteine thiols for the formation of thioether linkages between the vinyl groups of heme and the polypeptide (**Fig. 1**). A typical heme binding motif is CXXCH, where the first cysteine forms a link with the side chain at position C3 (2-vinyl) of the porphyrin ring while the second cysteine is bonded to the side chain at position C8 (4-vinyl). Because heme is asymmetric, the chemical reaction of the cysteine thiols with the $\alpha$ or $\beta$ carbons of the vinyl side chains of heme can theoretically produce eight possible products, out of which only one is favored in all cytochromes *c* in nature. In *c*-type cytochromes, the $\alpha$ carbons of 2-vinyl and 4-vinyl of heme are invariably linked to the first and second cysteines of the heme binding motif, a feature that led to the definition of the stereospecific attachment of heme to apocytochrome *c* (Barker and Ferguson, 1999). The two residues (X) in between the heme linking cysteines can be any residue except cysteine in naturally occurring cytochromes *c*. The iron atom in the heme prosthetic group is hexa-coordinated by the four nitrogen atoms of the porphyrin ring and two axial ligands. Two amino-acid side chains in the polypeptide provide proximal and distal axial ligation. The proximal ligand is the imidazole group of the histidine residue in the CXXCH motif while the distal ligand is nearly always a distant methionine or, less frequently, another residue (such as histidine, asparagine or tyrosine) (Bertini *et al.*, 2006). Variations of the heme binding site exist and usually occur in multiheme cytochromes *c* acting as enzyme subunits and not as electron carriers. In some bacterial cytochromes *c*, the heme iron is penta- instead of hexa-coordinated and a lysine in a CXXCK motif serves as the only axial ligand, such as the cytochrome *c* subunit of the nitrite reductase (Bamford *et al.*, 2002). In a few cases, bacterial cytochromes *c* carry unconventional binding motifs with a different spacing between the heme-linking cysteines (CXXXCH, CXXXXCH, and $CX_{15}CH$) (Bowman and Bren, 2008). A contracted heme binding motif (CXCH) with one instead of two intervening residues was recently recognized in multiheme cytochromes *c* occurring in several bacterial and archaeal phyla (Ferousi *et al.*, 2019). Other motif variations in organellar cytochromes *c* do occur but remain extremely rare. For instance, mitochondrial cytochromes *c* within the phylum Euglenozoa display an A/FXXCH motif instead of the classical CXXCH (Vilmos *et al.*, 2009; Allen *et al.*, 2004).

Cytochromes *c* can be further grouped in four classes based on their structural and redox properties (Ambler, 1991; Bowman and Bren, 2008). Class I, the largest group, is mainly defined by small soluble monoheme cytochromes *c* with a globular fold, an N-terminal heme binding site and a C-terminal methionine as the distal axial ligand (example, mitochondrial cytochrome *c*). Class II contains cytochromes *c* with a C-terminally located heme binding site and a typical four-helix bundle structure (example, cytochrome *c* in *Rhodobacter capsulatus*). Class III is defined by multiheme cytochromes *c* with a distal histidine ligand (example, cytochrome $c_3$ in *Desulfovibrio vulgaris*). Class IV contains cytochromes *c* with histidinyl-methionyl or bis-histidinyl coordination and additional non-heme prosthetic groups (e.g., flavin, molybdopterin). Since the seminal work of David Keilin that led to the first definition of the respiratory chain (Bendall, 2016), cytochromes *c* have been the subject of extensive investigation from their electron transfer properties to their three dimensional structures (Bowman and Bren, 2008; Ma and Ludwig, 2019; Bertini *et al.*, 2006). Building upon this work, the focus of this article describes the mechanistic details of their assembly.

## Assembly of Cytochromes *c*

### Common Principles for the Biogenesis of *c*-Type Cytochromes

Cytochrome *c* assembly or maturation is the conversion of apocytochromes *c* to their corresponding holoforms by the stereospecific attachment of the heme moiety via formation of the thioether bond linkages at the CXXCH motif. Unlike some metalloproteins that can be translocated across the membrane to the *p*-side as already assembled molecules with their prosthetic groups (e.g., FeS cluster) (Palmer and Stansfeld, 2020), all natural *c*-type cytochromes mature via a post-translational process on the *p*-side of the energy-transducing membrane. Because the *c*-type cytochromes are assembled in a different compartment from the site of synthesis of the apoprotein and heme, their biogenesis demands a mechanism for transport of both apocytochrome and heme substrates across at least one biological membrane (Hamel *et al.*, 2009). Most apocytochromes *c* are synthesized with a cleavable N-terminal pre-sequence acting as a specific targeting signal and required for import across one or several membranes (Owji *et al.*, 2018). One notable exception is the soluble cytochrome *c* in mitochondria (Dumont, 1996). In bacteria and organelles, apocytochromes *c* cross the membranes as unfolded molecules via general translocation machineries (Avendaño-Monsalve *et al.*, 2020; Richardson and Schnell, 2019; Frain *et al.*, 2016). In most cases, the terminal step of heme synthesis occurs on the *n*-side of the membrane and heme needs to be transported across the lipid bilayer to the *p*-side (Swenson *et al.*, 2020; Chambers *et al.*, 2021). In eukaryotes, heme must also be trafficked from its site of synthesis (chloroplast stroma or mitochondrial matrix) to several hemoproteins located throughout the cell (Chambers *et al.*, 2021; Swenson *et al.*, 2020). The description of bacterial and eukaryotic importers for heme assimilation and exporters for efflux of cytotoxic heme lends credence to the operation of specific heme delivery mechanisms for cytochrome *c* assembly (Chambers *et al.*, 2021; Swenson *et al.*, 2020). Translocation routes for heme from the *n*-side to the *p*-side that are specific to cytochrome *c* maturation

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have remained elusive until the discovery of the CcsBA heme exporter (Frawley and Kranz, 2009) (see Section "A Heme Exporter in the CCS Pathway").

## Chemical Requirements for Thioether Bond Formation

While thioether bond formation in holocytochrome *c* appears simple by comparison, attaching heme in a stereospecific manner is not a trivial chemical process (Bowman and Bren, 2008). Several studies of spontaneous thioether bond formation have provided some insight into the chemistry of the heme ligation reaction. (Daltrop *et al.*, 2002; Daltrop and Ferguson, 2003, 2004; Barker *et al.*, 1993; Metcalfe *et al.*, 2007; Daltrop *et al.*, 2003). The divalent state of the iron in heme appears critical for stereospecific thioether bond formation in vitro. If ferriheme ($Fe^{3+}$ heme) is provided instead of ferroheme ($Fe^{2+}$ heme), a radical-based chemistry, presumed to take place, promotes incorrectly attached heme (Barker *et al.*, 1993). The need for reductant in the context of the heme ligation reaction also extends to the apocytochrome substrate. In vitro thioether link formation does not proceed if the heme binding cysteines are oxidized into disulfides, irrespective of the redox state of the iron in the heme group (Daltrop *et al.*, 2002; Daltrop and Ferguson, 2003, 2004). That heme and apocytochrome *c* in the reduced form are sufficient to achieve stereospecificity implies that the heme-polypeptide interactions are probably the key determinants for selecting the correct heme orientation. Conceivably, the unfolded apocytochrome *c* provides sufficient structural features so the incorrect rotational isomer of heme is excluded from the reaction. The detection of a non-covalent heme-protein complex in which the iron atom is coordinated by two amino acid side chains from the protein in the in vitro reaction supports this scenario (Daltrop *et al.*, 2002, 2003; Tomlinson and Ferguson, 2000). A complex between apocytochrome *c* and non-covalently bound heme is a productive intermediate formed during the initial steps of the in vitro reaction, followed by spontaneous thioether bond formation between the vinyls and the CXXCH free thiols. A non-covalent heme-apoprotein intermediate is still detected when the CXXCH cysteines are substituted by alanine, an indication that heme linking thiols are probably not strictly required for the initial heme-polypeptide recognition in vitro (Tomlinson and Ferguson, 2000). The heme-apoprotein complex intermediate is presumed to form by preferential binding (or retention) of heme in one rotational isomeric position. The conserved histidine that acts as the proximal ligand of heme in the CXXCH motif is probably essential in selecting the correctly oriented heme to yield a productive apocytochrome-heme intermediate (Daltrop *et al.*, 2002). Intuitively, the distal axial ligand in apocytochrome must act as the second site for iron coordination in the apoprotein-heme complex prior to covalent attachment but this still awaits experimental testing. With reduced heme in the correct stereochemical orientation and the thiols optimally positioned for reacting with the vinyl moieties, thioether bridges can be "simply" promoted without any enzymatic catalysis. That the appropriate redox state of apocytochrome and heme substrates is essential for thioether bond formation led to the view that cytochrome *c* maturation is probably an enzymatically assisted process in vivo. While there are a few examples of heme and apocytochrome *c* spontaneously forming thioether bonds in vitro, a soluble form of a natural or artificial monoheme cytochrome *c* is the protein substrate in all of them (Daltrop *et al.*, 2002; Daltrop and Ferguson, 2003; Daltrop *et al.*, 2003). The slow rates of the in vitro reaction and the fact that assembling multiheme cytochromes *c* (Salgueiro and Dantas, 2016) constitutes a considerably more challenging process are arguments speaking to the necessity for enzyme-assisted catalysis in vivo (Daltrop *et al.*, 2002; Daltrop and Ferguson, 2003; Daltrop *et al.*, 2003).

## Three Distinct Systems for Maturation of Cytochrome c

Extensive studies of cytochrome *c* assembly in several genetically tractable model systems uncovered an unsuspected complexity for the regulation of the heme attachment reaction in vivo. At least three distinct assembly machineries or maturation systems, System I or CCM (for cytochrome *c* maturation), System II or CCS (for cytochrome *c* synthesis) and System III or HCCS (for holocytochrome *c* synthase) operate for the conversion of apoforms of cytochrome *c* to their holoforms (Mavridou *et al.*, 2013b; Verissimo and Daldal, 2014; Kranz *et al.*, 2009; Babbitt *et al.*, 2015; Travaglini-Allocatelli, 2013; Gabilly and Hamel, 2017; Simon and Hederstedt, 2011). This diversity came as a surprise considering the fundamental nature of the in vivo reaction is assumed to be the same as that of the in vitro reaction. While a few factors are shared among the different systems, specifically between System I and II, each maturation pathway can still be distinguished based on the occurrence of a signature assembly factor. However, there is a considerable difference in the number of assembly factors and their deduced biochemical activities among the different Systems. Another intriguing feature about the maturation pathways is their distribution across the different domains of life. While bacteria use only System I or System II, there is no obvious explanation for the selection of either system in a given bacterial phylum (Bertini *et al.*, 2007; Lee *et al.*, 2007). Systems I and II are believed to have a common ancestor and possible evolutionary scenarios for their origin have been proposed (Lee *et al.*, 2007).

While all archaeal and chloroplast cytochromes *c* appear to be matured via System I and System II, respectively (Gabilly and Hamel, 2017; Kletzin *et al.*, 2015), mitochondrial cytochromes *c* can be assembled by System I or III (Allen *et al.*, 2008). The taxonomic distribution of System I and III in the plant kingdom is even more puzzling as closely-related lineages contain different systems (Allen *et al.*, 2008; Allen, 2011; Giegé *et al.*, 2008). Except for some species that rely on System III, most terrestrial plants use the CCM pathway. Among green algae, Chlorophytes use System III while Charophytes, which are evolutionarily closer to land plants, use System I or III. A recent study shows that, in plants, a System I-dependent cytochrome *c* maturation pathway can be

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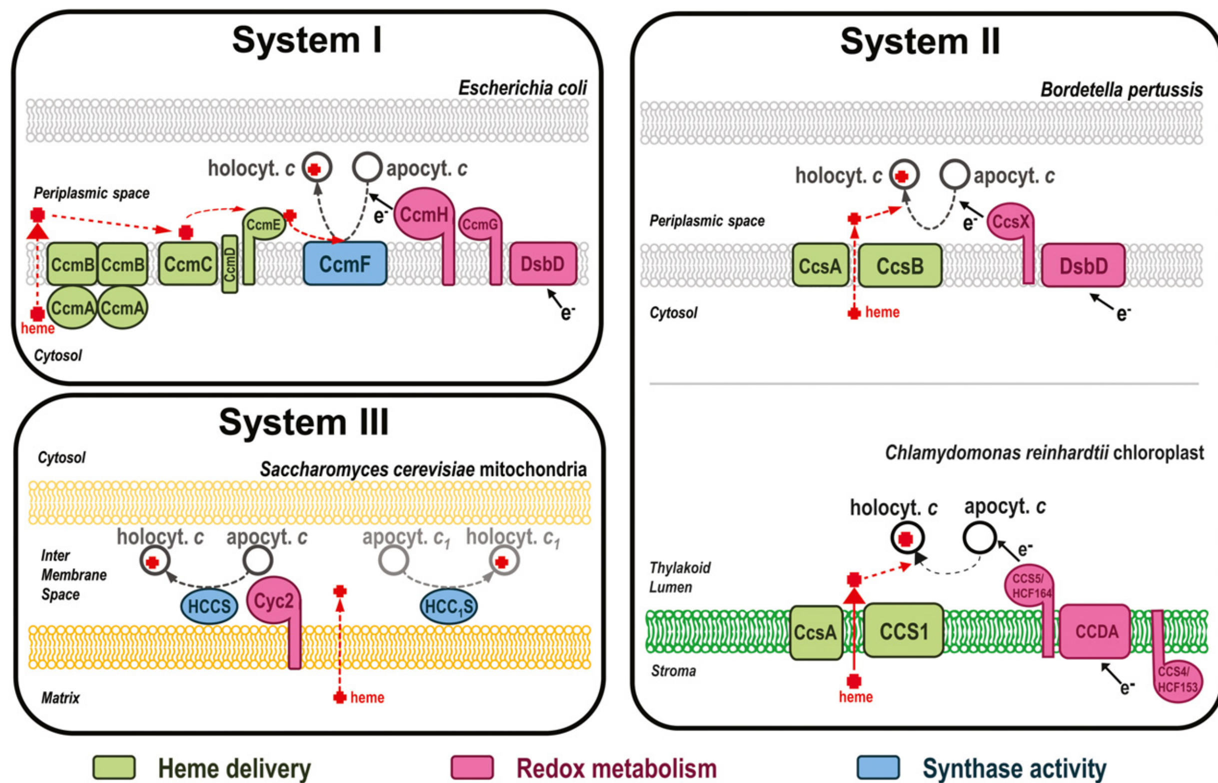


**Fig. 2** *Overview of systems I, II and III, the cytochromes c assembly pathways*. The cytochrome *c* assembly components of bacterial System I (e.g., *E. coli*), bacterial System II (e.g., *B. pertussis*), chloroplast System II (e.g., *Chlamydomonas reinhardtii*) and System III (e.g., mitochondria of *S. cerevisiae*) are represented using a color code for assigned function. Green components are involved in heme delivery, the pink components are implicated in redox chemistry (thiol-based chemistry or heme reduction), and the blue components are postulated to carry the holocytochrome *c* synthase function. In grey are the bacterial inner and outer membranes. In orange are the mitochondrial membranes, the outer membrane (light orange) and the inner membrane (dark orange). The thylakoid membrane is shown in green. Some components carry more than one function. For instance, CcmF is a component that carries the heme reduction and the holocytochrome *c* synthase functions. CcsA/ResC and CCS1/CcsB/ResB displays the heme delivery and holocytochrome *c* synthase activities. Adapted from Hamel, P., Corvest, V., Giege, P., Bonnard, G., 2009. Biochemical requirements for the maturation of mitochondrial *c*-type cytochromes. Biochim. Biophys. Acta 1793, 125–138.

substituted by System III (Kolli *et al.*, 2020). This finding suggests there is, a priori, no obvious reason why System I or III was retained in each plant lineage. In the three sections below, we provide an overview of the different maturation systems (**Fig. 2**) and highlight the unresolved questions. We follow with a discussion on the major advances in our understanding of the biochemical activities required to complete cytochrome *c* maturation, with a particular emphasis on the recent discoveries.

## The CCM Pathway or System I

If the CCM pathway stands among the most complicated maturation systems due to the number of its components, extensive dissection in bacterial models such as *E. coli* and *R. capsulatus* has now provided a mechanistically elaborate picture of this multicomponent machinery (Kranz *et al.*, 2009; Verissimo and Daldal, 2014; Stevens *et al.*, 2011; Travaglini-Allocatelli, 2013). The intellectual appeal of System I resides in the fact that the various Ccm components are now categorized in the separable functions that we view necessary to complete apo- to holocytochrome *c* conversion (**Fig. 2**). Such functions can be divided into (1) heme delivery/handling (CcmABCDEF) on the *p*-side, (2) preparation of apocytochrome *c* substrate via maintenance of the heme-linking thiols in a reduced form (CcmG and CcdA or DsbD), (3) heme reduction (CcmF) and (4) thioether bond formation (CcmFH). The heme chaperone CcmE is the signature component of System I and based on its conservation, the CCM-dependent maturation of cytochromes *c* takes place in $\alpha$, $\gamma$, a subset of $\delta$-proteobacteria (e.g., *Desulfivibrio desulfuricans*), some Gram-positive bacteria (such as *Deinococcus*), archaea, mitochondria of some plant phyla and several flagellates and ciliates among protozoans (e.g., *Reclinomonas americana*, *Paramecium* spp.). Variations of the CCM pathway occur in some bacteria where the C-terminal of CcmH that displays features of a TPR (tetratricopeptide repeat) domain occurs as a separate component encoded by the *CcmI* gene (Travaglini-Allocatelli, 2013). In a subset of bacteria and most archaea, the absence of CcmG and CcmH co-occur with a CcmE variant carrying a cysteine instead of the invariant heme-binding histidine residue (Stevens *et al.*, 2011). In plant mitochondria using the CCM pathway, CcmF is split into

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multiple polypeptides corresponding to orthologous domains in bacterial CcmF, all encoded in the mitochondrial genome (Giegé *et al.*, 2008). In addition, CcmD and the redox components maintaining the heme binding thiols in the reduced form (CcmG and DsbD or CcdA) cannot be detected. It is possible the plant orthologs are missing or have diverged considerably and hence are not easily detected via bioinformatics (Giegé *et al.*, 2008). In System I, the mechanisms for transmembrane movement of heme from the *n*- to the *p*-side are still unknown.

## The CCS Pathway or System II

The CCS pathway or System II first emerged from the analysis of chloroplast *c*-type cytochrome assembly in the green alga *Chlamydomonas reinhardtii* (Gabilly and Hamel, 2017). System II prototypical assembly factor is chloroplast CCS1, also called CcsB/ResB in bacteria (Simon and Hederstedt, 2011; Kranz *et al.*, 2009) (**Fig. 2**). Using CcsB/ResB as the signature assembly factor, the CCS pathway was recognized as a cytochrome *c* maturation system in cyanobacteria, the presumed bacterial ancestor of the chloroplast and several other bacteria, such as most Gram-positive (e.g., *Bacillus subtilis*, *Mycobacterium* spp.) and proteobacteria of the *β*- (e.g., *Neisseria* and *Bordetella* spp.), ε-type (e.g., *Helicobacter pylori*) and most representatives of the *δ*-type (e.g., *Geobacter metallireducens*). There is no evidence for the operation of System II outside bacteria and chloroplasts. CCS1/CcsB/ResB is a poorly conserved protein and co-occurs with the CcsA/ResC protein. In photosynthetic eukaryotes, CcsA/ResC is always plastid-encoded and used as a marker for the CCS-controlled assembly of chloroplast cytochromes *c*. In photosynthetic protozoans (e.g., Euglenids and Dinoflagellates), a CcsA-encoding gene cannot be detected (Hallick *et al.*, 1993; Waller and Kořený, 2017). While it is conceivable that another pathway operates in photosynthetic protozoans, a more likely explanation is that the CcsA-encoding gene has relocated to the nucleus. In *Euglena gracilis*, the *petA* gene encoding the membrane-bound cytochrome *c*, typically in the plastid genome of photosynthetic eukaryotes, is in the nucleus (Santillán Torres *et al.*, 2003).

In bacteria, the biochemical activities of heme transfer from *n*- to the *p*-side and linkage to apocytochromes *c* are carried by polytopic proteins CcsA/ResC and CcsB/ResB, while provision of reduced CXXCH motif is under the control of redox catalysts ResA/CcsX and CcdA or DsbD (Simon and Hederstedt, 2011; Kranz *et al.*, 2009) (**Fig. 2**). In plastids, orthologous proteins (CcsA, CCS1, CCS5 and CCDA) operate in the assembly of cytochrome *c* but genetic screens suggest the maturation pathway might be more complicated with the additional components, CCS2, CCS3, CCS4 and CCS6 (Gabilly and Hamel, 2017). While CCS2 appears to be a regulator of the expression of the *ccsA* gene and is unlikely to participate directly in the heme attachment reaction (Cline *et al.*, 2017), CCS4 functions in the control of in vivo redox state of the heme binding cysteines and has no counterpart in the bacterial System II (Gabilly *et al.*, 2011) (see Section "Thiol-Based Chemistry for Apocytochrome *c* Preparation"). The molecular identity of *CCS3* and *CCS6* remains unknown. Unlike in System I, there is no evidence for components controlling the reduction of heme in the CCS-dependent heme linkage to apocytochromes *c* (see Section "Chemical Reduction of Ferriheme to Ferroheme").

## The HCCS Pathway or System III

Historically, the first cytochrome *c* maturation system to be uncovered from genetic screens in fungi, System III or the HCCS pathway was named from the only identified component, HCCS (holocytochrome *c* synthase). HCCS was also formerly and incorrectly referred to as CCHL (for cytochrome *c* heme lyase, the lyase catalyzes the opposite reaction, i.e., detachment of heme from holocytochrome *c*) (Allen, 2011). Based on the occurrence of HCCS-like proteins, System III is present in the mitochondria of fungi, apicomplexan parasites (e.g., *Plasmodium falciparum*), some red and green algae, some land plant species, amoeba among protozoans (e.g. *Dictyostelium discoideum*) and all animals (Giegé *et al.*, 2008; Allen, 2011; Mavridou *et al.*, 2013b; Babbitt *et al.*, 2015). No HCCS-like encoding gene can be detected in any bacterial genome, a surprising finding considering the ancestral mitochondrion is believed to have originated from an α-proteobacterium, a bacterial phylum using System I (Allen, 2011; Giegé *et al.*, 2008). Because System III is restricted to mitochondria, it is thought the CCM pathway from the α-proteobacterium progenitor of the mitochondria was lost. As a result, HCCS became a new evolutionary solution to assemble soluble cytochrome *c* and membrane-bound cytochrome $c_1$, the only two monoheme cytochromes *c* residing in mitochondria (Babbitt *et al.*, 2015). That HCCS displays very little specificity towards bacterial monoheme cytochromes *c* and cannot assemble multiheme cytochromes *c* is consistent with a model where System III "adapted" to simpler apocytochromes *c* substrates (Babbitt *et al.*, 2016; San Francisco *et al.*, 2013; Richard-Fogal *et al.*, 2012).

In fungi, IMS-facing membrane-anchored HCCS and $HCC_1S$ were initially reported to be specific for the maturation of cytochrome *c* and $c_1$, respectively (**Fig. 2**). However, if $HCC_1S$ specificity towards cytochrome $c_1$ appears strict, HCCS reactivity towards apocytochrome $c_1$ is inherently weak but can be promoted by amino acid substitutions in HCCS or cytochrome $c_1$ (Bernard *et al.*, 2003).

In yeast, distinct heme reduction requirements for the assembly of cytochrome $c_1$ (NADH and FMN) and cytochrome *c* (NADPH and FAD) were inferred from the reconstitution of the heme attachment reaction in isolated mitochondria (Bonnard *et al.*, 2010; Hamel *et al.*, 2009). The identity of the reductase(s) had remained elusive until the isolation of Cyc2p in a suppressor screen (Bernard *et al.*, 2005; Corvest *et al.*, 2012; Bernard *et al.*, 2003). Cyc2p, an IMS-facing membrane-anchored FAD-containing protein, is essential for the HCCS-dependent assembly of cytochrome *c* and $c_1$. A role for Cyc2p as a HCCS-dedicated heme reductase was postulated based on the fact recombinant Cyc2p is active in reducing ferriheme in an NADPH-dependent manner (**Fig. 2**). Because $HCC_1S$-dependent assembly does not require Cyc2p, a distinct NADH-dependent FMN-containing heme reductase must operate. The requirement of specific heme reductases in fungi is puzzling as in multicellular organisms, a single

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HCCS can assemble both cytochrome *c* and $c_1$, and no Cyc2p ortholog is detected. This variation of System III in fungi remains obscure and needs to be further investigated.

With a single assembly factor, System III appears deceptively simple and hardly fits the definition of a pathway in comparison to System I and II, which are defined by multiple components with distinct biochemical activities. Saturating mutant screens failed to unveil the components controlling heme transport from the matrix to the IMS and it is plausible that heme exporters eluded genetic identification because of redundancy (Hamel *et al.*, 2009). If heme is relayed as ferroheme from matrix-localized membrane-associated ferrochelatase across the mitochondrial inner membrane and handed directly to HCCS, a mechanism for heme reduction might not be necessary. In yeast, exogenously added heme supports mitochondrial cytochrome *c* assembly in the absence of matrix localized ferrochelatase, an indication heme is still delivered to HCCS (Kim *et al.*, 2016).

Except for Cyc2p, components controlling the redox state of the apocytochrome and heme substrates in System III also escaped molecular identification. When IMS-resident HCCS is localized to the bacterial periplasm, the enzyme becomes dependent on the disulfide reducing components for assembling its cognate cytochrome *c* substrate (Richard-Fogal *et al.*, 2012). One possible interpretation is the maintenance of heme-linking cysteines under a reduced state is not a strict biochemical requirement in the mitochondrial IMS. If the mitochondrial IMS and the bacterial periplasm are typically viewed as bioenergetically analogous, they might not be equivalent compartments in terms of redox environments.

## Thiol-Based Chemistry for Apocytochrome *c* Preparation

In vitro studies showed that apocytochrome *c* with a disulfide bond between the cysteine residues in the CXXCH– motif is not a competent substrate for thioether bond formation (see Section "Chemical Requirements for Thioether Bond Formation"). *In vivo*, the redox state of the heme binding thiols is under the control of a membrane-bound *p*-side facing thioredoxin (Trx)-like protein. The *p*-side facing Trx-like protein specifically reduces the disulfide bonded cysteines of the heme binding site into free thiols that can subsequently react with the vinyl groups of heme (Gabilly and Hamel, 2017; Simon and Hederstedt, 2011). This dedicated redox catalyst is required for the assembly of all bacterial and chloroplast cytochromes *c*, further underscoring the importance of thiol-based chemistry for the preparation of apocytochrome *c* as a competent substrate for the heme ligation reaction. Named CcmG in bacterial System I, ResA/CcsX in bacterial System II and CCS5/HCF164 in chloroplasts, this redox component was shown to directly reduce a disulfide bond formed between the heme-linking cysteines of apocytochrome *c* (Cline *et al.*, 2016; Gabilly and Hamel, 2017). The Trx-like protein is maintained reduced via a member of the family of transmembrane thiol-disulfide oxidoreductases (TDOR), which acts as a transducer of reducing power across the energy-transducing membranes (**Fig. 3**) (Bushweller, 2020; Cho and Collet, 2013). The provision of reductants occurs via sequential thiol-disulfide exchanges across the membrane involving the *n*-side classical Trx, a member of the TDOR family and the *p*-side Trx-like protein dedicated to cytochrome *c* assembly

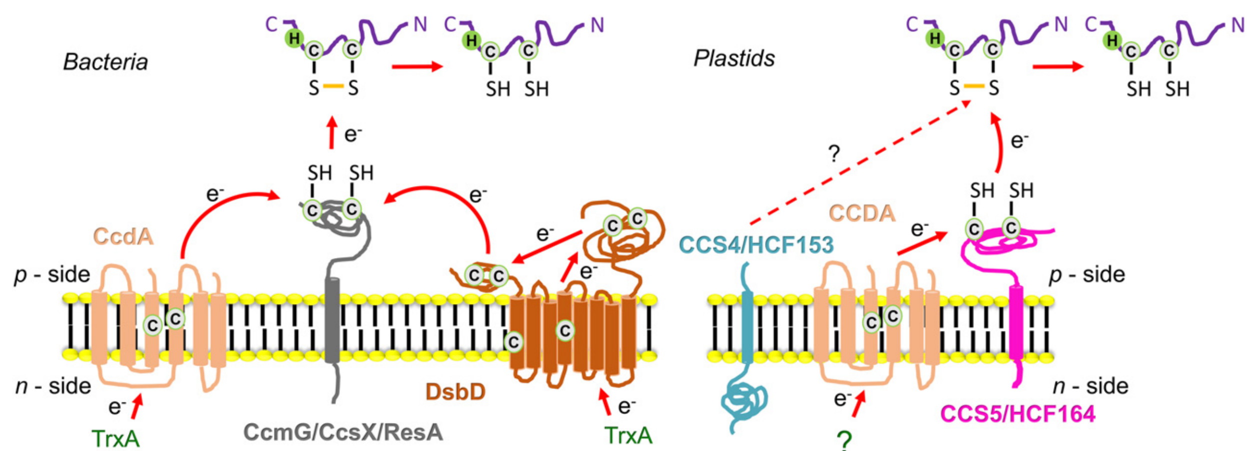


**Fig. 3** *Thiol – based chemistry for the preparation of the apocytochrome c* substrate. Heme attachment requires the heme binding cysteines in the CXXCH motif to be reduced. *Left:* In bacterial Systems I and II, disulfide-bonded apocytochrome *c* is reduced on the *p*-side of the membrane by a thioredoxin-like protein, named CcmG (gray) in System I (e.g., *E. coli*), CcsX/ResA in System II (e.g., *B. pertussis*, *B. subtilis*). This thioredoxin-like protein is maintained in a reduced state via a member of the TDOR (transmembrane thiol disulfide oxidoreductases) family, namely CcdA (e.g., *R. capsulatus*) or DsbD (e.g., *E. coli*), which occurs in System I or II and relays electrons across the membrane via thiol-disulfide exchange reactions. *Right:* In plastids, apocytochrome *c* is maintained in a reduced state by CCS5 in *C. reinhardtii* or HCF164 in *A. thaliana* via CCDA, the plastid ortholog of bacterial CcdA. In both Systems, electron transfer occurs from the *n*-side to the *p*-side of the membrane and a classical thioredoxin is the physiological electron donor. The physiological electron donor is known in bacterial System I and II (TrxA) but remains unidentified in plastids (?). CCS4 (HCF153 in *Arabidopsis*) is another component involved in the delivery of reductants to disulfide-bonded apocytochrome *c* in chloroplasts, however, its mechanism of action is unclear. Conserved cysteine and histidine residues are shown in gray and green circles respectively; disulfide bonds are indicated by yellow bonds; electron transfers are indicated by red arrows. Reduction of the disulfide requires 2 electrons and 2 protons.

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(Bushweller, 2020; Cho and Collet, 2013; Davey *et al.*, 2016). Members of the TDOR family involved in cytochrome *c* biogenesis are DsbD and CcdA, which display a critical pair of cysteines located within transmembrane domains and involved in the transfer of reducing power. DsbD displays eight transmembrane segments with N-terminal and C-terminal redox domains, both exposed to the *p*-side, exhibiting an immunoglobulin- and Trx-like fold, respectively. With six transmembrane domains resembling the central hydrophobic domain of DsbD and no additional redox domains, CcdA is a "stripped-down" version of DsbD (**Fig. 3**). Electrons are transferred within DsbD by a cascade of disulfide bond reduction steps from the central- to the C-terminal and, finally, to the N-terminal domain, which reduces the Trx-like protein (CcmG or ResA/CcsX) dedicated to cytochrome *c* assembly. In CcdA, the electrons move from the central domain directly to the Trx-like component (CcmG or ResA/CcsX). In bacterial System I and II DsbD or CcdA supply reducing power to the periplasm but CcdA appears to be the most represented TDOR member in all bacterial species. Despite their structural differences, DsbD and CcdA are interchangeable in their role in cytochrome *c* assembly (Katzen *et al.*, 2002). In chloroplasts, CCDA, an ortholog of bacterial CcdA, appears to be the only TDOR member involved in transferring the electrons to the Trx-like component involved in cytochrome *c* maturation (CCS5/HCF164) (**Fig. 3**). The *n*-side electron donor to the pathway is a classical Trx involved in several thiol-disulfide exchange reactions, and maintained reduced by Trx reductase at the expense of NADPH (Cline *et al.*, 2016). If the identity of the *n*-side Trx is established in bacteria, it has not been ascertained in the plastid (Gabilly and Hamel, 2017; Hamel *et al.*, 2009).

Loss of function of the chloroplast (CCS5/HCF164) or bacterial (CcmG or ResA/CcsX) Trx-like cytochrome *c* assembly factor can be rescued by provision of exogenous reductants, a finding consistent with the function of this component in a disulfide reducing pathway (Cline *et al.*, 2016). In bacterial System I and II, inactivation of the disulfide bond forming pathway restores cytochrome *c* assembly when the disulfide-reducing pathway is no longer operating (Cline *et al.*, 2016; Gabilly and Hamel, 2017). This suggests that the thiols in the CXXCH motif are first targeted by the disulfide forming pathway when apocytochrome is translocated on the *p*-side, and then subsequently reduced to produce a competent substrate for the heme ligation reaction. In the chloroplast lumen, a disulfide bond forming system has been described but it is not known if apocytochrome *c* is first acted upon by this system or if it is oxidized by a yet-to-be-defined lumen resident oxidant (Karamoko *et al.*, 2013; Gabilly and Hamel, 2017).

The function of the *p*-side Trx-like component as a mere apocytochrome *c* CXXCH disulfide reductase is probably an over-simplification of the protein activity. Features revealed from three dimensional structures in combination with numerous genetic studies suggest the Trx-like protein also functions as a redox-independent chaperone or a holdase, presumably to facilitate the presentation of reduced apocytochrome *c* substrate to the heme ligation components (Turkarslan *et al.*, 2008; Verissimo and Daldal, 2014; Simon and Hederstedt, 2011).

In mitochondria relying on Systems I or III, a Trx-like protein dedicated to cytochrome *c* maturation has not been identified. Maintenance of the free thiols in the CXXCH motif is conceivably under the control of another redox component. Another scenario is that apocytochrome *c* and $c_1$ are never targets of the Mia40/Erv1 disulfide bond forming pathway operating in the IMS and are translocated in the reduced form. If the apocytochrome *c* substrates are never oxidized, there is no chemical requirement for disulfide reduction. This is a plausible explanation considering most IMS resident proteins with disulfide bonds carry a specific twin cysteine motif ($CX_3C$ or $CX_9C$) specifically recognized by the Mia40 disulfide bond forming enzyme (Chatzi *et al.*, 2016).

In plastids, the transmembrane delivery of reducing power also relies on CCS4/HCF153, a small protein conserved in the green lineage (Gabilly and Hamel, 2017) (**Fig. 3**). While CCS4/HCF153 lacks any residue or motif speaking to a function in thiol-based chemistry, the reductant-dependent rescue of *ccs4*-null mutant categorizes this component as a participant in the thiol-based chemistry required for apocytochrome *c* preparation. The mode of action of CCS4 remains enigmatic as this membrane-anchored protein is *n*-side facing and disulfide reduction of the CXXCH motif is a process taking place on the *p*-side (**Fig. 3**). A role for CCS4 as a stabilizer of the CCDA protein is a possible function since *CCDA* overexpression restores cytochrome *c* assembly to a *ccs4* mutant. However, this is unlikely to be the sole function as genetic analysis points to the involvement of CCS4 in a CCDA-independent route for the control of the redox state of apocytochrome heme linking cysteines.

## Pathways for Heme Delivery and Handling

### Heme Relay in the CCM Pathway

The best characterized pathway in terms of mechanistic details is involved in the supply of heme to the apocytochrome *c* substrate in System I. This is also probably the most sophisticated in terms of number of participating components and the nature of the biochemical reactions. Purification of protein complex intermediates followed by spectroscopic analysis enabled the delineation of a heme relay pathway on the *p*-side involving a unique heme chaperone discovered over two decades ago (Schulz *et al.*, 1998). The heme delivery pathway is defined by proteins interacting directly with heme, namely CcmC, CcmE and CcmF and three other components CcmA, CcmB and CcmD, which are required for the transfer of heme to the apocytochrome but do not physically interact with heme (Babbitt *et al.*, 2015; Kranz *et al.*, 2009). CcmC and CcmF are polytopic membrane proteins belonging to the HHP (Heme Handling Protein) family, with the so-called heme interacting "WWD" domain (WGXΩWXWE/D) and flanking histidine residues exposed to the *p*-side (Babbitt *et al.*, 2015; Kranz *et al.*, 2009) (**Fig. 4**). If the mechanisms by which heme is delivered from the *n*-side to the *p*-side are still not established in the CCM pathway, there is no evidence that CcmC functions as a heme exporter (Richard-Fogal and Kranz, 2010). Ccm-dependent cytochrome *c* maturation can still proceed in a bacterial heme-deficient mutant supplemented with exogenous heme (Richard-Fogal *et al.*, 2007). That heme is endogenously produced on the *n*-side does not appear to be a pre-requisite in the CCM

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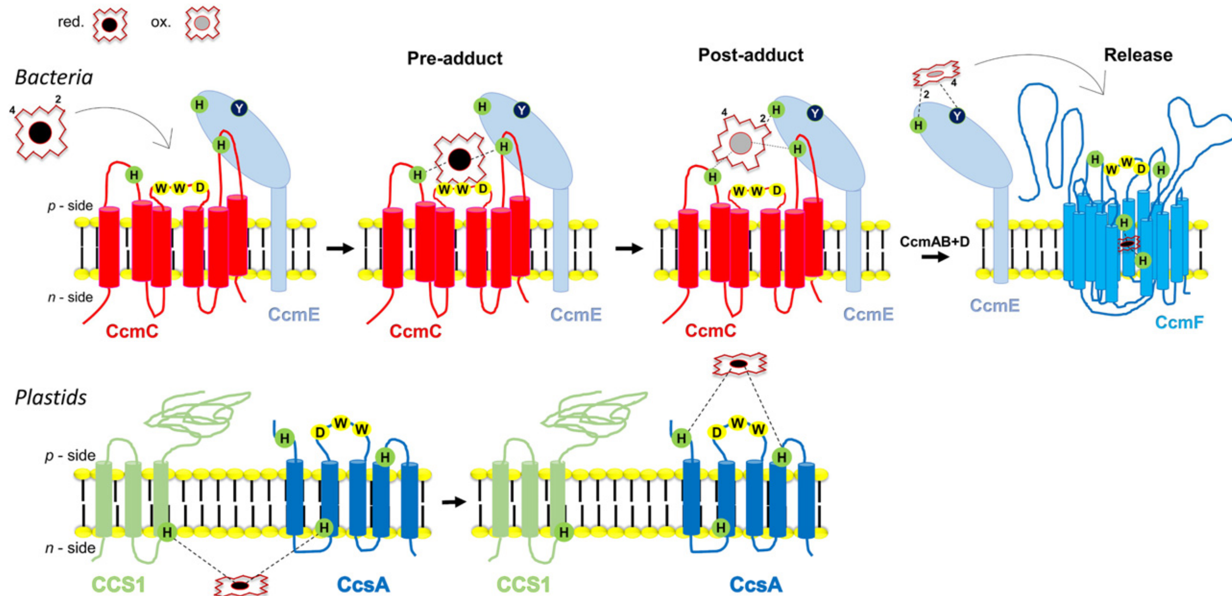


**Fig. 4** *Heme delivery pathway*. The top panel shows the *p*-side heme trafficking pathway in System I. The pathway comprises CcmC, CcmE and CcmF, which directly interact with heme, CcmD and CcmAB (not shown) that do no interact with heme but are involved in heme trafficking. First, reduced heme is loaded onto the "pre-adduct" complex formed by CcmCDE, where heme interacts via "WWD" motif of CcmC. The conserved histidines of CcmC serve as axial ligands for coordinating the Fe atom of heme and CcmE also provides surfaces of interaction with heme. Once heme is loaded, a 'post-adduct' complex of CcmCDE is formed where heme is oxidized and covalently linked to a conserved histidine of CcmE via, presumably, the 2-vinyl, while still axially ligated by CcmC histidines. Next, heme-containing CcmE is presumed to be released from CcmC by the activity of CcmAB and this step is also dependent upon CcmD. Released heme-containing CcmE is presented to CcmF, which contains a structural heme *b* buried inside the membrane. The heme contained in released CcmE is still covalently bound but no longer coordinated by CcmF histidines. In released CcmE, a conserved tyrosine provides axial ligation. The bottom panel shows the heme trafficking pathway in plastids, where CCS1 and CcsA function as a complex to deliver heme across the thylakoid membrane and also as a holocytochrome *c* synthase. The plastid heme trafficking pathway is inferred from the analysis of a natural CcsBA fusion in bacteria where CcsB corresponds to CCS1. The *n*-side histidines form the internal heme binding site. They coordinate heme and provide an entry site into the CCS heme exporter. Heme is translocated across the lipid bilayer and handled by the "WWD" motif and coordinated by the *p*-side histidines that form the external heme binding site. CcmC, CcmF and CcsA are heme handling proteins (HHP family) that contain conserved "WWD" motif, represented by yellow circles. Conserved histidines that participate in axial ligation of heme are represented by green circles. The tyrosine residue providing axial ligation in released heme-containing CcmE is shown in black. In heme, "2" and "4" indicate the 2-vinyl and 4-vinyl, respectively.

pathway. The first step of the heme trafficking pathway involves the formation of a ternary CcmCDE "pre-adduct" complex with the "WWD" domain acting as a heme interacting platform and the *p*-side histidines serving as axial ligands of the iron atom (**Fig. 4**) (Richard-Fogal *et al.*, 2009). If the "WWD" domain is the defining sequence feature of HHPs, a direct interaction with the porphyrin ring was lacking until chemical cross-linking of heme to CcmC "WWD" domain could be demonstrated (Sutherland *et al.*, 2018a). Because the presence of CcmE is necessary for heme binding onto CcmC, a buried heme binding site in CcmE also serves as an additional surface of interactions to form the heme-containing "pre-adduct" complex (Harvat *et al.*, 2009). Cross-linking experiments yielded a refined map of the heme binding cavity in CcmE, where distinct pockets accommodating the positioning of each of the vinyl groups of heme could be evidenced (Sutherland *et al.*, 2018a). Following this first step, a "post-adduct" complex forms where heme is under the oxidized form and covalently linked to a conserved histidine on CcmE, with CcmC histidines still providing axial ligation to iron in the heme moiety (**Fig. 4**). The covalent adduct of heme with CcmE has generated considerable interest because the chemical bond is between the $\beta$ carbon of the vinyl (postulated to be 2-vinyl) and a nitrogen atom on a conserved histidine in CcmE (Lee *et al.*, 2005). While the chemistry of this reaction is still an enigma, there is evidence the heme iron needs to be oxidized to react with this carbon on the vinyl side chain (Kranz *et al.*, 2009; Travaglini-Allocatelli, 2013). Hence, an unknown oxidant must catalyze oxidation of the heme iron as it is highly improbable that the chemistry of the reaction is left to chance in vivo. In a subset of organisms where CcmE histidine is replaced by a cysteine, heme is covalently attached to the CcmE cysteine (Goddard *et al.*, 2010; Mavridou *et al.*, 2013a). Because CcmE cysteine variant co-occurs with the absence of thiol-disulfide oxido-reductases CcmG and CcmH in some archaea and sulfate-reducing bacteria, it is possible the redox environment on the *p*-side in such organisms dictates a different chemistry for the CcmE-dependent relay of heme.

The current view is that heme-containing CcmE is released from CcmC via the activity of the CcmA and CcmB, the two components of an ABC (ATP binding cassette)-type transporter. This ATP-driven energy input is considered as a necessary step to communicate conformational changes in order to position CcmE towards the heme accepting side defined by the CcmFH ligase (**Figs. 4** and **5**). Released heme-containing CcmE is no longer coordinated by the *p*-side CcmC histidines and a conserved tyrosine residue in CcmE provides axial ligation. CcmD, a small *n*-side facing membrane anchored protein is essential for the

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CcmAB-dependent release of heme-containing CcmE. CcmD does not bind heme but, based on protein modeling, is predicted to interact with CcmC "WWD" domain via a conserved *p*-side facing conserved tyrosine to dislodge the heme onto the "WWD" platform during the ATP-driven conformational change (Richard-Fogal and Kranz, 2010; Sutherland *et al.*, 2018a). CcmAB is phylogenetically related to the exporter subfamily of ABC transporter and an alternative view favors a model where CcmAB functions as a classical transporter and translocates an unknown reductant required for detaching heme from CcmE (Kranz *et al.*, 2009). However, all the biochemical evidence provided so far supports a function for CcmAB in releasing heme-containing CcmE from CcmC and this is the more generally accepted view in the field.

### A Heme Exporter in the CCS Pathway

In System II, the delivery of heme to apocytochrome *c* proceeds through a different mechanism, which appears, on the surface, simple and requires the CcsA/ResC and CcsB/ResB/CCS1 proteins (Kranz *et al.*, 2009; Travaglini-Allocatelli, 2013; Mavridou *et al.*, 2013b) (**Fig. 4**). Initial functional dissection of CcsA and CCS1 in chloroplasts led to the proposition that the two proteins work as a complex in a transmembrane heme delivery pathway from *n*-side to *p*-side (Gabilly and Hamel, 2017). This proposal was supported by topological studies, site-directed mutagenesis of putative heme-interacting residues such as a "WWD" motif in CcsA, strictly conserved histidine residues in CcsA and CCS1, and molecular description of existing mutant alleles (Gabilly and Hamel, 2017) (**Fig. 4**). This hypothetical biochemical function was substantiated by elegant genetic and biochemical investigations using a naturally occurring CcsB-CcsA fusion protein in a System II bacterium, referred to as CcsBA, as a model of study (Frawley and Kranz, 2009). The role of CcsBA was demonstrated via complementation of an *E. coli* mutant lacking the entire CCM pathway and expressing the *ccsBA* gene from *Helicobacter hepaticus*. In the absence of any other Ccm factors, CcsBA-dependent cytochrome *c* assembly could proceed, demonstrating the bifunctional activity of the CcsBA as heme translocase and holocytochrome *c* synthase (Richard-Fogal *et al.*, 2012; Frawley and Kranz, 2009). Spectroscopic analyses of the purified functional CcsBA indicate the presence of heme in an external heme binding site formed by the "WWD" motif and the *p*-side histidines (**Fig. 4**). Binding of heme at the external site is not dependent upon the apocytochrome *c* substrate, and physical interaction of heme to the "WWD" domain was documented via chemical cross-linking (Sutherland *et al.*, 2018b). An internal heme binding site with two key *n*-side histidines in the CcsB and CcsA domain of the fusion protein was mapped in CcsBA (**Fig. 4**). Mutations of either *n*-side histidine abolish cytochrome *c* assembly and CcsBA can no longer be purified with bound heme. This was interpreted as an indication that heme enters the CcsBA protein via an internal heme pocket containing the two *n*-side histidines and is translocated to the *p*-side. Chemical correction of the *n*-side histidine mutations by application of exogenous imidazole was taken as experimental evidence for the bis-histidinyl axial ligation of the heme substrate entering the channel through the internal heme binding site (Kern *et al.*, 2010; Frawley and Kranz, 2009; Sutherland *et al.*, 2018b).

## Chemical Reduction of Ferriheme to Ferroheme

### CcmF-Dependent Heme Reduction

If chemical reduction of ferriheme to ferroheme was expected to be an enzymatically-assisted process, the identity of the catalyst(s) controlling this process has long remained elusive until the spectroscopic analysis of the Ccm heme delivery protein complexes (Kranz *et al.*, 2009). Surprisingly, through this study, one non-covalently bound heme was detected in CcmF (Richard-Fogal *et al.*, 2009). This structural heme was present in stoichiometric amount and spectral signature suggested the prosthetic group was of the *b*-type. Because heme iron is oxidized in the CcmCDE post-adduct complex (**Fig. 4**) and must undergo reduction to detach from CcmE, an attractive scenario is that CcmF transfers electrons to the heme substrate via the structural heme as an intermediate (**Fig. 5**). The source of reducing power in vivo is still unidentified but quinol-dependent reduction of CcmF structural heme can be achieved in vitro (Richard-Fogal *et al.*, 2009). A putative quinol binding pocket was recognized in CcmF but alteration of key residues does not abolish CcmF function in cytochrome *c* assembly (Mavridou *et al.*, 2013a). The measurement of the midpoint potential of CcmF structural heme is compatible with the idea that several "natural" reductants (e.g., NADH or flavin) can serve as physiological electron donors (Babbitt *et al.*, 2017). The reducing power could be transferred directly or indirectly via additional redox intermediates (Sutherland *et al.*, 2018b). The identity of the ligands for the structural heme *b* in CcmF was examined and two strictly conserved within-membrane histidines (**Fig. 5**), one of which is required for binding of heme, were postulated to provide axial coordination (Richard-Fogal *et al.*, 2009). Additionally, a *b*-heme embedded within CcmF was recently identified in the solved structure of the *Thermus thermophilus* protein, and the within-membrane histidines were found to be positioned within an expected distance for iron coordination (Brausemann, 2017). Bound Cu and Zn ions were also detected and while this awaits further experimental validation, it is tempting to hypothesize that Cu acts as an additional redox center for the transfer of electrons to reduce the heme substrate.

### CCS-Dependent Heme Reduction?

In the CCS pathway, there is no experimental support for the recruitment of a ferriheme reductase prior to the thioether reaction. Replacement of either of the two *p*-side heme-coordinating histidines in the external heme binding site of the CcsBA heme channel

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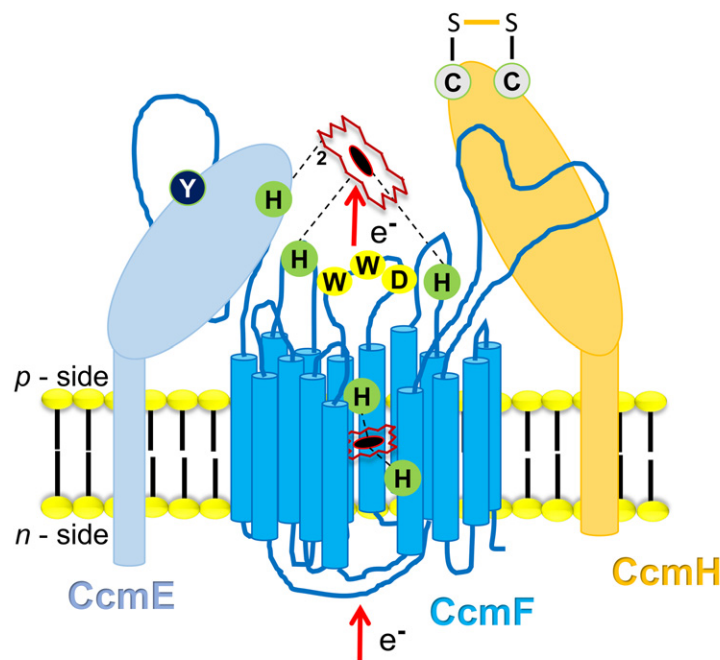


**Fig. 5** *CcmF-dependent Heme reduction.* CcmF contains a non-covalently bound structural heme *b* coordinated axially by conserved histidines present in its transmembrane domains. CcmF transfers electrons to the ferriheme bound to CcmE via the *b*-type heme, resulting in its reduction to ferroheme and presumed release from CcmE. The source of electrons to the structural *b*-heme in CcmF is not known. In complex with CcmH, CcmF is postulated to present heme to apocytochrome *c* via "WWD" motif for covalent attachment. Heme is shown coordinated via the CcmF histidines and still covalently attached to CcmE. The disulfide bond in CcmH is shown in yellow, and the electron transfer is shown by red arrows. In heme, "2" indicates the 2-vinyl.

changes the redox state of the bound heme substrate from reduced to oxidized (Frawley and Kranz, 2009) (**Fig. 4**). This led to the hypothesis that heme is protected from oxidation via bis-histidinyl ligation and is maintained reduced in this manner. An attractive model is that once reduced, iron ($Fe^{2+}$) is incorporated into the porphyrin ring via the activity of ferrochelatase on the *n*-side and, is delivered directly onto the *n*-side histidines forming the internal heme binding site of the CcsBA heme channel (see Section "A Heme Exporter in the CCS Pathway"). For heme to be channeled, physical interaction between ferrochelatase and the CCS heme exporter must take place. Alternatively, considering the heme pool needs to be distributed to several different hemoproteins (some of which are located in different cellular compartments in the case of heme synthesized in the plastid), dedicated factors controlling ferroheme allocation upon release from ferrochelatase to the CCS heme exporter could exist. In *Arabidopsis* chloroplasts, a distribution of task with one ferrochelatase producing heme for the photosynthetic proteins and the other supplying heme to the rest of the cell has been suggested (Woodson *et al.*, 2011).

## Thioether Bond Formation

### CcmF-Dependent Heme Ligation

In System I, there are several biochemical evidences that a CcmFH complex (or CcmFHI in instances where CcmI is a separate component) catalyzes the terminal step of cytochrome *c* maturation, i.e., formation of thioether bond linkages (Verissimo *et al.*, 2017; Sanders *et al.*, 2008; Ren *et al.*, 2002, San Francisco *et al.*, 2014). While the mechanistic details of this chemical reaction are not known, Ccm-dependent thioether bond formation is likely to be far more complicated than the one described for HCCS (see Section "HCCS-Dependent Heme Ligation"). How CcmF ligates heme is not known. A model is emerging where CcmF, with its 15 transmembrane domains, creates an ideal chemical environment to promote the release of heme from CcmE, followed by the presentation of heme to apocytochrome *c* via the WWD motif in interaction with CcmH (**Figs. 5** and **6**). CcmF displays several periplasmic domains, including a large C-terminal periplasmic domain, apparently devoid of recognizable sequence features, which appears flexible in the resolved structure (Brausemann, 2017). Such domains may mediate intermolecular recognition processes with apocytochrome *c* and one or several component(s) involved in the ligation process. The distinct CcmF polypeptides in mitochondrial System I might correspond to the separable functions of the protein in cytochrome *c* maturation (Giegé *et al.*, 2008).

CcmH is a membrane-tethered *p*-side facing thiol-disulfide oxido-reductase whose function in the maturation process is still a matter of considerable debate. The soluble domain of CcmH can form a cross-link between the second solvent-exposed cysteine of

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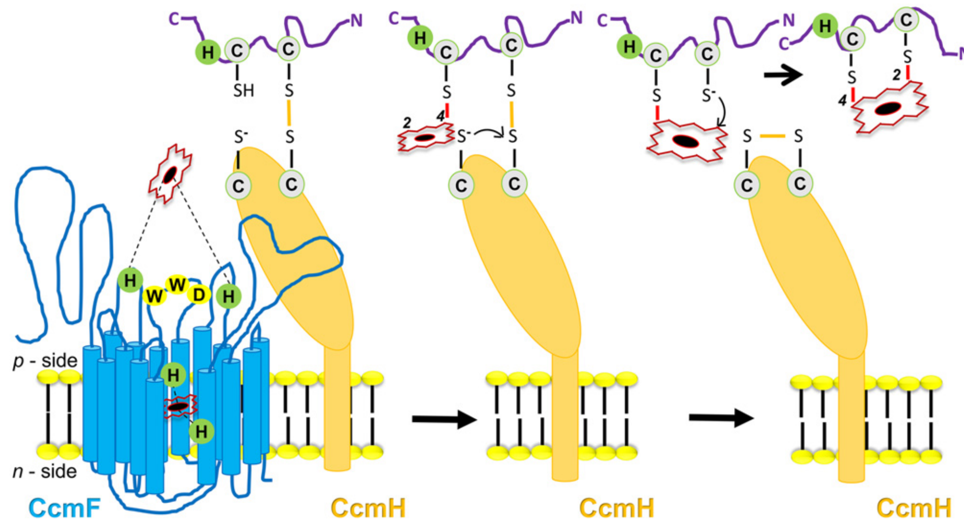


**Fig. 6** *CcmF-dependent heme ligation*. CcmF presents heme to apocytochrome *c* via the "WWD" domain. CcmH forms a disulfide link (yellow bond) with the first cysteine of apocytochrome *c* heme binding site, followed by formation of thioether linkage between the second cysteine of apocytochrome *c* CXXCH with 4-vinyl group of heme (red bond). The last step of heme ligation involves formation of the second thioether bond between the first cysteine of apocytochrome *c* heme binding site and 2-vinyl of heme, and reoxidation of CcmH. CcmH is re-oxidized via resolution of the CcmH-apocytochrome *c* disulfide via CcmG (not shown) or the second cysteine of CcmH as shown on the figure. Disulfide bonds are indicated in yellow and thioether bonds are depicted in red. In heme, "2" and "4" indicate the 2-vinyl and 4-vinyl, respectively.

its redox motif and the first cysteine of the CXXCH of an apocytochrome *c* substrate in vitro (Verissimo *et al.*, 2017; Di Matteo *et al.*, 2007) (**Fig. 6**). Surprisingly, CcmG appears very efficient in resolving this mixed disulfide in vitro. In *Rhodobacter*, the purification of a CcmFHI complex containing CcmG and the tight interaction between CcmG and CcmH supports the direct involvement of CcmH in the heme ligation reaction. To form a disulfide link with reduced apocytochrome *c*, CcmH must be in the oxidized form and this was experimentally verified by trapping the in vivo redox state of its cysteines (Verissimo *et al.*, 2017; Monika *et al.*, 1997). In accordance with this result, a *Rhodobacter ccmH*-null mutant can be rescued for cytochrome *c* assembly by cysteine or cystine supplementation, indicating that provision of an oxidant (if cysteine is assumed to be oxidized to cystine inside the cell) is probably the chemical basis for the phenotypic rescue (Cox *et al.*, 2001). A preliminary "model" where CcmH-apocytochrome *c* disulfide is required as a mechanism to block the first heme-binding thiol to allow thioether bond formation with the second heme-binding thiol and the 4-vinyl of heme (presented by CcmE) is now emerging (**Fig. 6**). In vitro, the mixed disulfide between CcmH and apocytochrome *c* could be resolved efficiently by CcmG or less efficiently by the second cysteine of CcmH (Verissimo *et al.*, 2017) (**Fig. 6**). It is now apparent that CcmG reductase activity extends beyond the disulfide bonded CXXCH motif in apocytochrome c, its cognate substrate.

This model suggests there is a preferred order in the formation of the two thioether links at the CXXCH, with the second cysteine thiol in the motif reacting first to heme. Interestingly, studies of HCCS-dependent heme attachment to single cysteine heme binding motif is also suggestive of the same sequence of reaction for the vinyl groups (Babbitt *et al.*, 2017). This sequential attachment of the vinyl side chains could be a universal feature in cytochrome *c* assembly, but this warrants further experimental exploration.

## HCCS-Dependent Heme Ligation

Our understanding of thioether bond formation is still incomplete but the elaboration of the biochemical activity of HCCS, the prototypical System III component provided considerable insights into this reaction. The study of HCCS enzymology was greatly facilitated by the fact that HCCS-dependent assembly of mitochondrial cytochrome *c* could be achieved in the bacterial cytoplasm without any additional assembly factors (Babbitt *et al.*, 2014a; San Francisco *et al.*, 2013; Babbitt *et al.*, 2014b). The use of multiple HCCS and apocytochrome substrate variants enabled the purification of different intermediates and for the first time, a detailed step-by-step molecular mechanism underlying heme attachment could be proposed (Alvarez-Paggi *et al.*, 2017; Babbitt *et al.*, 2015).

If ferroheme and reduced apocytochrome *c* are the substrates of HCCS, there is an order in which they are recognized by HCCS. Heme is first bound by HCCS via a strictly conserved histidine and heme interacting residues, mapping to two different domains (I and II). HCCS variants lacking the conserved histidine in domain II are non-functional but can be restored for cytochrome *c* assembly by provision of exogenous imidazole. This chemical rescue was taken as evidence of the histidine residue lying within a heme binding cavity. Inspection of the residues in domain II in HCCS revealed a sequence relationship to the "WWD" domain present in CcmC, CcmF and CcsA/ResC. In all four proteins, the spacing and the identity of functional important residues appear to be remarkably conserved. The HRM motif (Heme Regulatory Motif, CPX, where X is preferentially a hydrophobic residue),

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recognized in HCCS and initially postulated to interact with heme lie outside domains I or II and a possible function in heme binding appears unlikely. After heme binding by HCCS, apocytochrome *c* is recognized to form a ternary complex where the proximal histidine of the CXXCH motif provides a second coordination to the heme iron. The bis-histidinyl coordination of heme is likely to contribute to stabilize the apocytochrome *c*-heme complex. HCCS specificity toward its cognate apocytochrome *c* substrates is strict, and regions within the apoprotein must play a role in facilitating the recognition by the heme containing HCCS. One obvious determinant shown to be required for apocytochrome interaction with HCCS is the conserved heme binding motif. HCCS-dependent maturation of cytochromes *c* probably needs extended regions in the apoprotein beyond the CXXCH. A structurally conserved alpha-helix common to the fold of all *c*-type cytochromes and preceding the CXXCH motif was evidenced to be an essential determinant for the HCCS-dependent assembly of cytochrome *c*. Specifically, this structural determinant is not required for apocytochrome recognition by HCCS but appears key for positioning the first cysteine of the CXXCH motif when bound to HCCS (Babbitt *et al*., 2016). Yeast apocytochrome $c_1$ with changes in the CXXCH intervening residues becomes a strict substrate of HCCS and can no longer be acted upon by $HCC_1S$ (Corvest *et al*., 2010). In a separate study, mutating one of the variable *X* residues was found to cause profound changes in the local flexibility of the CXXCH motif (Bowman and Bren, 2008). Hence, the conformational propensity of the heme binding site could be a key feature for interaction between HCCSs and their cognate substrates, either for the recognition step and/or for the correct positioning of the heme-linking cysteines. There is no information available concerning the region of HCCS involved in the recognition and binding of the apocytochrome *c* substrate. The specificity of yeast HCCS towards apocytochrome $c_1$ is enhanced by altering residues adjacent to the conserved histidine in domain II, an indication that the heme binding domain might also play a role in substrate-enzyme interaction (Bernard *et al*., 2003). Provided the cysteines and the heme group are in the correct spatial disposition, thioether bond formation probably occurs via a spontaneous chemical reaction. The designated activity of HCCS might not be the direct catalysis of thioether bond formation, but possibly the selection of the correct rotational isomer of heme followed by the stabilization of a productive ternary heme-polypeptide complex allowing the chemistry of thioether bond linkage. There appears to be a mechanism for the release of holocytochrome *c* from HCCS and residues accelerating or slowing down this process have been identified (Mendez *et al*., 2017). The process is still not entirely deciphered but a conformational change due to the distortion of attached heme, which can be monitored spectroscopically, appears to provide a reasonable molecular basis. This change might induce a ligand switch between the histidine in HCCS and the distal ligand in apocytochrome, which is a methionine in all mitochondrial cytochromes *c*. There is compelling chemical evidence that the covalent attachment of heme influences the interaction between histidine and the iron of the heme molecule (Bowman and Bren, 2008). Hence, the displacement of the histidine imidazole (providing axial ligation to heme in HCCS) by the apocytochrome methionine sulfur following thioether bond formation is a reasonable proposal. Following its release from HCCS, apocytochrome *c* acquires its native structure through spontaneous folding. HCCS-dependent heme attachment to single heme-linking cysteine in apocytochrome *c* variants was also achieved in the bacterial cytoplasm (San Francisco *et al*., 2013; Mendez *et al*., 2017; Rosell and Mauk, 2002). HCCS-like proteins are not recognized in Euglenozoans that assemble mitochondrial cytochromes *c* with heme attached via a single thioether to a A/FXXCH motif. Hence, heme attachment to this unique type of cytochromes *c* must be dependent on assembly components that are distinct from HCCS (Allen, 2011). At the present time, there is no biochemical evidence that HCCS can function in the reverse direction, i.e., breaking the thioether bonds to release heme and apocytochrome *c*. Such an activity must exist in vivo as *Caenorhabditis elegans*, a natural heme auxotroph, is able to grow using a variety of natural and artificial *c*-type cytochromes as their only dietary source (Murphey *et al*., 2018).

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## References

Allen, J.W., Ginger, M.L., Ferguson, S.J., 2004. Maturation of the unusual single-cysteine (XXXCH) mitochondrial c-type cytochromes found in trypanosomatids must occur through a novel biogenesis pathway. Biochem. J. 383, 537–542.

Allen, J.W., Jackson, A.P., Rigden, D.J., *et al*., 2008. Order within a mosaic distribution of mitochondrial *c*-type cytochrome biogenesis systems? FEBS J. 275, 2385–2402.

Allen, J.W.A., 2011. Cytochrome *c* biogenesis in mitochondria – Systems III and V. FEBS J. 278, 4198–4216.

Alvarez-Paggi, D., Hannibal, L., Castro, M.A., *et al*., 2017. Multifunctional cytochrome *c*: Learning new tricks from an old dog. Chem. Rev. 117, 13382–13460.

Ambler, R.P., 1991. Sequence variability in bacterial cytochromes *c*. Biochim. Biophys. Acta 1058, 42–47.

Avendaño-Monsalve, M.C., Ponce-Rojas, J.C., Funes, S., 2020. From cytosol to mitochondria: The beginning of a protein journey. Biol. Chem. 401, 645–661.

Babbitt, S.E., San Francisco, B., Bretsnyder, E.C., Kranz, R.G., 2014a. Conserved residues of the human mitochondrial holocytochrome c synthase mediate interactions with heme. Biochemistry 53, 5261–5271.

Babbitt, S.E., San Francisco, B., Mendez, D.L., *et al*., 2014b. Mechanisms of mitochondrial holocytochrome c synthase and the key roles played by cysteines and histidine of the heme attachment site, Cys-XX-Cys-His. J. Biol. Chem. 289, 28795–28807.

Babbitt, S.E., Hsu, J., Kranz, R.G., 2016. Molecular basis behind inability of mitochondrial holocytochrome *c* synthase to mature bacterial cytochromes: Defining a critical role for cytochrome c α Helix-1. J. Biol. Chem. 291, 17523–17534.

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Babbitt, S.E., Hsu, J., Mendez, D.L., Kranz, R.G., 2017. Biosynthesis of single thioether c-type cytochromes provides insight into mechanisms intrinsic to holocytochrome c synthase (HCCS). Biochemistry 56, 3337–3346.

Babbitt, S.E., Sutherland, M.C., Francisco, B.S., Mendez, D.L., Kranz, R.G., 2015. Mitochondrial cytochrome *c* biogenesis: No longer an enigma. Trends Biochem. Sci. 40, 446–455.

Bamford, V.A., Angove, H.C., Seward, H.E., *et al.*, 2002. Structure and spectroscopy of the periplasmic cytochrome *c* nitrite reductase from *Escherichia coli*. Biochemistry 41, 2921–2931.

Barker, P.D., Ferguson, S.J., 1999. Still a puzzle: Why is haem covalently attached in c-type cytochromes? Structure 7, 281–290.

Barker, P.D., Ferrer, J.C., Mylrajan, M., *et al.*, 1993. Transmutation of a heme protein. Proc. Natl. Acad. Sci. USA 90, 6542–6546.

Bendall, D.S., 2016. Keilin, cytochrome and its nomenclature. In: Cramer, W.A., Kallas, T. (Eds.), Cytochrome Complexes: Evolution, Structures, Energy Transduction, and Signaling. Dordrecht: Springer Netherlands.

Bernard, D.G., Gabilly, S.T., Dujardin, G., Merchant, S., Hamel, P.P., 2003. Overlapping specificities of the mitochondrial cytochrome *c* and $c_1$ heme lyases. J. Biol. Chem. 278, 49732–49742.

Bernard, D.G., Quevillon-Cheruel, S., Merchant, S., Guiard, B., Hamel, P.P., 2005. Cyc2p, a membrane-bound flavoprotein involved in the maturation of mitochondrial *c*-type cytochromes. J. Biol. Chem. 280, 39852–39859.

Bertini, I., Cavallaro, G., Rosato, A., 2006. Cytochrome *c*: Occurrence and functions. Chem. Rev. 106, 90–115.

Bertini, I., Cavallaro, G., Rosato, A., 2007. Evolution of mitochondrial-type cytochrome *c* domains and of the protein machinery for their assembly. J. Inorg. Biochem. 101, 1798–1811.

Bonnard, G., Corvest, V., Meyer, E.H., Hamel, P.P., 2010. Redox processes controlling the biogenesis of *c*-type cytochromes. Antioxid. Redox Signal. 13, 1385–1401.

Bowman, S.E.J., Bren, K.L., 2008. The chemistry and biochemistry of heme *c*: Functional bases for covalent attachment. Nat. Prod. Rep. 25, 1118–1130.

Brausemann, A., 2017. Crystal Structure of CcmF: The Cytochrome c Maturation Heme Lyase. Universitätsbibliothek Freiburg.

Bushweller, J.H., 2020. Protein disulfide exchange by the intramembrane enzymes DsbB, DsbD, and CcdA. J. Mol. Biol. 432, 5091–5103.

Chambers, I.G., Willoughby, M.M., Hamza, I., Reddi, A.R., 2021. One ring to bring them all and in the darkness bind them: The trafficking of heme without deliverers. Biochim. Biophys. Acta 1868, 118881.

Chatzi, A., Manganas, P., Tokatlidis, K., 2016. Oxidative folding in the mitochondrial intermembrane space: A regulated process important for cell physiology and disease. Biochim. Biophys. Acta 1863, 1298–1306.

Cho, S.-H., Collet, J.-F., 2013. Many roles of the bacterial envelope reducing pathways. Antioxid. Redox Signal. 18, 1690–1698.

Cline, S.G., Laughbaum, I.A., Hamel, P.P., 2017. CCS2, an octatricopeptide-repeat protein, is required for plastid cytochrome c assembly in the green alga Chlamydomonas reinhardtii. Front. Plant Sci. 8, 1306.

Cline, S.G., Gabilly, S.T., Subrahmanian, N., Hamel, P.P., 2016. Cofactor assembly of cytochrome $bc_1$-$b_6f$ complexes. In: Cramer, W.A., Kallas, T. (Eds.), Cytochrome Complexes: Evolution, Structures, Energy Transduction, and Signaling. Dordrecht: Springer Netherlands.

Colbert, C.L., Wu, Q., Erbel, P.J., Gardner, K.H., Deisenhofer, J., 2006. Mechanism of substrate specificity in *Bacillus subtilis* ResA, a thioredoxin-like protein involved in cytochrome *c* maturation. Proc. Natl. Acad. Sci. USA 103, 4410–4415.

Corvest, V., Murrey, D.A., Bernard, D.G., *et al.*, 2010. *c*-type cytochrome assembly in *Saccharomyces cerevisiae*: A key residue for apocytochrome $c_1$/lyase interaction. Genetics 186, 561–571.

Corvest, V., Murrey, D.A., Hirasawa, M., *et al.*, 2012. The flavoprotein Cyc2p, a mitochondrial cytochrome c assembly factor, is a NAD(P)H-dependent haem reductase. Mol. Microbiol. 83, 968–980.

Cox, R.L., Patterson, C., Donohue, T.J., 2001. Roles for the *Rhodobacter sphaeroides* CcmA and CcmG proteins. J. Bacteriol. 183, 4643–4647.

Crow, A., Acheson, R.M., Le Brun, N.E., Oubrie, A., 2004. Structural basis of redox-coupled protein substrate selection by the cytochrome *c* biosynthesis protein ResA. J. Biol. Chem. 279, 23654–23660.

Daltrop, O., Ferguson, S.J., 2003. Cytochrome *c* maturation. The in vitro reactions of horse heart apocytochrome *c* and *Paracoccus dentrificans* apocytochrome $c_{550}$ with heme. J. Biol. Chem. 278, 4404–4409.

Daltrop, O., Ferguson, S.J., 2004. In vitro studies on thioether bond formation between *Hydrogenobacter thermophilus* apocytochrome $c_{552}$ with metalloprotoporphyrin derivatives. J. Biol. Chem. 279, 45347–45353.

Daltrop, O., Smith, K.M., Ferguson, S.J., 2003. Stereoselective in vitro formation of *c*-type cytochrome variants from *Hydrogenobacter thermophilus* containing only a single thioether bond. J. Biol. Chem. 278, 24308–24313.

Daltrop, O., Allen, J.W., Willis, A.C., Ferguson, S.J., 2002. *In vitro* formation of a *c*-type cytochrome. Proc. Natl. Acad. Sci. USA 99, 7872–7876.

Davey, L., Halperin, S.A., Lee, S.F., 2016. Thiol-disulfide exchange in gram-positive firmicutes. Trends Microbiol. 24, 902–915.

de Vitry, C., 2011. Cytochrome *c* maturation system on the negative side of bioenergetic membranes: CCB or System IV. FEBS J. 278, 4189–4197.

Deshmukh, M., May, M., Zhang, Y., *et al.*, 2002. Overexpression of *ccl1-2* can bypass the need for the putative apocytochrome chaperone CycH during the biogenesis of c-type cytochromes. Mol. Microbiol. 46, 1069–1080.

Di Matteo, A., Gianni, S., Schinina, M.E., *et al.*, 2007. A strategic protein in cytochrome c maturation: Three-dimensional structure of CcmH and binding to apocytochrome c. J. Biol. Chem. 282, 27012–27019.

Dumont, M.E., 1996. Mitochondrial import of cytochrome c. In: Hart, F.-U. (Ed.), Advances in Molecular and Cell Biology. Greenwich, JAI Press.

Ferousi, C., Lindhoud, S., Baymann, F., *et al.*, 2019. Discovery of a functional, contracted heme-binding motif within a multiheme cytochrome. J. Biol. Chem. 294, 16953–16965.

Frain, K.M., Gangl, D., Jones, A., Zedler, J.A., Robinson, C., 2016. Protein translocation and thylakoid biogenesis in cyanobacteria. Biochim. Biophys. Acta 1857, 266–273.

Frawley, E.R., Kranz, R.G., 2009. CcsBA is a cytochrome *c* synthetase that also functions in heme transport. Proc. Natl. Acad. Sci. USA 106, 10201–10206.

Gabilly, S.T., Hamel, P.P., 2017. Maturation of plastid *c*-type cytochromes. Front. Plant. Sci. 8, 1313.

Gabilly, S.T., Kropat, J., Karamoko, M., *et al.*, 2011. A novel component of the disulfide-reducing pathway required for cytochrome *c* assembly in plastids. Genetics 187, 793–802.

Giegé, P., Grienenberger, J.M., Bonnard, G., 2008. Cytochrome *c* biogenesis in mitochondria. Mitochondrion 8, 61–73.

Goddard, A.D., Stevens, J.M., Rao, F., *et al.*, 2010. *c*-Type cytochrome biogenesis can occur via a natural Ccm system lacking CcmH, CcmG, and the heme-binding histidine of CcmE. J. Biol. Chem. 285, 22882–22889.

Hallick, R.B., Hong, L., Drager, R.G., *et al.*, 1993. Complete sequence of *Euglena gracilis* chloroplast DNA. Nucleic Acids Res. 21, 3537–3544.

Hamel, P., Corvest, V., Giege, P., Bonnard, G., 2009. Biochemical requirements for the maturation of mitochondrial *c*-type cytochromes. Biochim. Biophys. Acta 1793, 125–138.

Harvat, E.M., Redfield, C., Stevens, J.M., Ferguson, S.J., 2009. Probing the heme-binding site of the cytochrome c maturation protein CcmE. Biochemistry 48, 1820–1828.

Karamoko, M., Gabilly, S.T., Hamel, P.P., 2013. Operation of trans-thylakoid thiol-metabolizing pathways in photosynthesis. Front. Plant Sci. 4, 476.

Katzen, F., Deshmukh, M., Daldal, F., Beckwith, J., 2002. Evolutionary domain fusion expanded the substrate specificity of the transmembrane electron transporter DsbD. EMBO J. 21, 3960–3969.

Kern, M., Scheithauer, J., Kranz, R.G., Simon, J., 2010. Essential histidine pairs indicate conserved haem binding in epsilon proteobacterial cytochrome *c* haem lyases. Microbiology 156, 3773–3781.

Kim, H.J., Jeong, M.Y., Parnell, T.J., *et al.*, 2016. The plasma membrane protein Nce102 implicated in eisosome formation rescues a heme defect in mitochondria. J. Biol. Chem. 291, 17417–17426.

Kletzin, A., Heimerl, T., Flechsler, J., *et al.*, 2015. Cytochromes *c* in Archaea: Distribution, maturation, cell architecture, and the special case of *Ignicoccus hospitalis*. Front. Microbiol. 6, 439.

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Kolli, R., Engstler, C., Akbaş, Ş., *et al*., 2020. The OXA2a insertase of arabidopsis is required for cytochrome c maturation. Plant Physiol. 184, 1042–1055.
Kranz, R.G., Richard-Fogal, C., Taylor, J.-S., Frawley, E.R., 2009. Cytochrome *c* biogenesis: Mechanisms for covalent modifications and trafficking of heme and for heme-iron redox control. Microbiol. Mol. Biol. Rev. 73, 510–528.
Lee, D., Pervushin, K., Bischof, D., Braun, M., Thöny-Meyer, L., 2005. Unusual heme-histidine bond in the active site of a chaperone. J. Am. Chem. Soc. 127, 3716–3717.
Lee, J.-H., Harvat, E.M., Stevens, J.M., Ferguson, S.J., Saier, M.H., 2007. Evolutionary origins of members of a superfamily of integral membrane cytochrome *c* biogenesis proteins. Biochim. Biophys. Acta 1768, 2164–2181.
Ma, S., Ludwig, R., 2019. Direct electron transfer of enzymes facilitated by cytochromes. ChemElectroChem 6, 958–975.
Matias, P.M., Coelho, A.V., Valente, F.M.A., *et al*., 2002. Sulfate respiration in *Desulfovibrio vulgaris* Hildenborough: Structure of the 16-heme cytochrome c HmcA at 2.5-Å resolution and a view of its role in transmembrane electron transfer. J. Biol. Chem. 277, 47907–47916.
Mavridou, D.A.I., Clark, M.N., Choulat, C., Ferguson, S.J., Stevens, J.M., 2013a. Probing heme delivery processes in cytochrome c biogenesis system I. Biochemistry 52, 7262–7270.
Mavridou, D.A.I., Ferguson, S.J., Stevens, J.M., 2013b. Cytochrome *c* assembly. IUBMB Life 65, 209–216.
Mendez, D.L., Babbitt, S.E., King, J.D., *et al*., 2017. Engineered holocytochrome *c* synthases that biosynthesize new cytochromes *c*. Proc. Natl. Acad. Sci. USA 114, 2235.
Metcalfe, C.L., Daltrop, O., Ferguson, S.J., Raven, E.L., 2007. Tuning the formation of a covalent haem-protein link by selection of reductive or oxidative conditions as exemplified by ascorbate peroxidase. Biochem. J. 408, 355–361.
Monika, E.M., Goldman, B.S., Beckman, D.L., Kranz, R.G., 1997. A thioreduction pathway tethered to the membrane for periplasmic cytochromes *c* biogenesis: In vitro and in vivo studies. J. Mol. Biol. 271, 679–692.
Murphey, A.C., Mavridou, D.A.I., Hodgkin, J., Ferguson, S.J., 2018. The heme auxotroph *Caenorhabditis elegans* can cleave the thioether bonds of *c*-type cytochromes. FEBS Lett. 592, 928–938.
Owji, H., Nezafat, N., Negahdaripour, M., Hajiebrahimi, A., Ghasemi, Y., 2018. A comprehensive review of signal peptides: Structure, roles, and applications. Eur. J. Cell Biol. 97, 422–441.
Palmer, T., Stansfeld, P.J., 2020. Targeting of proteins to the twin-arginine translocation pathway. Mol. Microbiol. 113, 861–871.
Ren, Q., Ahuja, U., Thony-Meyer, L., 2002. A bacterial cytochrome c heme lyase. CcmF forms a complex with the heme chaperone CcmE and CcmH but not with apocytochrome c. J. Biol. Chem. 277, 7657–7663.
Richard-Fogal, C., Kranz, R.G., 2010. The CcmC:Heme:CcmE complex in heme trafficking and cytochrome *c* biosynthesis. J. Mol. Biol. 401, 350–362.
Richard-Fogal, C.L., Frawley, E.R., Bonner, E.R., *et al*., 2009. A conserved haem redox and trafficking pathway for cofactor attachment. EMBO J. 28, 2349–2359.
Richard-Fogal, C.L., Frawley, E.R., Feissner, R.E., Kranz, R.G., 2007. Heme concentration dependence and metalloporphyrin inhibition of the system I and II cytochrome c assembly pathways. J. Bacteriol. 189, 455–463.
Richard-Fogal, C.L., San Francisco, B., Frawley, E.R., Kranz, R.G., 2012. Thiol redox requirements and substrate specificities of recombinant cytochrome *c* assembly Systems II and III. Biochim. Biophys. Acta 1817, 911–919.
Richardson, L.G.L., Schnell, D.J., 2019. Origins, function, and regulation of the TOC–TIC general protein import machinery of plastids. J. Exp. Bot. 71, 1226–1238.
Rosell, F.I., Mauk, A.G., 2002. Spectroscopic properties of a mitochondrial cytochrome C with a single thioether bond to the heme prosthetic group. Biochemistry 41, 7811–7818.
Salgueiro, C.A., Dantas, J.M., 2016. Multiheme Cytochromes. Berlin and Heidelberg: Springer.
San Francisco, B., Bretsnyder, E.C., Kranz, R.G., 2013. Human mitochondrial holocytochrome *c* synthase's heme binding, maturation determinants, and complex formation with cytochrome *c*. Proc. Natl. Acad. Sci. USA 110, E788–E797.
San Francisco, B., Sutherland, M.C., Kranz, R.G., 2014. The CcmFH complex is the System I holocytochrome *c* synthetase: Engineering cytochrome *c* maturation independent of CcmABCDE. Mol. Microbiol. 91, 996–1008.
Sanders, C., Turkarslan, S., Lee, D.W., *et al*., 2008. The cytochrome *c* maturation components CcmF, CcmH, and CcmI form a membrane-integral multisubunit heme ligation complex. J. Biol. Chem. 283, 29715–29722.
Sanders, C., Boulay, C., Daldal, F., 2007. Membrane-spanning and periplasmic segments of CcmI have distinct functions during cytochrome *c* biogenesis in *Rhodobacter capsulatus*. J. Bacteriol. 189, 789–800.
Sanders, C., Deshmukh, M., Astor, D., Kranz, R.G., Daldal, F., 2005. Overproduction of CcmG and CcmFH(Rc) fully suppresses the *c*-type cytochrome biogenesis defect of *Rhodobacter capsulatus* CcmI-null mutants. J. Bacteriol. 187, 4245–4256.
Santillán Torres, J.L., Atteia, A., Claros, M.G., González-Halphen, D., 2003. Cytochrome *f* and subunit IV, two essential components of the photosynthetic *bf* complex typically encoded in the chloroplast genome, are nucleus-encoded in *Euglena gracilis*. Biochim. Biophys. Acta 1604, 180–189.
Schulz, H., Hennecke, H., Thöny-Meyer, L., 1998. Prototype of a heme chaperone essential for cytochrome c maturation. Science 281, 1197–1200.
Simon, J., Hederstedt, L., 2011. Composition and function of cytochrome *c* biogenesis System II. FEBS J. 278, 4179–4188.
Stevens, J.M., Mavridou, D.A.I., Hamer, R., *et al*., 2011. Cytochrome *c* biogenesis System I. FEBS J. 278, 4170–4178.
Sutherland, M.C., Jarodsky, J.M., Ovchinnikov, S., Baker, D., Kranz, R.G., 2018a. Structurally mapping endogenous heme in the CcmCDE membrane complex for cytochrome c biogenesis. J. Mol. Biol. 430, 1065–1080.
Sutherland, M.C., Tran, N.L., Tillman, D.E., *et al*., 2018b. Structure-function analysis of the bifunctional CcsBA heme exporter and cytochrome c synthetase. mBio 9. doi:10.1128/mBio.02134-18.
Swenson, S.A., Moore, C.M., Marcero, J.R., *et al*., 2020. From synthesis to utilization: The ins and outs of mitochondrial heme. Cells 9.
Thöny-Meyer, L., 1997. Biogenesis of respiratory cytochromes in bacteria. Microbiol. Mol. Biol. Rev. 61, 337–376.
Tomlinson, E.J., Ferguson, S.J., 2000. Conversion of a *c* type cytochrome to a *b* type that spontaneously forms *in vitro* from apo protein and heme: implications for *c* type cytochrome biogenesis and folding. Proc. Natl. Acad. Sci. USA 97, 5156.
Travaglini-Allocatelli, C., 2013. Protein machineries involved in the attachment of heme to cytochrome *c*: Protein structures and molecular mechanisms. Scientifica 2013, 505714.
Turkarslan, S., Sanders, C., Ekici, S., Daldal, F., 2008. Compensatory thio-redox interactions between DsbA, CcdA and CcmG unveil the apocytochrome *c* holdase role of CcmG during cytochrome *c* maturation. Mol. Microbiol. 70, 652–666.
Verissimo, A.F., Daldal, F., 2014. Cytochrome *c* biogenesis System I: An intricate process catalyzed by a maturase supercomplex? Biochim. Biophys. Acta 1837, 989–998.
Verissimo, A.F., Khalfaoui-Hassani, B., *et al*., 2017. The thioreduction component CcmG confers efficiency and the heme ligation component CcmH ensures stereo-specificity during cytochrome c maturation. J. Biol. Chem. 292, 13154–13167.
Vilmos, F., Katharine, A.S., Stuart, J.F., Michael, L.G., James, W.A.A., 2009. Structure of a trypanosomatid mitochondrial cytochrome *c* with heme attached via only one thioether bond and implications for the substrate recognition requirements of heme lyase. FEBS J. 276, 2822–2832.
Waller, R.F., Kořený, L., 2017. Chapter four – Plastid complexity in dinoflagellates: A picture of gains, losses, replacements and revisions. In: Hirakawa, Y. (Ed.), Advances in Botanical Research. Academic Press.
Woodson, J.D., Perez-Ruiz, J.M., Chory, J., 2011. Heme synthesis by plastid ferrochelatase I regulates nuclear gene expression in plants. Curr. Biol. 21, 897–903.
Zito, F., Alric, J., 2016. Heme $c_i$ or $c_n$ of the cytochrome $b_6f$ complex: A short retrospective. In: Cramer, W.A., Kallas, T. (Eds.), Cytochrome Complexes: Evolution, Structures, Energy Transduction, and Signaling. Dordrecht: Springer Netherlands.