Biochimica et Biophysica Acta 1793 (2009) 125–138

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

# Biochemical requirements for the maturation of mitochondrial *c*-type cytochromes

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ARTICLE INFO

*Article history:*
Received 11 March 2008
Received in revised form 18 June 2008
Accepted 26 June 2008
Available online 4 July 2008

*Keywords:*
Mitochondria
Cytochrome *c* assembly
Thioether linkage
Heme transport
Heme lyase
Redox chemistry

ABSTRACT

Cytochromes *c* are metalloproteins that function in electron transfer reactions and contain a heme moiety covalently attached via thioether linkages between the co-factor and a CXXCH motif in the protein. Covalent attachment of the heme group occurs on the positive side of all energy-transducing membranes (bacterial periplasm, mitochondrial intermembrane space and thylakoid lumen) and requires minimally: 1) synthesis and translocation of the apocytochromes *c* and heme across at least one biological membrane, 2) reduction of apocytochromes *c* and heme and maintenance under a reduced form prior to 3) catalysis of the heme attachment reaction. Surprisingly, the conversion of apoforms of cytochromes *c* to their respective holoforms occurs through at least three different pathways (systems I, II and III). In this review, we detail the assembly process of soluble cytochrome *c* and membrane-bound cytochrome $c_1$, the only two mitochondrial *c*-type cytochromes that function in respiration. Mitochondrial *c*-type cytochromes are matured in the intermembrane space via the system I or system III pathway, an intriguing finding considering that the biochemical requirements for cytochrome *c* maturation are believed to be common regardless of the energy-transducing membrane under study.

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## 1. Introduction

### 1.1. Definition of c-type cytochromes

Cytochromes are redox active molecules that contain heme as a prosthetic group. Cytochromes are commonly recruited in electron transfer reactions and their redox activity is determined by the valence change of the iron atom in the heme co-factor ($Fe^{3+}$ to $Fe^{2+}$). This review is concerned with *c*-type cytochromes also generically referred to as cytochromes *c*, a distinct group of hemoproteins characterized by the covalent attachment of heme to the apoprotein. Cytochromes *c* constitute a structurally diverse family of molecules that are found on the so-called positive side (*p*-side),[1] of the energy-transducing membrane systems [1]. In all *c*-type cytochromes the heme is covalently attached to the polypeptide via two (or very rarely one) thioether linkages between the vinyl groups of heme and the cysteines sulfhydryls in the apocytochrome *c* [1,2] (Fig. 1). A CXXCH[2] sequence in the apocytochrome, also referred to as the heme binding site provides the sulfhydryls for formation of the thioether bonds, and the imidazole of the histidine residue serves as one of the axial ligands of the heme iron which is hexacoordinated. Four ligands of the heme iron are provided by the nitrogen atoms of the tetrapyrrole ring and an additional ligand is usually a methionine or a histidine residue in the protein [3,4] (Fig. 1). Exceptions to the CXXCH sequence are found in some bacterial cytochromes *c* such as diheme cytochrome $c_{552}$ (NirB) where CXXXCH is one of the heme binding sites [5]. Variation of the axial ligand also occurs in some bacterial *c*-type cytochromes such as the nitrite reductase NrfA that contains the unusual CXXCK heme binding site [6]. At the heme binding site, the first cysteine of the sequence always forms a stereospecific link with the side chain at the C3 position on the porphyrin ring while the second cysteine is invariably bonded to the side chain at the C8 position (Fig. 1). One intriguing exception is found in trypanosomatid mitochondria where heme is attached to the *c*-type apocytochromes via a single thioether bond at a F/AXXCH motif [7]. By analogy with the CXXCH motif, it is believed that the cysteine in the F/AXXCH motif is bonded to the vinyl at the C8 position but this has not been demonstrated. This view is reinforced by the finding that the vinyl in C3 is attached to a CXXAH apocytochrome *c* variant while the vinyl in C8 is linked to a AXXCH variant in an in vitro reconstitution of the heme ligation reaction [8].

Soluble and membrane-anchored cytochromes *c* are the two types of cytochromes *c* that are found in energy-transducing membrane systems [2,9]. Soluble *c*-type cytochromes, such as cytochrome *c* in mitochondria and cytochrome $c_6$ in plastids, display some structural and functional similarities and are involved in shuttling electrons from the cytochrome $bc_1$ (in bacteria and mitochondria) or $b_6f$ (cyanobacteria and plastids) complexes to an electron acceptor [2,10,11]. The

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[1] The *p*-side corresponds to the plastid lumen, mitochondrial intermembrane space, or bacterial periplasm. The *n*-side faces the plastid stroma, mitochondrial matrix or the bacterial cytoplasm.

[2] Where *X* can be any amino acid except cysteine.

0167-4889/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.bbamcr.2008.06.017

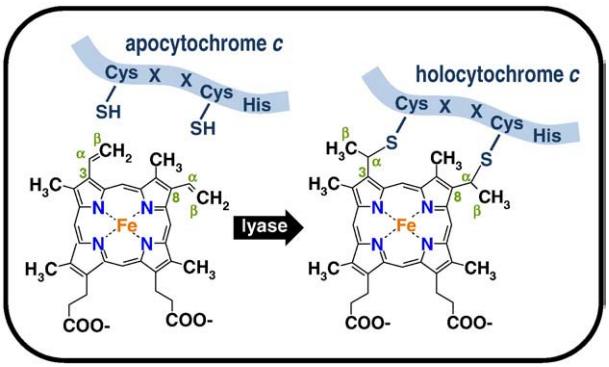


**Fig. 1.** Stereospecific heme attachment reaction. The lyase is the enzyme responsible for the stereospecific addition of the cysteinyl thiols from the apocytochrome C*XX*CH motif (shown in blue) to the α carbons at the vinyl group in positions C3 and C8 of Fe-protoporphyrin IX (heme, shown in black with iron in orange and nitrogen in blue). This reaction occurs on the *p*-side of the energy-transducing membranes. The numbering of the carbons atoms in the porphyrin rings follows the rules of the International Union of Pure and Applied Chemistry (IUPAC).

second major group of *c*-type cytochromes is defined by membrane-bound cytochromes $c_1$ (bacteria and mitochondria) and cytochromes *f* (cyanobacteria and plastids) of the $bc_1/b_6f$ complexes [2,11]. Cytochromes $c_1/f$ are structurally and evolutionarily distinct monotopic membrane proteins with a large hydrophilic N-terminal domain harboring the heme binding domain that is exposed to the *p*-side compartment. In mitochondria, membrane-bound cytochrome $c_1$ in the $bc_1$ complex and soluble cytochrome *c* that transfers the electrons from the $bc_1$ to the terminal cytochrome *c* oxidase are the only *c*-type cytochromes that participate in electron transfer in the intermembrane space (IMS) [5].

### 1.2. Three pathways for c-type cytochrome assembly

If *c*-type cytochromes stand among the best characterized molecules from structural analyses and mechanistic studies of electron transfer reactions [1,2], little is known about the biochemistry of their maturation. All holocytochromes *c* reside in a different location than the site of synthesis of their apoproteins and co-factor heme. Thus, they represent an attractive model to address the question of co-factor-protein assembly. Key questions in defining the steps and the biochemical requirements to complete holocytochrome *c* maturation have been addressed experimentally in the different membrane systems and the general conclusion is that heme and apocytochromes need to be transported independently to the *p*-side of the energy-transducing membrane and maintained in a reduced form prior to catalysis of the stereospecific addition of heme, the terminal step of the maturation process (for review [12–14]).

Extensive biochemical and genetic investigations have revealed the existence of three distinct systems (systems I, II and III, see Fig. 2) for assembling bacterial and organellar *c*-type cytochromes (for review [12,13–15]), an unexpected discovery for what appears to be on the surface a rather simple biochemical process (i.e. formation of thioether bonds at a conserved C*XX*CH heme binding site motif). The definition of the three systems is based on the occurrence of specific assembly components that are unique to each maturation pathway [12]. Mitochondrial cytochromes *c* are assembled via the system I or system III pathway (Fig. 2) and a distinct system (system V) is suspected to operate in trypanosomatid mitochondria based on the observation that no ortholog of the prototypical assembly factor of systems I, II or III is present in the genomes of *Trypanosoma* and *Leishmania* [7]. The CCB[3] pathway also referred to as system IV is a recently discovered maturation pathway required for the covalent attachment of heme $c_i$ to cytochrome $b_6$ in the $b_6f$ complex of cyanobacteria and photosynthetic eukaryotes [16–18]. Similarly to the heme bound in trypanosomatid mitochondrial cytochromes *c* and $c_1$, heme $c_i$ is attached to cytochrome $b_6$ via a single thioether bond. System IV is distinct from systems I, II and III in the sense that heme $c_i$ is not attached at a *XXX*CH motif and the iron atom has no axial amino acid ligand [19,20]. In addition, the heme $c_i$ is located on the *n*-side of the thylakoid membrane. Hence, the CCB pathway should not be regarded as a cytochrome *c* maturation pathway.

Although the focus of this review is the maturation pathways of mitochondrial cytochromes *c*, we will first describe the three systems for cytochrome *c* assembly, their prototypical components and their proposed activity in the maturation process. Then, we will discuss the specific biochemical steps required for the conversion of apocytochromes *c* and $c_1$ to their respective holoforms in the context of the system I and III assembly pathways which are the two routes required for mitochondrial cytochrome *c* assembly.

#### 1.2.1. System I/CCM

This pathway also referred to as the "CCM" (for *C*ytochrome *C* *M*aturation) has been extensively studied in α and γ-proteobacteria microbial models where it was initially discovered. On the basis of nuclear and mitochondrial genomes analyses and recent experimental investigation in the *Arabidopsis* plant model [21–23], it is now clear that system I is prevalent in land plants. It is also believed to operate in some red algae and protozoal mitochondria [1]. Extensive functional investigations in various bacteria, including *Escherichia coli* and *Rhodobacter capsulatus*, led to the identification of as many as 12 genes products (CcmA to CcmH, CcmI, DsbA, DsbB and DsbD/CcdA) whose activities are required to complete the conversion of multiple apocytochromes *c* to their respective holoforms. Typically, the cytochrome *c* assembly machinery is formed of 9 membrane and periplasmic assembly proteins (CcmA to CcmH and DsbD/CcdA, see Fig. 2). In some bacteria, the C-terminal of CcmH occurs as a separate protein (Ccm I). The implication of DsbA and DsbB in cytochrome *c* maturation is still questionable (see Section 5).

A detailed view of the system I apparatus for cytochrome *c* maturation in the bacterial periplasm has now emerged and the various components can be functionally categorized as follows: 1) A heme delivery system including an ABC transporter (CcmAB) whose role is still actively debated in the field [24] and a periplasmic heme relay with the operation of an heme interacting protein (CcmC), a "helper" protein (CcmD) and a unique heme chaperone (CcmE) (reviewed in [25–27]), 2) a transmembrane thio-reduction pathway defined by a thiol-disulfide transporter (DsbD or CcdA), a thioredoxin-like protein (CcmG) and a thiol-disulfide oxido-reductase (CcmH) involved in conveying reducing power across the membrane for maintenance of apocytochrome cysteine sulfhydryls in a reduced state prior to the heme lyase reaction (reviewed in [15,27–29]) and 3) a candidate heme lyase (CcmF) believed to facilitate the catalysis of the two thioether linkages [30–33].

System I in mitochondria displays some striking difference from its bacterial counterpart (for review [27]). For instance, CcmD, CcmI (corresponding to the C-terminal part of *E. coli* CcmH) and the prototypical component of the thio-reduction pathway DsbD/CcdA and CcmG, appear to be missing. It is conceivable that CcmD, CcmI, DsbD/CcdA and CcmG proteins are completely absent from the system I assembly machinery in mitochondria. Alternatively, these assembly factors might have evolved considerably so that they can no longer be recognized on the basis of sequence similarity. Interestingly, in the mitochondrial genome of land plants, CcmF occurs as split in multiple genes; each of which encodes a domain ($AtCcmF_{N1}$, $AtCcmF_{N2}$ and $AtCcmF_C$, see Fig. 2) showing similarity to a corresponding domain in the bacterial CcmF ortholog (for review [27]) (see Fig. 2).

[3] (C for Cofactor binding, C for Cytochrome $b_6f$ complex, and B for PetB subunit which is cytochrome $b_6$).

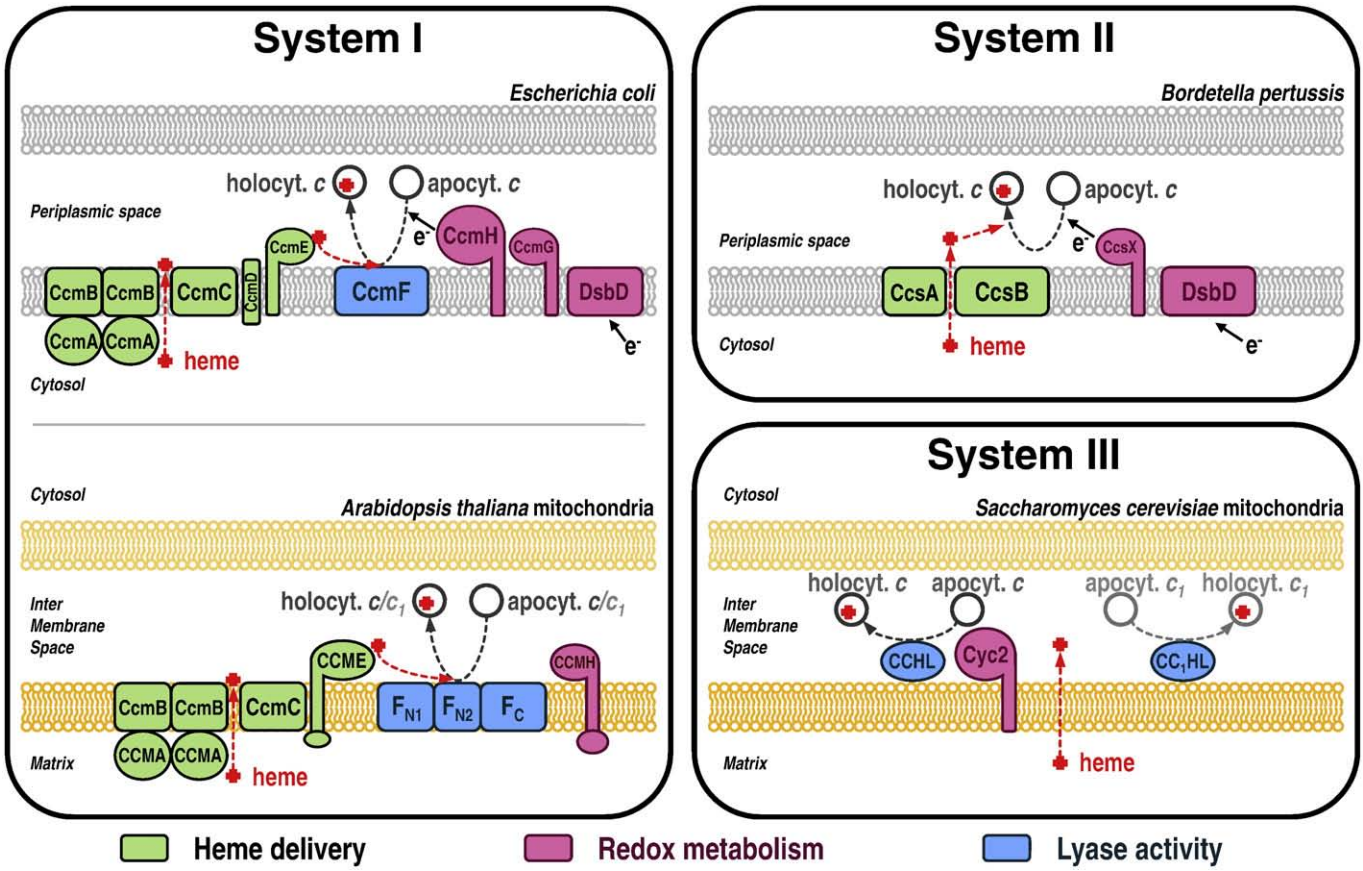


**Fig. 2.** Overview of systems I, II and III *c*-type cytochromes assembly pathways. The green components are involved in heme delivery, the pink components are implicated in redox activity, and the blue components are proposed to have a heme lyase function. In orange are the mitochondrial membranes, the outer membrane (light orange) and the inner membrane (dark orange). System I operates in plant and protist mitochondria and Gram− (α, γ) bacteria. System II occurs in chloroplasts, Gram+ and Gram− (β, δ, ε) bacteria, and cyanobacteria. System III is only restricted to mitochondria of fungi, vertebrates, invertebrates, apicomplexan parasites, green algae and diatoms. CcdA or DsbD occurs in bacterial system I and in system II. Cyc2p is restricted to organisms where both CCHL and $CC_1HL$ are present. In animals, only one heme lyase, HCCS is present. Note the complexity of the mitochondrial pathway with some organisms using system I (e.g. plants) while others have evolved a system III (animals).

*1.2.2. SystemII/CCS*

System II also referred to as the "CCS" pathway (for Cytochrome *C* Synthesis) was originally discovered in the green alga *Chlamydomonas reinhardtii* through studies of plastid cytochrome *c* assembly [34–36]. CcsA and Ccs1/CcsB which are the defining components of this system [37–41] co-occur in cyanobacterial, β, δ, ε-proteobacterial and most Gram+ bacterial genomes [39–41]. While only four cytochrome *c* assembly factors were revealed in bacterial models for the system II pathway [40,41] (Fig. 2), investigations in *Chlamydomonas* and *Arabidopsis* have implicated the participation of nine components in the plastid cytochrome *c* maturation process [34–36]. It is conceivable that, because of the compartmentalization of the thylakoid lumen, the cytochrome *c* assembly pathway which evolved in plastids relies on several players and is therefore more complex than its bacterial counterpart. The working model is that CcsA and Ccs1/CcsB together form a transmembrane heme delivery system from stroma/cytosol to the thylakoid lumen/periplasm for the heme attachment reaction [40,42–45]. The requirement for a thiol-reducing pathway in system II was established through classical and reverse genetic approaches in both the plastid and bacterial systems. Components of this pathway include a thiol/disulfide membrane transporter of the CcdA/DsbD family whose implication in cytochrome biogenesis was first demonstrated in the bacteria *Bacillus subtilis* and *Bordetella pertussis* [46–48] and more recently in the plant *Arabidopsis* [36]. Another component of the system II thioreduction pathway is the membrane-anchored *p*-side facing thioredoxin-like HCF164 in chloroplasts and ResA/CcsX in bacteria, required for accumulation of the chloroplast cytochrome $b_6f$ complex in *Arabidopsis* and for multiple bacterial cytochromes *c* [40,49,50].

*1.2.3. System III/CCHL*

The defining component of this system is CCHL, the prototypical cytochrome *c* *h*eme *l*yase. The first CCHLs were discovered during the genetic analysis of mitochondrial cytochrome *c* and $c_1$ biogenesis in fungi, especially *Saccharomyces cerevisiae* and *Neurospora crassa* [51–54]. The CCHL pathway seems to be restricted to mitochondria of fungi, animals, apicomplexan parasites, diatoms and green algae as gauged from genome sequence analysis [55]. Two related proteins, the cytochrome *c* heme lyase (CCHL) and cytochrome $c_1$ heme lyase ($CC_1HL$) are required for the assembly of cytochrome *c* and cytochrome $c_1$, respectively but their activity in the pathway remains elusive (reviewed in [56,57]). The genomes of multicellular eukaryotes, including human, reveal only one heme lyase (referred to as HCCS for holocytochrome *c* synthase), which exhibits both CCHL and $CC_1HL$ activity [55,58]. System III appears deceptively simple in terms of composition in contrast to the complexity of systems I and II because saturating genetic screens in fungi failed to identify the components involved in heme delivery and provision of reductants to the site of assembly [12,59]. There is biochemical evidence for the involvement of both pyridine and flavin nucleotides in the holocytochrome *c* and $c_1$ assembly reaction *in organello* [60,61] but the identity of the specific redox components implicated in this process is not known. In yeast mitochondria, Cyc2p is a NADPH-dependent flavoprotein required for the CCHL-catalyzed assembly of cytochrome

*c* and $c_1$. One attractive hypothesis is that Cyc2p maintains the sulfhydryls at the CXXCH motif and/or the heme co-factor under a reduced state prior to the ligation reaction catalyzed by CCHL [55,62].

Because cytochrome *c* and cytochrome $c_1$ are assembled in the IMS while both apocytochrome and heme substrates originate from a distinct compartment, the process of cytochrome *c* maturation requires consideration of the temporal order of 1) synthesis of the apocytochrome *c* and heme substrates, 2) delivery of the substrates to the IMS and maintenance under a reduced state prior to 3) catalysis of the heme ligation reaction. We discuss below each of these different steps.

## 2. Import of apocytochrome *c* and $c_1$

The translocation of apocytochrome *c* across energy-transducing membranes proceeds, in general, separately from the heme attachment reaction. Consequently, in situations where the cytochrome *c* assembly process is impaired, apoforms of cytochrome *c* are still synthesized, translocated, processed but failed to be converted to their holoforms. Exception to this rule occurs in system III mitochondria where the import of apocytochrome *c* is controlled by its cognate assembly factor, the cytochrome *c* heme lyase (CCHL).

### 2.1. Import of apocytochrome *c*

In all mitochondria, soluble cytochrome *c* and membrane-bound cytochrome $c_1$ are encoded by nuclear genes and are imported post-translationally as apoforms to the mitochondrial IMS via distinct mechanisms. Apocytochrome *c* is an unusual protein in the sense that it does not display a typical N-terminal extension for mitochondrial targeting but uses instead internal signals to reach its final destination in the IMS [57]. Interestingly, CCHL is also reaching its IMS location via the presence of internal targeting signals [63]. The apocytochrome *c* import pathway is unique and requires components of the TOM machinery on the cytosolic side of the outer membrane [64,65] and CCHL that acts as a receptor on the IMS side of the outer membrane [66]. The role of CCHL in the import of its apocytochrome *c* substrate was deduced from the observation that *S. cerevisiae* and *N. crassa* mutants in the CCHL-encoding gene cannot import apoforms of cytochrome *c* that are rapidly degraded on the cytosolic side of the mitochondrial outer membrane [67–70]. It is believed that the interaction of apocytochrome *c* with CCHL followed by the subsequent folding of the newly formed holoprotein are driving the import of the apocytochrome substrate into the IMS [56,57]. However, heme attachment is not absolutely required for import of cytochrome *c* because a cytochrome *c* variant that can no longer be acted upon by CCHL due to a mutation in the CXXCH motif is still imported in the mitochondria in a CCHL-dependent manner [69].

It is not known if HCCS participates in the import of apocytochrome *c* in system III mitochondria where HCCS is the only heme lyase acting on both apocytochromes *c* and $c_1$ substrates. However, based on the observation that HCCS can substitute for the absence of CCHL in yeast and restore holocytochrome *c* assembly, it is reasonable to postulate that HCCS is also involved in the import of apocytochrome *c* [55,71]. Note that in system I mitochondria, it is not known if the presumptive lyase CcmF is also implicated in the import of its apocytochrome $c/c_1$ substrate.

### 2.2. Import of apocytochrome $c_1$

In almost all eukaryotes, cytochrome $c_1$ is targeted to mitochondria by virtue of a bipartite sequence in the N-terminal part of the protein that undergoes a two step sequential processing in the organelle. Experimental work in *S. cerevisiae* has determined the order of processing and defined the implication of cognate lyase $CC_1HL$ in the proteolytic cleavage of the precursor form of cytochrome $c_1$. If it is clear that pre-apocytochrome $c_1$ is imported by the TOM and TIM translocation machinery into the mitochondria, there is still no consensus as to the sorting of the bipartite sequence. In one model the entire bipartite sequence in pre-apocytochrome $c_1$ is imported in the matrix and "pulled-back" into the inner membrane [72]. In a second model, the hydrophobic domain of the bipartite sequence acts as a stop-transfer signal and anchors the precursor in the mitochondrial inner membrane [73,74]. There is agreement however on the order and location of the processing events. The first part of the presequence of pre-apocytochrome $c_1$ is proteolytically removed by MPP in the matrix to generate an intermediate size apocytochrome $c_1$ which is then acted upon by Imp2, an IMS-localized protease to generate apocytochrome $c_1$ [55,58,75,76]. $CC_1HL$ catalyzes the covalent attachment of heme to intermediate size apocytochrome $c_1$ and this is a prerequisite for the second proteolytic cleavage of the signal sequence by Imp2 peptidase [77,78]. It is also established that the C-terminal part that contains the transmembrane segment serving as an anchor for cytochrome $c_1$ is inserted from the IMS side of the membrane [72].

So unlike CCHL, $CC_1HL$ is not required for the import of its cognate substrate but is needed for processing of the precursor form. That the processing of apoform of *c*-type cytochrome is depending upon heme attachment was also noted in the case of cyanobacterial cytochrome *f*, a *c*-type cytochrome involved in photosynthesis [39]. One interpretation is that the heme attachment to the apocytochrome produces a conformational change that exposes the cleavage site to the protease. Another possibility is that heme attachment and processing of the precursor are coupled reactions. This is however unlikely in the case of mitochondrial cytochrome $c_1$ because inactivation of the Imp2 protease results in the accumulation of intermediate size apocytochrome $c_1$ with covalently attached heme [55,76].

Mitochondrial cytochrome $c_1$ from *Trypanosoma brucei* is distinct for other eukaryotic cytochrome $c_1$ because it lacks a typical targeting sequence and is not processed upon import in the mitochondria [79]. Note that trypanosomatid mitochondria define a distinct pathway for the covalent attachment of heme and the cytochrome *c* and $c_1$ heme lyase in this system, yet to be discovered, are likely to be very distant from their system III counterparts.

## 3. Heme synthesis

Heme is a prosthetic group that consists of an iron atom coordinated in the centre of a large heterocyclic organic ring called a porphyrin (Fig. 1). It is one of the end products of the branched tetrapyrrole biosynthetic pathway that involves several enzyme-catalyzed steps. In animals, heme is the most abundant cyclic tetrapyrrole while in plants, it is chlorophyll the other product resulting from the activity of the branched tetrapyrrole pathway [80] (Fig. 3A). In this section, we aim to provide an overview of the different heme synthesis routes operating in eukaryotes. In particular we will detail the sub-cellular distribution of the different enzymatic steps and discuss the different proposed pathways of heme delivery in the context of cytochrome *c* maturation in the mitochondria.

### 3.1. Chemistry of heme synthesis

Three key products namely 5-amino-levulinate (ALA), uroporphyrinogen III and protoporphyrin IX are produced by the activity of the tetrapyrrole synthesis pathway (Fig. 3A). Uroporphyrinogen III and protoporphyrin IX are intermediates for the synthesis of other tetrapyrroles such as siroheme and chlorophyll, respectively. ALA is synthesized via two different ways: the C5 pathway and the Shemin pathway. The C5 pathway is housed in the plastid of photosynthetic eukaryotes, in archeae and in most bacteria including cyanobacteria. This pathway consists of a three-step reaction where glutamate (a five carbon chain amino acid) loaded on $tRNA^{Glu}$ is converted to ALA. In the

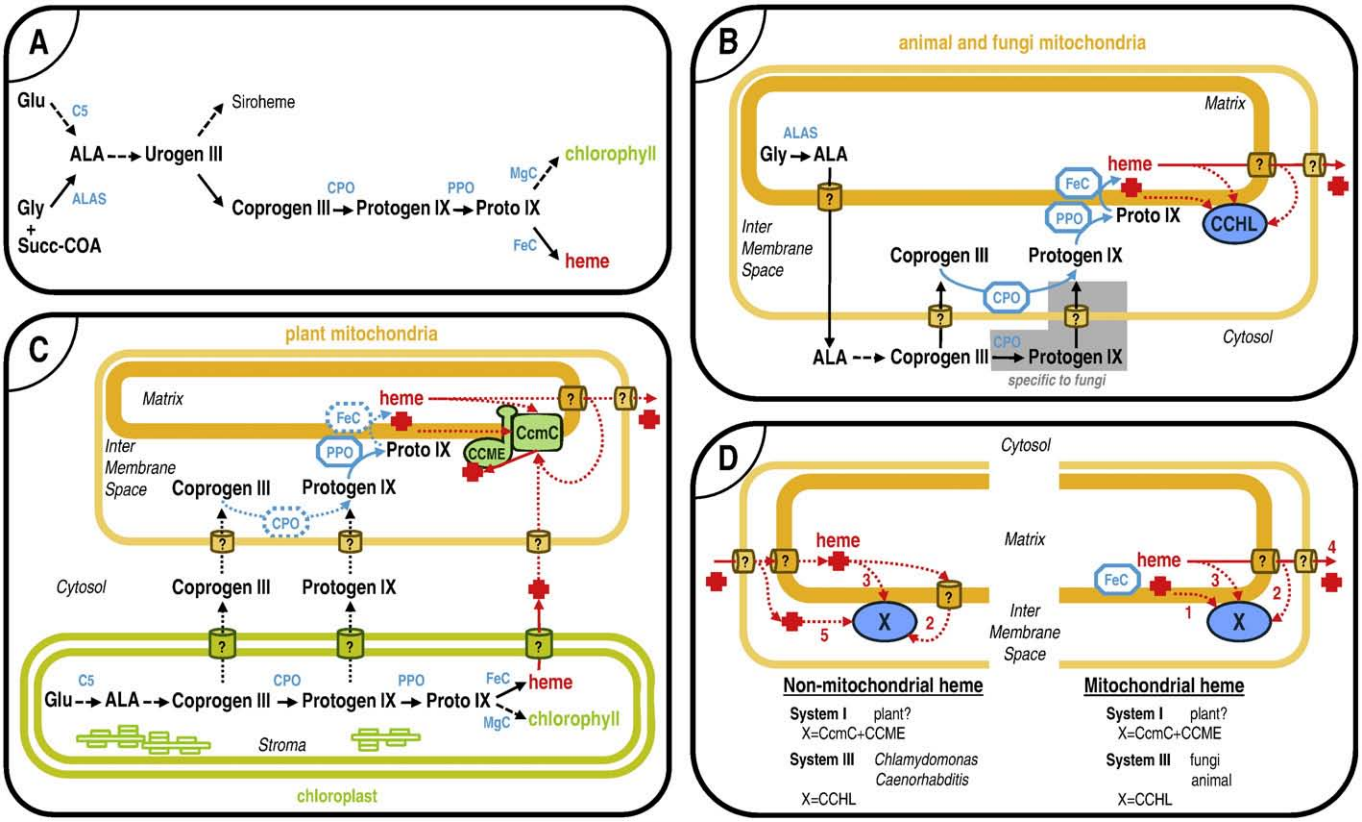


**Fig. 3.** Heme biosynthesis and trafficking routes in eukaryotes. (A) Shows the part of the tetrapyrrole biosynthetic pathway leading to the synthesis of chlorophyll and heme. C5 stands for the C5 pathway, ALAS for 5-amino-levulinate synthase, CPO for coproporphyrinogen oxidase, PPO for protoporphyrinogen oxidase, MgC for magnesium chelatase and FeC for ferrochelatase. Glu is an abbreviation for glutamate, Gly is for glycine, and Succ-CoA is for succinyl-CoA. Urogen III is an abbreviation for uroporphyrinogen III, Coprogen III for coproporphyrinogen III, Protogen IX for protoporphyrinogen IX and Proto IX for protoporphyrin IX. Siroheme is an iron-containing modified tetrapyrrole similar in structure to both heme and chlorophyll and is synthesized in bacteria and plastids. (B) Shows the localization of the heme synthesis intermediates and enzymes in yeast and animals. (C) Gives the localization of the heme synthesis intermediates and enzymes as well as heme delivery proteins in plant mitochondria. For chloroplasts, the sub-organellar localization of the enzymes is not represented. (D) Proposes alternative routes for the trafficking of heme to its site of assembly with *c*-type cytochromes in the absence or in presence of FeC in mitochondria. The different routes are proposed for the maturation pathways I and III in different model organisms. The numbers 1 to 5 refer to possible heme delivery routes as described in the text. Broken line arrows represent enzymatic pathways performed in multiple steps, whereas dotted arrows represent hypothetical movements of heme or of heme precursors. Standard arrows represent either single enzymatic steps or accepted heme or heme precursor movements. Cylinders with a question mark symbolize the various transporters, importers or exporters, envisaged for the trafficking of heme or of heme precursors in the respective proposed models.

Shemin pathway, ALA is formed in a single step by the condensation of succinyl-CoA and glycine by ALA synthase (ALAS). The Shemin reaction takes place in animal and fungal mitochondria and in α-proteobacteria. The chemistry of the subsequent reactions, from ALA to protoporphyrin IX, is common to all organisms.

The condensation of two molecules of ALA followed by a polymerization and a conversion reaction leads to the formation of uroporphyrinogen III, which is decarboxylated into coproporphyrinogen III. The oxidation of coproporphyrinogen III into protoporphyrinogen IX is catalyzed by the coproporphyrinogen III oxidase (CPO), and the protoporphyrinogen IX oxidase (PPO) mediates the conversion of protoporphyrinogen IX to protoporphyrin IX. Finally, the ferrochelatase (FeC) catalyzes the insertion of one atom of ferrous iron into protoporphyrin IX to form protoheme (heme B). Heme B is the final product of the heme biosynthesis pathway and corresponds to the prosthetic group found in organellar cytochrome of *b*- and *c*-type, cytochrome P450 and a number of hemoproteins (globin, catalase, peroxidase…). In photosynthetic organisms protoporphyrin IX is a main branch point compound since it is also leading to the synthesis of chlorophyll (Fig. 3A) (for review [81]).

### 3.2. Cellular localization of heme biosynthesis in eukaryotes

In animals and fungi, mitochondria are playing a major role in heme synthesis, while in plants, plastids are believed to be the essential if not unique site for heme production in the cell. The occurrence of hemoproteins that are located outside mitochondria and plastids such as for instance peroxisomal catalase, cytochrome $b_5$ and P450 at the ER membrane, implies that mechanisms of heme export from the site of synthesis and systems of distribution/delivery to the relevant targets must exist. The identity of the specific components involved in heme transport in the cell is largely unknown but candidate proteins have been suggested.

#### 3.2.1. Animals and fungi

In animals and fungi, the heme synthesis starts and ends in mitochondria with some intermediate reactions occurring in the cytosol. The first enzyme of heme synthesis, ALA synthase (ALAS), is located in the mitochondrial matrix (Fig. 3B). ALA comes out of mitochondria and the synthesis proceeds in the cytosol up to the coproporphyrinogen III product in animals or to the protoporphyrinogen IX intermediate in fungi (Fig. 3B). This is supported by the experimentally deduced localization of coproporphyrinogen III oxidase (CPO) and protoporphyrinogen IX oxidase (PPO). In animals, CPO resides in the IMS [82,83], whereas it is a cytosolic protein in *S. cerevisiae* [84] (Fig. 3B). In animals, protoporphyrinogen IX is synthesized in the mitochondrial IMS from the coproporphyrinogen III substrate originating from the cytosol. In yeast, cytosolic protoporphyrinogen IX crosses the mitochondrial outer membrane and the molecular bases of such a transport are not uncovered (Fig. 3B). The

next enzymatic step is catalyzed by PPO while the following and final step of heme synthesis is catalyzed by FeC. Both enzymes are localized in the mitochondrial inner membrane. PPO is an IMS-facing protein while FeC is exposed to the matrix. A channeling of protoporphyrinogen IX and protoporphyrin IX through a complex formed by PPO and FeC has been proposed, based on the crystal structure of the two enzymes that are embedded in the opposite sides of the membrane [85]. Heme B, the final product, is delivered on the matrix side of the inner membrane or possibly directly to the inner mitochondrial lipid bilayer. It is not known whether heme chaperones are involved in the release of heme from FeC or if it is naturally ejected from the chelating enzyme once synthesized. It is possible that heme which is a hydrophobic molecule is diffusing through the lipid layer to reach the IMS where it is captured by CCHL as soon as it emerges (Fig. 3B). Alternatively a flippase could be responsible for heme transfer from the inner to the outer lipid layer. Finally, it is also plausible that a specific transporter is involved in the translocation of heme from the matrix to the IMS. Since mitochondria in animals and fungi are the unique source for cytosolic heme, a heme delivery export system must exist. How heme leaves mitochondria and is delivered to other cellular compartments for incorporation into apoprotein targets is not understood yet. The necessity of heme transport in the cell is extreme in organisms that do not synthesize heme such as the free-living worm *Caenorhabditis elegans* [86]. In that case, dedicated heme transport systems must have evolved to acquire exogenous dietary heme and distribute it to the different hemoproteins in the cell (including apocytochrome in mitochondria).

### 3.2.2. Photosynthetic eukaryotes

In photosynthetic eukaryotes, the complete tetrapyrrole synthesis pathway, including heme synthesis, takes place in plastids (Fig. 3A). This location can be rationalized by considering that chlorophyll is the major end product of the activity of this pathway in plants and is only needed in the light-harvesting complexes (LHC) in the thylakoid membrane. However in the non-photosynthetic alga *Polytomella*, plastids that no longer synthesize chlorophyll remain the major source of heme synthesis [87]. Although this matter is still very much debated, it is generally believed that heme synthesis is not taking place in plant mitochondria. Hence, the current thinking is that mitochondrial heme originates from outside the organelle. In plants, the heme biosynthesis starts with the formation of ALA through the C5 route. This reaction and all subsequent steps to the production of coproporphyrinogen III are taking place exclusively in plastids. The last three steps of heme synthesis catalyzed by CPO, PPO and FeC in plastids have been proposed to be also occurring in mitochondria (Fig. 3C).

While PPO and FeC activities were measured in mitochondrial extracts, CPO activity could not be detected in mitochondria [88,89]. The possibility that CPO could be a cytosolic enzyme similarly to the yeast situation has not been excluded [89]. In many plants, CPO, PPO and FeC are encoded by two genes. In *Arabidopsis*, one CPO encoding gene is likely to be a pseudo-gene, and the other one encodes a protein only present in the plastid [90]. In maize, one of the two CPO isoforms is targeted to mitochondria [91] and CPO was detected in a proteomic analysis of mitochondria from symbiotic nodule [92].

The existence of a mitochondrial PPO in addition to its plastid isoform is not controversial. Plants use different strategies for targeting PPO to the mitochondria and the plastid. In tobacco, two different nuclear genes encode the plastid and mitochondrial PPOI and PPOII isoforms, respectively [93]. In other plants, dual targeting of PPO to mitochondria and chloroplasts was explained by the alternative use of two in-frame initiation codons from a single transcript [94,95].

The presence of FeC in plant mitochondria has been long debated. *Arabidopsis* FeCI, one of the two FeC isoforms, was first reported to be dually targeted and processed into plastid and mitochondria [96]. These results were later disputed since the pea mitochondria which was used as an in vitro import system is not always specific and can, in some instances, import proteins that are known to be solely resident in the plastid [97]. Furthermore, *Arabidopsis* mitochondria could not import FeCI [98]. In cucumber, both immunodetection analysis and transient expression assays with ferrochelatase-GFP fusion proteins support a plastid localization for both FeCI and FeCII but no mitochondrial localization could be demonstrated [99]. A corollary of the absence of FeC in plant mitochondria is that mitochondrial PPO is probably not implicated in the heme biosynthetic pathway but rather fulfills a new and unknown function. Nevertheless, mitochondrial FeC activity was quantified in different plant tissues and the possibility of plastid contamination was ruled out by the experimentalists [88]. Consequently, it is not completely excluded that FeC is indeed present in plant mitochondria but there is no firm evidence supporting this proposal at the present time. Therefore, based on the presence or the absence of mitochondrial FeC, two main scenarios can be considered (Fig. 3C): First, FeC is present in mitochondria and protoporphyrinogen IX must be the circulating porphyrin intermediate between plastids and mitochondria. If CPO is also present in the IMS, coproporphyrinogen III is presumably imported in mitochondria (similarly to animal mitochondria, Fig. 3B). In the second scenario, FeC is absent from mitochondria and heme has to be transported from plastid to mitochondria.

The later scenario is most likely used by the green alga *Chlamydomonas*. In this unicellular organism, PPO and FeC, each encoded by a single gene, are located only in chloroplasts [100]. In such a situation, plastids become the only source of heme for other cellular compartments including mitochondria. A heme transport system must exist through the cytosol, similarly to the situation in fungi and animals where mitochondria are the heme reservoir for the rest of the cell (Fig. 3D). It is also possible that direct contacts between the unique *Chlamydomonas* chloroplast and the closely associated mitochondria mediate the exchange of molecules between the two organelles. Interestingly, in apicomplexans, physical contacts between the apicoplast, a plastid-derived organelle, and the mitochondrion have been observed [101] and it appears to be impossible to physically separate the two organelles by fractionation [102]. In apicomplexans, the fact that heme is only produced in the mitochondria while the biosynthetic pathway is shared between the mitochondrion and the apicoplast indicates that exchange of porphyrin intermediates between the two organelles must exist [103].

## 3.3. Porphyrins or heme transport to mitochondria

The transport of heme and porphyrins is not well understood. Interestingly, three mitochondrial proteins, two located in the outer membrane (PBR, ABCB6) and one in the inner membrane (2-oxoglutarate carrier), have been proposed to transport porphyrins [104]. The available data concern mammalian cells were coproporphyrinogen III is synthesized in the cytosol and imported into mitochondria (Fig. 3B). These proteins should be regarded as candidate transporters as there is still no direct demonstration for a transport activity.

The mammalian peripheral-type benzodiazepine receptor (PBR) that mediates mitochondrial cholesterol uptake was also shown to bind protoporphyrinogen IX and hemin [105]. PBR receptors share a common motif with bacterial tryptophan-rich sensory proteins shown to be involved in tetrapyrrole metabolism. A plant PBR homologue was proposed to be involved in the uptake of protoporphyrinogen IX into the IMS but this awaits experimental verification [106].

The mammalian ATP-binding cassette transporters ABCB6 are up regulated by increased levels of cellular porphyrins and are required for mitochondrial porphyrin uptake [107]. ABCB6 was shown to be localized in the outer membrane and was hypothesized to transport coproporphyrinogen III from the cytoplasm to the IMS. The *ABCB6* gene, similar to its close relative *ABCB7*, can complement yeast cells that are defective in the *ATM1* gene, a gene encoding a mitochondrial inner membrane ABC that is involved in the transport of Fe/S cluster

precursors from mitochondria to the cytosol [108]. These data are unexpected considering the sublocalization of Atm1 vs. ABCB6/7 in the mitochondria and the precise role of mammalian ABCB6/7 needs to be further explored.

The human 2-oxoglutarate inner membrane carrier was also reported to bind porphyrin [109]. Consistent with a postulated function in porphyrin transport for this protein, uptake of 2-oxoglutarate into mitochondria was inhibited by several porphyrin derivatives in a competitive manner. This is the only mitochondrial inner membrane protein that has been proposed to have a function in porphyrin import into mitochondria. Additional work is needed to demonstrate the biological role of this transporter in heme synthesis.

### 3.4. Heme delivery to the mitochondrial intermembrane space

In systems I and III, heme has to reach the CCHL or CCM proteins in the mitochondrial IMS in order to be inserted into apocytochromes *c* and yield the corresponding holoforms. Although a heme transport function has been postulated for some proteins involved in cytochrome *c* maturation system, no experimental proof has been provided to consolidate this hypothesis. In mitochondria different routes of heme transport can be imagined (Fig. 3D). If heme is synthesized via mitochondrial FeC, it can either diffuse across lipid membrane to the IMS side of the inner membrane (route 1) or be actively transported to the IMS (route 2). This IMS transport system could be specific to the cytochrome *c* maturation system (route 3) or be part of a heme export system to the cytosol through both IM and OM (route 4). If mitochondria have lost FeC activity, heme might reach directly the IMS compartment through an outer membrane importer (route 5). Alternatively heme could be first delivered to the matrix and then distributed to the IMS.

One obvious question is whether a mitochondrial site for heme synthesis (i.e. presence of FeC in the mitochondria) correlates with system I or system III? Most organisms using system III synthesize heme in mitochondria. Notable exceptions are the green alga *Chlamydomonas* because heme is of plastid origin and helminths such as *C. elegans* because they are natural heme auxotrophs and require dietary heme for survival. Consequently, system III is not necessarily linked to the presence of a mitochondrial FeC. The lack of a definite proof for the presence of mitochondrial FeC in vascular plants makes it difficult to draw a general conclusion for mitochondrial system I. However, system I can be linked to the presence of a mitochondrial site for heme synthesis in non-photosynthetic eukaryotes like *Paramecium* and *Reclimonas* that contain FeC. So it seems that there is no correlation between the occurrence of mitochondrial FeC and the system evolved for cytochrome *c* maturation.

## 4. Heme delivery pathways dedicated to cytochrome *c* maturation

In system I bacteria, periplasmic *b*-type cytochromes are still formed in the absence of all Ccm proteins [110,111]. This observation supports the view that Ccm proteins are not involved in a general heme export from cytosol to periplasm. System I and system III components have been postulated to bind or transport heme across the membrane and it is believed that their function is restricted to the cytochrome *c* maturation process. However, there are now indications that in some bacteria, the activity of system I components involved in heme binding/handling might extend to additional processes that are unrelated to cytochrome *c* maturation [112,113]. This chapter aims to summarize and critically evaluate the experimental data that support the operation of heme delivery/handling pathways in mitochondrial cytochrome *c* maturation.

### 4.1. CCHL, the prototypical component of system III

Eukaryotic cytochrome *c* heme lyases are responsible for the attachment of heme to the apoforms of cytochrome *c* and $c_1$. Conserved regions in the C-terminal part of these proteins define a heme lyase signature domain presumed to be important for their function (Pfam 01265). In addition, CP*X* (X=V, M, I, L) motifs or HRM (for heme responsive motifs) are usually found in the less conserved N-terminal domains of these proteins. *Chlamydomonas* and *Plasmodium* $CC_1HL$ are exceptions because no CP*X* motifs are present in the protein sequence predicted from the genome sequence [55]. The CP*X* motifs have been defined as transient heme binding sites. The cysteine-proline dipeptide is strictly conserved in the HRMs found in heme-regulated proteins. Through this motif, heme controls the activity of transcription factors that function as activator [114–116] or repressor [117,118], the initiation of translation (e.g. eIF-2 alpha kinase) [119] and protein import into mitochondria (mammalian ALA synthase) [120]. In vitro binding of heme to a consensus HRM peptide was demonstrated by spectrophotometry [121]. Analysis of mutated peptides further shows that, in the CP*X* motif, the cysteine is critical for heme binding and that the proline promotes the affinity. However no structural model of this heme/peptide interaction is currently available. In yeast, a single HRM motif is found in $CC_1HL$ while two HRMs are present in CCHL [55]. There are only indirect evidences supporting the proposal that yeast $CC_1HL$ binds heme via its unique CPV motif [78]. In vivo, mutation or deletion of the CPV motif of yeast $CC_1HL$ affects but does not abolish the maturation of cytochrome $c_1$ (Merchant and Hamel, unpublished and Ref. [78]). Similarly, a yeast CCHL carrying an in-frame deletion of the two HRM motifs only produces reduced levels of holocytochrome *c* (Merchant and Hamel, unpublished data). The HRM motif is therefore considered crucial but not essential for the function of CCHL and $CC_1HL$. The existence of other heme binding elements such as residues in the heme lyase signature motif should be envisaged. The complete understanding of how heme binds to CCHLs would answer the question of whether CCHLs function as true enzymes or as a "scaffold" for the attachment of heme to apocytochrome (see Section 6).

### 4.2. Heme delivery components in system I: a conserved system from bacteria to plant mitochondria

In contrast to system III, a complex heme delivery pathway has been described for system I. In system I bacteria, this pathway involves five proteins CcmABCDE that are conserved in plant mitochondria with the exception of CcmD (Fig. 2). Most of our knowledge on the function of these CCM proteins in mitochondria is derived from studies of bacterial system I. The discovery that bacterial CcmE is able to bind heme covalently has been a major breakthrough in our understanding of the cytochrome *c* assembly process [122].

#### 4.2.1. CcmE

In bacteria, CcmE is a periplasmic protein anchored to the cytoplasmic membrane via an N-terminal hydrophobic domain. Experiments in *E. coli* showed that heme is transiently bound to CcmE and delivered to apocytochrome *c*, hence the terminology of heme chaperone for the CcmE protein. In *ccm* mutants or a strain overexpressing the *ccmE* gene, CcmE accumulates as a hemoprotein referred to as "holo-CcmE" due to the presence of a covalently bound heme at a conserved histidine residue. Holo-CcmE is thought to be a transient intermediate in the heme delivery pathway for *c*-type cytochromes [26]. The soluble periplasmic domain of CcmE was analyzed by NMR spectroscopy and its structure revealed a compact β-barrel core with a flexible C-terminal domain [123,124]. The key residue, $His^{130}$, is located at the interface of these two domains and is exposed to the surface of the protein near conserved hydrophobic residues proposed to act as a heme docking-platform [125]. While the covalent heme adducts are typically formed at the α carbon of the vinyl as in the case of *c*-type cytochromes, the chemical bond between heme and CcmE is of a novel type because it occurs between the β carbon of the heme vinyl and the $N^{\delta 1}$ on the histidine [126] (Fig. 1). If holo-CcmE

is a genuine intermediate in the maturation process, an obvious question that has currently no answer is how this unusual bond between heme and CcmE is resolved during cytochrome *c* maturation?

Even if CcmC is necessary and sufficient to load heme onto CcmE, the presence of all the other Ccm proteins is required to transfer heme from CcmE to apocytochrome *c* [127,128]. The current model predicts that CcmE shuttles between CcmC and CcmF for heme delivery and that CcmF, together with CcmH promotes heme ligation to apocytochrome *c* [32].

#### 4.2.2. CcmC and CcmF

CcmC and CcmF are membrane proteins that possess a conserved tryptophan-rich, "WWD" motif (also present in CcsA, a system II protein). This motif is located in a periplasmic loop and surrounded by conserved and essential histidines in the adjacent loops. This arrangement has been originally considered as a heme binding hydrophobic platform where the conserved histidines could serve as axial ligands of the heme group. Mutant analyses show that the WWD motif of CcmC is involved in a direct interaction with CcmE and not in heme binding [127]. Although this has not been experimentally tested, it is tempting to propose that, similarly, the WWD motif of CcmF is involved in interaction with CcmE. Several functions have been postulated for CcmC. In one model, CcmC takes up heme from the outer layer of the membrane and delivers it to the periplasmic heme chaperone CcmE. Alternatively, CcmC could be responsible for heme translocation from the cytoplasm to the periplasm either on its own (as proposed for CcsA in system II) or as a subunit of a putative heme ABC transporter.

#### 4.2.3. Ccm ABC transporter

The exact subunit composition of the Ccm ABC transporter and its activity as a heme transporter has been everlastingly controversial [25]. Typically, a bacterial ABC transporter is a four module complex composed of two ATP-binding cassettes and two membrane proteins with six transmembrane helices. While CcmA harbors a canonical nucleotide-binding domain (NBD), both CcmB and CcmC are good candidates for the transmembrane domains (TMD) of the ABC transporter. Several lines of evidence suggest a distinct role of CcmC versus CcmAB [129].

The obvious and early proposal was that heme is the substrate of the Ccm ABC transporter. In the absence of CcmAB, holo-CcmE is not detected and no holocytochrome *c* is synthesized. Overexpression of CcmC leads to the accumulation of holo-CcmE in the absence of CcmAB but does not restore holocytochrome *c* formation [128]. These data indicate that CcmC is sufficient for heme loading onto CcmE and that CcmAB (and the transported substrate) are necessary for the subsequent heme release from CcmE onto the apocytochromes *c*. The ATPase activity of CcmA is essential for cytochrome *c* maturation. Studies on ABC transporter revealed that ATPase activity can be stimulated by the substrate [130]. However, heme does not stimulate the ATPase activity of purified CcmAB complex [131]. In addition, no difference in heme uptake could be evidenced with everted membrane vesicles prepared from wild-type and *ccmA* deletion strain [132]. The debate on the nature of the substrate was recently stimulated by experiments showing that the ATPase activity of the ABC transporter is necessary for the release of holo-CcmE from CcmC but not for heme incorporation into CcmE [24,131]. Two models for the Ccm ABC transporter are now envisioned. In one model, the ABC complex is proposed to act as a CcmE chaperone releaser rather than a true transporter. ATP hydrolysis induces conformational changes in the ABC complex, which in turn promotes the release of the associated holo-CcmE, allowing the subsequent delivery of heme to apocytochrome. In a second model, the release of heme from holo-CcmE is dependent on a molecule transported by the CcmAB transporter and required for the chemistry of the heme ligation reaction. In the absence of such a molecule, the maturation process is aborted and heme cannot be released from holo-CcmE for subsequent ligation to the apocytochromes *c*. A reductant has been proposed as a substrate of the ABC transporter, although, as for heme, no direct evidence is supporting this hypothesis [131]. Interestingly, transfer of heme from holo-CcmE to apocytochrome *c* could be reconstituted in vitro only in reducing conditions [133]. Note that the reduced state of apocytochrome cysteines and heme iron (ferrous) is an essential biochemical requirement for holocytochrome *c* formation and it is therefore expected that there is a mechanism for heme reduction in vivo [134,135]. In fact, a precedent for the operation of an ABC transporter whose substrate is a reductant already exists in *E. coli*. CydDC is an ABC transporter that mediates the delivery of glutathione to the periplasm, an essential reductant required for the assembly of cytochrome *bd* but also *c*-type cytochromes [136]. It is believed that the *c*-type cytochrome deficiency in the absence of CydDC is caused by a modified redox balance in the periplasm that impairs the activity of the redox factors involved in cytochrome *c* maturation (see Section 5). The implication of CydDC in cytochrome *c* maturation might not be universal as a *cydDC*-null mutant in a *R. capsulatus* is deficient for cytochrome *bd* but remains proficient for *c*-type cytochromes (F. Daldal, personal communication). So it is possible that CcmAB transports a reductant or a compound required to resolve the linkage between heme and holo-CcmE.

#### 4.2.4. Heme delivery components in plant mitochondria

Plant mitochondria have kept all the Ccm proteins involved in heme delivery except the small CcmD [137] that was recently proposed to be required in the release of holo-CcmE from a CcmABCD complex in bacteria [138]. The genome of plant mitochondria contains *ccmB* and *ccmC* genes that encode hydrophobic intrinsic inner membrane proteins [27,139,140]. In some unicellular eukaryotes such as the jakobid flagellate *Reclinomonas americana* or the unicellular red alga *Cyanidioschyzon merolae*, *ccmA* is also found in the mitochondrial genome. In *Arabidopsis thaliana*, CCMA (AtCCMA) and CCME (AtCCME) are encoded by single nuclear genes and imported into mitochondria [21,23]. *Arabidopsis* CcmABCE proteins are clear orthologs of their bacterial counterparts. The conservation of their size, motifs and proposed topological arrangement within the membrane strongly suggest that their function in heme delivery is also conserved in plant mitochondria. For instance, the WWD motif and the important histidines in CcmC are conserved in the plant mitochondrial proteins. Another example is given by AtCCME that was shown to be anchored in the mitochondrial inner membrane with its C-terminal domain exposed to the IMS, a similar topology than its bacterial counterpart. Furthermore, functional heterologous complementation of the *E. coli ccmE* mutant showed that AtCCME has the capability to bind heme on its conserved histidine [21].

AtCCMA is located to the matrix side of the mitochondrial inner membrane and behaves like an integral membrane protein. The solubilization of AtCCMA is facilitated by the presence of ATP and the protein exhibits an ATPase activity. One possible interpretation is that AtCCMA interaction with the transmembrane domains of the ABC transporter can be modulated by its ATPase activity through conformational changes. Yeast two-hybrid assays demonstrate that AtCCMA can interact with AtCcmB. In addition, only AtCcmB loops predicted to be oriented toward the matrix show interaction with AtCCMA while loops predicted to be located in the IMS do not interact with AtCCMA [23]. Using a similar method no interaction could be detected between AtCCMA and any of the AtCcmC domains that were tested (Hagenmuller, Giegé and Bonnard, unpublished data). Antibodies directed against AtCCMA reveal that the protein occurs in a 480 kDa mitochondrial complex whose subunit composition is unknown. In particular, the occurrence of AtCcmC or mitochondria specific system I components in this complex needs to be investigated.

Regardless of the presence of mitochondrial FeC, if heme reaches the IMS from the matrix side of the inner membrane (routes 2 and 3 in

Fig. 3D), then it is expected that the heme delivery machinery in plant and bacterial system could function in a very similar way. If heme is delivered directly to the IMS, there is no need for a putative translocation mechanism of heme via CcmC or the ABC transporter.

## 5. Redox chemistry in the context of cytochrome *c* assembly

The need for reducing conditions seems to be inherent to the heme attachment reaction. The implication of redox chemistry in the assembly process was first established through pioneer work in fungal mitochondria where *in organello* reconstitution of holocytochrome *c* and $c_1$ assembly indicates that both heme and apocytochrome *c* substrates need to be reduced in order to be acted upon by their cognate heme lyases [60,61,141,142]. In the case of heme, the chemical basis for such a requirement was understood from work on hemoprotein reconstitution [143]. The conclusion from such studies is that the initial oxidation state of the heme iron when it is presented to the apoprotein is a critical determinant in the formation of the thioether bond linkage. When the iron is initially oxidized, a radical-based mechanism is initiated and leads to the formation of alternative thiol adducts. When the heme iron is reduced, the dominant process appears to be the simple addition of the thiol across the vinyl double bond.

### 5.1. Bacterial redox components involved in cytochrome c assembly

In vivo, the involvement of redox chemistry was first rationalized in the context of bacterial cytochrome *c* assembly because the bacterial periplasmic space is also the compartment where oxidative folding of cysteine-containing proteins is promoted by the Dsb/Bdb system (reviewed in [144,145]). The current thinking is that apocytochrome *c* is first a substrate of the Dsb/Bdb machinery that introduces intramolecular disulfides between the two cysteines of the C*XX*CH sequences and is consequently reduced to provide free sulfhydryls for the heme ligation reaction. The early postulate that the formation of a disulfide bond is a biochemical requirement for cytochrome *c* maturation is derived from the observation that loss of the DsbA/DsbB catalysts involved in disulfide bond formation results in cytochrome *c* deficiency [146–148]. Since provision of oxidized thiols to *dsbA*/*dsbB* mutants rescues the cytochrome *c* biogenesis defect, it was believed that oxidation of the C*XX*CH sequence is a biochemical requirement in the assembly process [148]. However the DsbA/DsbB catalysts do not appear to be essential for cytochrome *c* maturation in all organisms and under all conditions and their participation in cytochrome *c* assembly needs to be re-examined [50,149–152].

Consistent with the view that reducing conditions are needed for the chemistry of the heme lyase reaction, the operation of specific oxido-reductases defining a transmembrane thiol-disulfide relay from cytosol to periplasmic space was discovered in system I and system II bacteria [1,28,29,145,153]. The proposal that the components of this pathway control the in vivo redox status of the sulfhydryls at the C*XX*CH sequence is inferred from the presence of motifs in their protein sequence that speak to redox chemistry [28] and the in vitro demonstration that their recombinant forms can participate in thiol-disulfide exchange reactions [154,155]. Moreover, the ability of exogenous thiol compounds to by-pass the lack of these factors in vivo substantiates the view that the redox components have a thio-reducing activity in the pathway [47,48,152,156,157]. However, a direct demonstration that bacterial apocytochromes *c* are the in vivo targets of action of redox factors with a reducing activity (CcmH in system I and CcsX in system II, see Fig. 2) has never been provided. This is likely to be technically challenging because apoforms of cytochromes *c* that fail to be converted to their respective holoforms are short-lived species that are rapidly degraded. In situations where the thio-reducing pathway is not functional, the prediction is that apocytochromes *c* are oxidized by the Dsb/Bdb components and are no longer competent for the heme ligation reaction. This scenario is supported by the finding that loss of CcdA/DbsD in the thio-reduction pathway can be by-passed by inactivation of the Dsb/Bdb component in the thio-oxidation pathway [46,48].

Recently, in vitro holocytochrome *c* reconstitution from apocytochrome *c* and heme substrates was achieved. The in vitro reaction appears to yield the correct end product, holocytochrome *c* only in conditions where both substrates are provided under a reduced form [8,158–160]. In the case of apocytochrome *c*, an intramolecular disulfide bond was shown to be formed at the C*XX*CH motif if reductants were omitted from the in vitro assay. The provision of ferrous heme and reduced apoprotein avoided formation of abortive side products that were observed when ferric heme was used instead, corroborating the initial observation from Barker et al. that the chemistry of thioether bond formation is absolutely dependent upon a reduced heme substrate [143].

### 5.2. CcmH, a redox component in system I mitochondria

In mitochondria, the identity of the redox components involved in cytochrome *c* assembly is now beginning to surface but their enzymology needs to be elaborated. In system I mitochondria, CCMH, an IMS facing, inner membrane-bound protein has emerged as a candidate redox assembly component through work in *Arabidopsis* [22]. *Arabidopsis thaliana* CCMH (AtCCMH) is an ortholog of bacterial CcmH whose function in the cytochrome *c* maturation process has been investigated in detail [32,33,161–163]. In vitro assays demonstrate that AtCCMH possess a RC*XX*C redox active site and can promote the reduction of an oxidized apocytochrome *c* mimic. The proposal that apocytochrome *c* is acted upon by AtCCMH is also strengthened by two-hybrid experiments showing that the two proteins can interact [22]. Recent work indicates that bacterial CcmH is able to bind to its apocytochrome *c* target with a moderate affinity and a mechanistic model for the mode of action was proposed based on the available crystal structure of the CcmH soluble domain [164]. In mitochondria, the reductant of AtCCMH is not known because orthologs of the prototypical bacterial redox components (DsbD/CcdA and CcmG) appear to be missing. In bacteria, CcmH is believed to be maintained reduced via the activity of thioredoxin-like CcmG which receives electrons from the cytosol via the DsbD/CcdA redox membrane transporter [31,154,161,162,165–167] (see Fig. 2). An alternative proposal is that CcmH does not function in a thio-redox relay but controls some redox aspect of the heme lyase reaction independently of DsbD/CcdA and CcmG.

### 5.3. Cyc2p, a system III redox component

In system III mitochondria, Cyc2p, a novel redox cytochrome *c* assembly factor was isolated as a partner of CCHL through a multicopy suppressor screen [55]. This molecule had been characterized previously by the Sherman group as a “general” factor in the biogenesis of mitochondria, but its function has remained obscure [57,168,169]. A direct role for Cyc2p in the redox chemistry of the cytochrome assembly pathway is based on the following lines of evidence: 1) the presence of a noncovalently bound FAD molecule in the C-terminal domain of Cyc2p, 2) the localization of Cyc2p in the inner membrane with the FAD binding domain exposed to the intermembrane space, the compartment where cytochrome *c* maturation takes place and 3) the ability of recombinant Cyc2p to carry the NADPH-dependent reduction of ferricyanide [62]. The relevant targets of Cyc2p action in the mitochondria are not known and Cyc2p was postulated to act as heme and/or apocytochrome *c* sulfhydryl reductase in vivo [62]. A clue to Cyc2p activity in vivo came from the observation that a *cyc2*-null mutation results in a synthetic respiratory deficient phenotype with loss of holocytochrome $c_1$ assembly when combined with a CAPCH heme binding

site in cytochrome $c_1$ instead of the wild-type CAACH site [62]. The genetic synthetic interaction between the *cyc2*-null mutation and the mutation at the C*XX*CH sequence of cytochrome $c_1$ suggests that the site of action of Cyc2p is the heme binding site. Conceivably, the A to P mutation could alter the reactivity of the cysteinyl thiols to redox chemistry so that apocytochrome $c_1$ heme binding site occurs in an oxidized form (i.e. the cysteines are engaged in an intramolecular disulfide bond) and is therefore no longer able to participate in the heme ligation reaction. If Cyc2p is implicated in the control of the redox state of the apocytochrome *c* sulfhydryls during the maturation process, its function is clearly redundant based on the fact that holocytochrome *c* assembly is only partially affected in the absence of Cyc2p [55,62]. Interestingly, Cyc2p displays weak similarities to members of the cytochrome $b_5$ reductase family but the significance of this finding is unclear [55,62]. The distribution of Cyc2p seems to be restricted to system III mitochondria where both CCHL and $CC_1HL$ are present. The observation that there is no structural homologue of Cyc2p in system III mitochondria where HCCS is the only heme lyase suggests that other redox components must operate.

The question of redox chemistry in the context of mitochondrial cytochrome *c* assembly has received little attention because of the accepted dogma that the IMS is a "naturally" reducing compartment. This perception is now challenged with the recent discovery that Erv1p, an IMS resident sulfhydryl oxidase is a key catalyst in a disulfide relay system driving the import of cysteine-rich proteins into the IMS [170].

## 6. Definition of a heme lyase

Formally, the cytochrome *c* heme lyase (CCHL) is defined as the enzyme catalyzing the terminal step in the holocytochrome synthesis pathway, that is, the formation of the thioether linkage between the heme vinyl groups and the C*XX*CH sulfhydryls in apocytochrome *c* (Fig. 1). Two candidate enzymes with a possible heme lyase activity are the mitochondrial so-called CCHLs in system III and bacterial/mitochondrial CcmF in system I.

### 6.1. CCHL/$CC_1$HL/HCCS, the system III heme lyases

The initial biochemical definition of the heme lyase originated from work in fungal mitochondria where the activity responsible for apo to holocytochrome *c* conversion was fractionated and found to be associated to the IMS side of the mitochondrial inner membrane [141,171–173]. The conversion of apoform of both cytochrome *c* and $c_1$ to their respective holoforms could be reconstituted in purified mitochondria and the cytochrome heme lyase activity measured [60,61,70,142,174]. The first genetic identification of the heme lyase came from the studies of yeast *cyc3* mutants that displayed a specific loss in holocytochrome *c* assembly and a deficiency in the heme lyase activity measured in reconstitution assays with mitochondria [59]. The *CYC3* gene was cloned and proposed to encode CCHL, the enzyme responsible for the heme attachment onto apocytochrome *c* [51]. Fungal orthologs of CCHL were also discovered in *N. crassa* and *Candida albicans* and a function in cytochrome *c* assembly was inferred from the analysis of the corresponding mutants [52,54,70]. The observation that holocytochrome $c_1$ assembly proceeds normally in a *cyc3* mutant led to the definition of $CC_1HL$, a separate heme lyase, specific for the apocytochrome $c_1$ substrate. The *CYT2* gene that encodes the $CC_1HL$ was discovered via the study of yeast mutants that display a specific block in holocytochrome $c_1$ accumulation [175]. Based on genetic analysis of CCHL- and $CC_1HL$-minus mutants, it was long assumed that CCHL and $CC_1HL$ displayed non-overlapping substrate specificity for their respective apocytochrome substrates [53,176–178]. However, the yeast CCHL displays intrinsic weak activity toward its non-cognate substrate, apocytochrome $c_1$ and this activity can be enhancced by point mutations in either the enzyme CCHL or the substrate apocytochrome $c_1$ [55]. On the other hand, $CC_1HL$ specificity toward its cognate apocytochrome $c_1$ appears strict [55]. In animals, system III is reduced to one heme lyase called HCCS that can act upon both apocytochrome *c* and $c_1$ substrates [55]. All CCHLs display sequence similarity (30% sequence identity) with one another and they do not appear to have bacterial orthologs. Yeast CCHL and $CC_1HL$ are localized at the mitochondrial inner membrane, facing the IMS [62,179].

Because CCHLs interact with apocytochromes [52,66] and also with heme via the HRM [58] (see Section 4), the present model is that these proteins participate in the biogenesis of cytochromes *c* by catalyzing thioether bond formation. The sequence requirements for recognition of apocytochrome *c* and $c_1$ substrate by their cognate heme lyases are not known. Based on the observation that yeast CCHL can attach heme to a 25 residue synthetic peptide and to mitochondrial apocytochrome *c* from different sources, it is likely that only a limited region encompassing the C*XX*CH motif is recognized [142,180]. This view is further corroborated by the fact that the specificity of CCHL for the apocytochrome $c_1$ substrate is increased by mutations at residues between the cysteines of the C*XX*CH motif [55]. Despite the fact that the CCHLs were the first cytochrome assembly factors to be discovered, the specific enzymatic function of the CCHLs remains obscure (reviewed in [56,57]) and ultimate biochemical proof of their function awaits a direct enzymatic assay of cytochrome *c* heme lyase activity of the purified proteins.

### 6.2. CcmF, the proposed heme lyase in system I mitochondria

CcmF in system I mitochondria and bacteria is a polytopic membrane protein whose biochemical activity in the assembly process remains undeciphered. In *E. coli*, co-immunoprecipitation experiments showed that CcmF interacts both with CcmE, the heme chaperone and CcmH, a redox component in the thio-reduction subpathway. This led to the proposal that CcmF acts as the bacterial cytochrome *c* heme lyase along with CcmH [181]. When expressed in *E. coli*, AtCCMH can also be co-immunoprecipitated with CcmF. Yeast two-hybrid assays suggest that $AtCcmF_{N2}$ can interact with $AtCcmF_{N1}$ and $AtCcmF_C$ but also with both apocytochrome *c* and $c_1$ (Giegé, unpublished data). Morever AtCCMH, $AtCcmF_{N1}$, $AtCcmF_{N2}$ and $AtCcmF_C$ occur in a high molecular complex suggesting interaction with other unknown cytochrome *c* assembly components. It is likely that this complex acts as a cytochrome *c* assembly machinery in the mitochondrial membrane [22]. Candidate assembly complexes for system II and system III have also been detected in the thylakoid membranes [43] and in the mitochondrial inner membranes of yeast (Bernard and Hamel, unpublished). Interestingly, the assembly of bacterial pentaheme cytochrome *c* NrfA requires dedicated CcmF and CcmH orthologs for the sole purpose to attach heme onto the atypical C*XX*CK motif [182]. Possibly, CcmF/CcmH functions as a heme lyase minimal unit in system I. The same concept was already formulated for CcsA/Ccs1, the prototypical components of system II in plastid [43]. Work in bacteria has now shown that CcsA/CcsB indeed functions as a minimal heme delivery system that also exhibits the heme lyase activity [45].

Reconstitution of holocytochrome *c* assembly in vitro indicates that the chemistry of thioether linkage formation does not require catalysts [8,158–160]. The implication of these findings is that mitochondrial cytochrome *c* heme lyases are probably not catalyzing the covalent attachment of heme to the apocytochrome c. The heme lyases could be involved in stabilizing specific conformations of apoproteins (i.e., cytochrome *c* or cytochrome $c_1$), maintaining the cysteines and/or heme under a reduced form, proximal binding of heme and subsequent presentation to the apocytochromes *c* for correct stereospecific heme ligation. The rate of the uncatalyzed in vitro cytochrome *c* formation is relatively slow [158] and it is still

conceivable that the stereospecificity is under the kinetic control of the mitochondrial heme lyases and possibly other factors (CCMH or Cyc2p). One function of the heme lyase in the assembly process could be to create a favorable microenvironment for the heme ligation to take place.

## Acknowledgements

Research projects in the Hamel laboratory are funded by a Muscular Dystrophy Association research grant (MDA 4247) and the College of Biological Sciences, the College of Medicine, and the Dorothy M. Davis Heart and Lung Institute at The Ohio State University. P.H. also wishes to acknowledge the Institute of Mitochondrial Biology at The Ohio State University for the intellectual support. V.C. is supported by an American Heart Association post-doctoral fellowship. G.B. and P.G. are supported by the Centre National de la Recherche Scientifique. P.G. was supported by a EU Marie Curie reintegration grant. The authors wish to thank Dr. F. Daldal for stimulating scientific exchanges and sharing unpublished results and also Dr. R. Stuart for scientific discussions.

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