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MINIREVIEW

Cytochrome *c* maturation system on the negative side of bioenergetic membranes: CCB or System IV

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Cytochromes of the *c*-type contain hemes covalently attached via one or, more generally, two thioether bonds between the vinyls of heme *b* and the thiols of cysteine residues of apocytochromes. This post-translational modification relies on membrane-associated specific biogenesis proteins, referred to as cytochrome *c* maturation systems. At least three different versions (i.e. Systems I–III) are found on the positive side of bioenergetic membranes in different organisms and compartments. The present minireview is concerned with systems on the negative side of the membranes. It describes System IV, also referred to as cofactor assembly on complex C subunit B, for heme binding on cytochrome b_6 through one thioether bond; this covalent heme is usually called c_i. This system is found in all organisms with oxygenic photosynthesis but not in Firmicutes, although they also have a cytochrome *b* protein with an additional heme c_i covalently attached via a single thioether bond.

Introduction

Cytochromes *c* are hemoproteins with covalently attached iron-protoporphyrin IX (or heme *b*) groups. They act as electron carriers, such as in photosynthetic and respiratory electron transport chains, as well as in a variety of other cellular processes [1,2]. Cytochromes of the *c*-type contain hemes attached via occasionally one or, more generally, two thioether bonds formed stereo-specifically between the vinyls at positions 2 and 4 of the tetrapyrrole ring of ferroheme *b* and the thiols of the N- and C-terminal cysteines, respectively, of a –CXXCH– heme-binding motif within apocytochromes *c* [3–6]. The number of residues between the cysteines and the histidine of this motif sometimes vary [$CX_nC(H/K)$] [5]. The amino acids serving as axial ligands to hexacoordinate the heme-iron commonly comprise the histidine or lysine residue of the motif together with another amino acid (e.g. methionine, tyrosine, histidine).

Because *c*-type cytochromes have covalently bound heme and are slowly formed *in vitro* without the assistance of maturation factors [7], catalysis of their biosynthesis was studied and genetic analyses have revealed a striking diversity of cytochrome *c* maturation systems [3,4,8–11]. Systems I–III are found on the positive (or *p*) side of bioenergetic membranes (bacterial periplasm, chloroplast lumen and mitochondrial intermembrane space), as is a likely additional system for Euglenozoa mitochondrial *c*-type cytochromes. In the latter case, as in other mitochondrial cytochromes *c*,

Abbreviations
CCB, cofactor assembly on complex C subunit B; Q, quinone.

the heme is hexacoordinated by amino acid axial ligands but, instead of the usual pair of thioether bonds, it has a single thioether bond between vinyl-4 of heme *b* and thiol of (A/F)XXCH motif [12–14].

The present minireview is concerned with systems on the negative (or *n*) side of bioenergetic membranes (bacterial cytoplasm and plastid stroma). It describes System IV, or cofactor assembly on complex C subunit B (CCB), for attaching a covalent heme onto cytochrome b_6. Attachment is via a single thioether bond and, thus, the covalent heme is regarded as a *c*-type. It is usually designated as c_i, although the names c_n and *x* (in the older literature) have been used. This system is found in all organisms with oxygenic photosynthesis but not in Firmicutes (e.g. *Bacillus subtilis*), although they also have a heme c_i as part of a cytochrome *b* protein.

Heme c_i in cytochrome b_6f complexes

Cytochromes b_6f and bc_1 are homologous protein complexes that play a major role in photosynthetic and respiratory electron transport chains. They contribute to building up the proton motive force via the quinone (Q)-cycle [15–17]. In cytochrome b_6f, this redox loop couples the consecutive oxidation of two quinols at the Q_o site (also called Q_p) to the reduction of one quinone at the Q_i site (also called Q_n) through the low potential chain involving hemes b_l (also known as b_p), b_h (also known as b_n) and c_i, and to the reduction of two plastocyanins along the high potential chain involving Rieske Fe_2S_2 protein and cytochrome *f* (Fig. 1A).

A salient feature of b_6f complexes differentiating them from bc_1 complexes is an extra heme *c* bound to cytochrome b_6, heme c_i, which has atypical binding and iron-coordination features. It is a high-spin heme *c* with a pentacoordinated central iron that lacks amino acid axial ligands but, instead, has a single axial ligand, either hydroxyl or water [18–20]. Furthermore, it is attached by a single thioether bond between the vinyl-2 of the tetrapyrrole ring of heme *b* and the thiol of Cys35 residue in cytochrome b_6. The structure of the protein shows that this cysteine is near the quinone-reducing site, Q_i, on the *n*-side of the membrane in close vicinity to heme b_h (Fig. 1B) [21]; see also Fig. 1C. In heme c_i binding cytochromes (discussed below), the consensus motifs CX_nC(H/K) or (A/F)XXCH are lacking (Fig. 1D).

Heme c_i corresponds to redox center 'G' observed *in vivo* to exchange one electron with heme b_h [22,23]. *In vitro*, the reduced minus oxidized absorption spectrum of heme c_i consists of a broad absorption

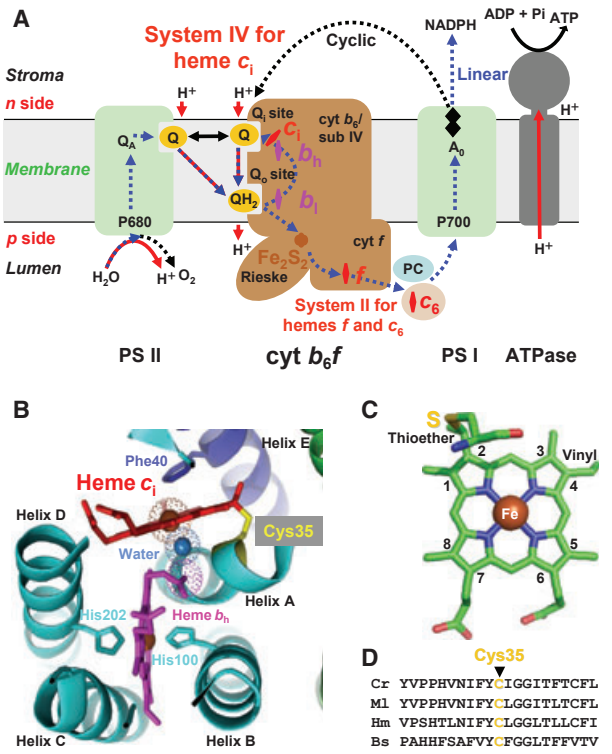

Fig. 1. Heme c_i in the photosynthetic membrane. (A) Schematic photosynthetic electron transport chain. Cytochrome *c* maturation system IV on *n*-side and system II on *p*-side. Hemes (diamonds), quinone (Q), quinol (QH_2), plastocyanin (PC), linear electron transfer from water to NADPH (dotted blue arrows), proton release or uptake (red arrows), and cyclic electron transfer with re-injection of electrons from photosystem I into the Q pool (dotted black arrow) are shown. (B) Heme c_i in *Chlamydomonas* b_6f structure (Protein Data Bank accession number: 1Q90). Heme c_i is covalently attached by a thioether bond to Cys35 of cytochrome b_6 and its iron is bridged to a heme b_h propionate through a water molecule. Heme c_i (red), heme b_h (pink), Cys35 (yellow), cytochrome b_6 (cyan), subunit IV (blue), and water (blue sphere) are shown. (C) Heme c_i and its thioether linkage between vinyl-2∼Cys35 (Protein Data Bank accession number: 1Q90). C (green), N (blue), O (red), S (yellow). (D) Sequence alignment of cytochrome regions around cysteine binding heme c_i. Cr, *Chlamydomonas reinhardtii*; Ml, *Mastigocladus laminosus*; Hm, *Heliobacterium modesticaldum*; Bs, *Bacillus subtilis*.

increase centered at 425 nm with no obvious change in the green region. The midpoint potential of heme c_i ($E_{m,7}$ = +100 mV) is shifted by −225 mV in the presence of Q_i site inhibitor 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide [24]. EPR studies suggest that 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide occupies the sixth ligand position and that there is a strong interaction of heme c_i with heme b_h [25,26]. The b_h/c_i pair may represent an alternative Q_i site that would, relative to the situation in the cytochrome bc_1 complex, facilitate the two-electron reduction of quinone, thereby minimizing the level

of reactive semiquinone intermediate, which could be an adaption of the cytochrome b_6f complex to an oxygenic environment. A role for heme c_i in cyclic electron transfer has also been proposed [18,19,27].

We identified an additional cytochrome *c* maturation system for heme c_i biogenesis: System IV or CCB.

Discovery of CCB or System IV by forward genetics in *Chlamydomonas*

Genetic studies in the unicellular green alga *Chlamydomonas reinhardtii* led to the identification of four nuclear *CCB* loci that control heme c_i maturation by complementation of *ccb* mutants with an indexed library and selection for restoration of phototrophy [20,28–30]. The signature of apocytochrome b_6 impaired in heme c_i binding was described for the *petB-C35V* mutant, where the unique covalent cysteine ligand to heme c_i was substituted in *petB* gene coding for cytochrome b_6 [20]. The signature consists of a double band in urea-SDS/PAGE, or a single band of lower apparent molecular mass than in the wild-type

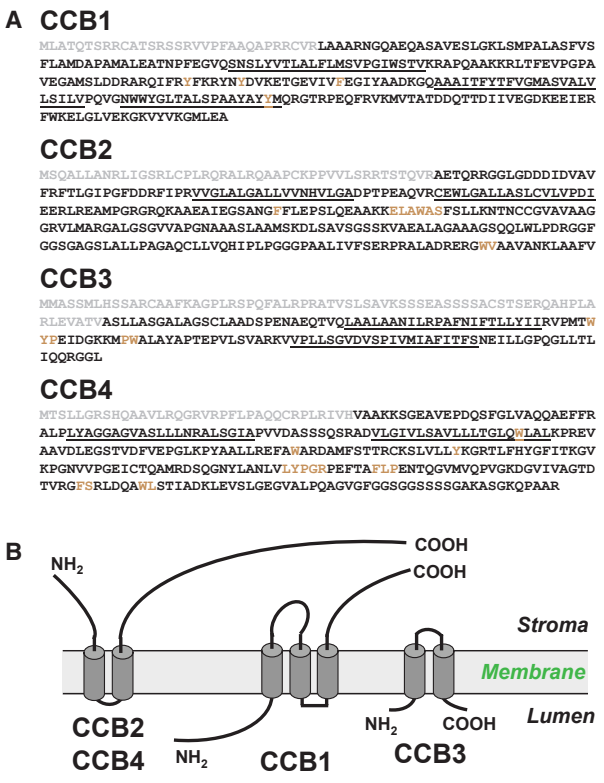

Fig. 2. CCB proteins. (A) CCB protein sequences in *Chlamydomonas*. Predicted chloroplast transit peptides (gray); transmembrane domains (underlined); conserved tryptophan, tyrosine and/or phenylalanine residues with conserved neighboring residues (brown). (B) Trans-membrane topology of CCB proteins.

in SDS/PAGE concomitant with the lack of peroxidase activity; the latter is an indicator of covalently attached heme on such gels. The same signature is observed in all *ccb* mutants, indicating that heme c_i binding is prevented in each case. This entirely new biogenesis pathway comprises at least four proteins (Fig. 2 and Table 1) for which no function had been ascribed previously [30]. Their chloroplast and transmembrane localization is consistent with their role in attaching heme covalently to cytochrome b_6. Transit peptides are indicated in Fig. 2A as predicted by CHLOROP [31]. The transmembrane topologies of CCB factors in Fig. 2B were predicted by TMAP [32] and the positive inside rule [33]. Confirmation of such topology came from subcellular localization, chaotropic extraction, protease accessibility and yeast two-hybrid split ubiquitin analysis [30,34]. CCB1 has three transmembrane domains, with its C terminus in the stroma. CCB2 and CCB4 are low similarity paralogs with two N-terminal transmembrane domains and a long C-terminal part in the stroma. CCB2 and CCB4 form a stable 70 kDa heterodimer in blue-native/PAGE, accumulate in a concerted manner and interact in two-hybrid experiments [34]. CCB3 has a horseshoe transmembrane configuration with N- and C-termini in the lumen, similar to some polypeptides shown to insert spontaneously in the thylakoid membrane [35,36].

Because heme c_i is located in the same compartment as the site of synthesis of the apoprotein and the heme, its maturation does not demand a mechanism for heme-translocation across the membrane. Most conserved regions in CCB proteins are found in regions oriented toward the *n*-side of the membrane, including tryptophan, tyrosine and/or phenylalanine residues (Fig. 2A) (CCB alignments are provided elsewhere [30]), and are expected to interact with heme based on what is known about heme-relay processes in other maturation systems [9] (e.g. WWD motif in CcsA) [37]. Some of these conserved residues might participate in heme-chaperoning and delivery to cytochrome b_6 on the stromal side of the membranes; their essential role remains to be tested experimentally. None of the four CCB proteins displays a conserved histidine residue, which is a residue found to be involved in heme-handling processes in other maturation systems [9] and, in particular, in covalent heme binding to CcmE [38]. In addition, no CCB intermediate carrying a covalently bound heme could be detected.

None of the four CCB proteins has conserved cysteine residues, which are critical for apocytochrome *c* thioredox or heme-iron redox control processes in other maturation systems [6,9,39] (e.g. in thioredoxin-like HCF164) [40]. We cannot exclude the existence of

Table 1. CCB homologs and mutant phenotypes. CPLD, conserved in Plantae and diatoms; CGLD, conserved in green lineage and diatoms (CGLD) [44].

Protein	*Chlamydomonas reinhardtii*	*Arabidopsis thaliana*	*Synechocystis* sp. CC 6803
CCB1	EF190472, 5723435[a], CPLD51	At3g26710	Slr0589
	Lacks c_i-heme, ε cytb_6f	Lacks c_i-heme, ε cytb_6f	Altered photosynthesis
CCB3	EF190474, 5715778[a], CPLD43	At5g36120	Ssl0353
	Lacks c_i-heme, ε cytb_6f	Not determined	Altered photosynthesis[b]
CCB4	EF190475, 5719843[a], CGLD23	At1g59840	CCB2/4 Slr0948
	Lacks c_i-heme, ε cytb_6f	Lacks c_i-heme, ε cytb_6f	Not determined
CCB2	EF190473, 5718457[a]	At5g52110	
	Lacks c_i-heme, ε cytb_6f	Lacks c_i-heme, ε cytb_6f	
Other YGGT	300853[a]	At5g21920, normal photosynthesis	Ssr2142
	516754[a]	At4g27990, normal photosynthesis	Normal photosynthesis
	380546[a]	At3g07430, altered nucleoid distribution	

[a] Protein identity at: http://genome.jgi-psf.org/Chlre4. [b] Incomplete segregation.

additional CCB proteins catalysing redox chemistry that have escaped our genetic screen. It is possible that the thiol of cytochrome b_6, Cys35, may form a disulfide that has to be reduced before heme c_i binds via a thioether bond; numerous species in the stroma, such as thioredoxins, could be involved in such the reduction of such a disulfide [41]. We cannot exclude either that heme-binding process by a unique thioether bond would not require the reduction of a pre-existing disulfide bond or that easier access of heme-delivery complexes to ferrous heme produced by the neighboring ferrochelatase suppresses the requirement of a heme reducing step before covalent binding to cytochrome b_6 [42].

System IV conservation in organisms performing oxygenic photosynthesis

The four CCB proteins identified in *Chlamydomonas* are conserved among all organisms performing oxygenic photosynthesis whose genome sequences are currently available [30,43–45]. CCB1 is present as a unique ortholog. As noted above, CCB2 and CCB4 are low similarity paralogs; they derive from a unique cyanobacterial ancestor (named CCB2/4 in Table 1). CCB4 is closer to the cyanobacterial protein than CCB2; a single ortholog is identified in red algae and diatoms [30]. CCB3 belongs to the large YGGT protein family (conserved domain cl00508, pfam02325) found in plastids and bacteria. Although bacteria often contain one YGGT member (two in cyanobacteria), photosynthetic eukaryotes contain up to four, and all are all chloroplast-localized [30,45–49]. The CCB3 phylogenetic tree (Fig. 3A) illustrates the conservation of CCB proteins in all plastid-containing eukaryotes and cyanobacteria and shows that a single member of each photosynthetic organism segregates with *Chlamydomonas* CCB3, clearly defining its functional orthologs [30].

The mutant phenotype of CCB orthologs in *Arabidopsis* and Cyanobacteria has been studied (Table 1). *Arabidopsis* mutants confirmed the role in the heme c_i binding of CCB1, CCB2 and CCB4 [48,50] and not of the other YGGTs [45,47,48]. Cyanobacteria *ccb1* and *ccb3* mutants showed impaired photosynthesis; however, the mutation in the *ccb3* strain was incompletely segregated [45]. Active cyanobacterial b_6f complex is essential because it participates in respiratory and photosynthetic electron transfer chains. It is expected that b_6f complex lacking heme c_i would accumulate more in cyanobacteria than in *Chlamydomonas* as a result of a lower quality control, as observed for crippled photosystem II complexes [51–53]. In conclusion, until now, the CCB proteins, and not the other YGGT proteins, have been implicated in heme c_i binding.

Waiting for an additional system for heme c_i binding in Firmicutes

The molecular phylogeny of 16S ribosomal sequences indicates that Gram-positive bacteria may be of photosynthetic ancestry [54], whereas similar analysis of Rieske/cytochrome *b* complexes suggests a green clade, including cyanobacteria, heliobacteria, Chlorobiaceae and many nonphototrophs [55]. Cytochrome *b* of Firmicutes share markers traits with cytochrome b_6, such as the cytochrome *b* component split into two subunits, the conservation of the cysteine binding heme c_i in helix A, the absence of the eighth helix found in the corresponding subunit of cytochrome bc_1 complexes, and the presence of 14 amino acid residues between the histidine ligands to hemes b_l and b_h in helix D (Fig. 3B). Covalent heme attachment to the conserved cysteine of cytochrome *b* in *B. subtilis* was demonstrated using a

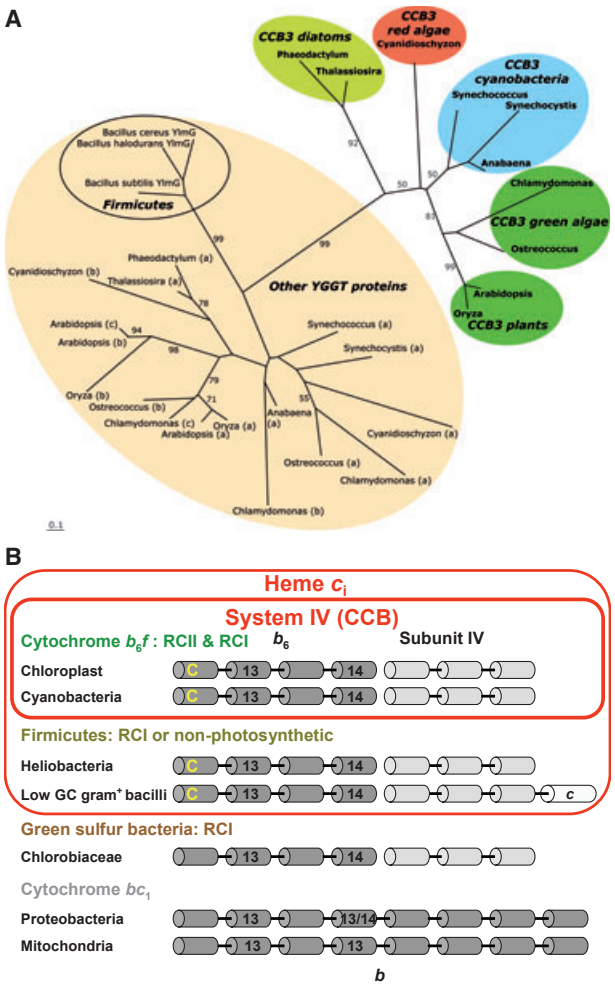

Fig. 3. System IV conservation in organisms performing oxygenic photosynthesis. (A) Unrooted maximum likelihood phylogenetic tree of CCB3 in green algae and plants (green), cyanobacteria (blue), red algae (red), diatoms (greenish) and of other YGGT proteins (beige). The scale bar represents the number of changes per site. Bootstrap values ≥ 50% are indicated. (B) More restrictive distribution of System IV than for heme c_i. Schematic cytochromes *b*. In helix A, cysteine binding heme c_i (yellow). In helices B and D, amino acid number between histidine ligands of hemes b_l and b_h. Cytochrome b_6 (b_6), cytochrome *b* (*b*), cytochrome *c* (*c*). The absence of the eighth helix found in the corresponding subunit of cytochrome bc_1 complexes also holds for *Bacillus subtilis*, which has a cytochrome *c* fused to the C-terminus of subunit IV.

mutagenesis approach [56]. Heliobacteria were shown to possess heme c_i by biochemistry and EPR spectroscopy [57]. However, bacilli and heliobacteria lack CCB proteins [30]. *Bacillus subtilis* YlmG, comprising part of a cell division gene cluster [58], is not a CCB3 functional ortholog (Fig. 3A) and did not appear to be required for heme c_i biogenesis [59]. Thus, in Firmicutes, System IV is absent and a different system for heme c_i binding is indicated.

Functional interactions between CCB components

We took advantage of the cysteine ligand mutant *petB-C35V* that contains the same cytochrome *c* maturation system components as in the wild-type but is blocked at the very last step of heme c_i binding to cytochrome b_6; hence, transiently-formed intermediates in the heme-delivery process are trapped. By blue-native/PAGE experiments, we identified an approximately 70–110 kDa CCB1/b_6 complex, a 170 kDa CCB2/CCB4/CCB3/b_6 complex and a CCB3/b_6 complex, the existence of which was confirmed by coimmunoprecipitation [34]. Heme c_i binding is independent of cytochrome b_6 assembly with other subunits of the cytochrome b_6f complex, and subunit IV is not found in complexes containing CCB proteins; thus, unassembled cytochrome b_6 is the substrate for the CCB machinery [34], which is consistent with the splitting of cytochrome *b* (recall that this is a single and larger polypeptide in cytochrome bc_1 complexes) into cytochrome b_6 and subunit IV (Fig. 3B) representing a heme c_i insertion constraint [18]. CCB1/b_6 complex formation, which still occurs in *ccb2*, *ccb3* and *ccb4* mutants, appears to be a prerequisite for CCB2/CCB4/CCB3/b_6 complex formation in the process of heme c_i binding to cytochrome b_6.

From the analysis of mutants lacking axial ligands of hemes b_l or b_h, we proposed that the formation of a heme b_l-dependent intermediate can be prevented by heme-depletion upon gabaculine treatment and that binding of heme b_l precedes that of heme b_h [29]. We extended our original model for cytochrome b_6 biogenesis with the sequential binding of hemes b_l, b_h and c_i [34].

In our model for a CCB-mediated heme attachment to cytochrome b_6 (with the two noncovalently hemes already bound) (Fig. 4), the formation of a transient complex comprising CCB1/b_6, tentatively assigned as chaperone complex, is followed by the formation of a transient complex comprising CCB3/b_6. The latter then recruits the stable CCB2/CCB4 heterodimer to form the heme c_i ligation complex comprising CCB2/CCB4/CCB3/b_6. Whether CCB3/b_6 is an intermediate before the formation or after dissociation of the CCB2/CCB4/CCB3/b_6 complex remains an open question. Concerning the specific roles of CCB2 and CCB4, it can be speculated that CCB2 (i.e. the less conserved CCB protein) would have evolved to adapt for heme-scavenging in different organisms performing oxygenic photosynthesis, whereas the better conserved CCB4 protein would pack closely with CCB3 and cytochrome b_6, whose sequences are very well

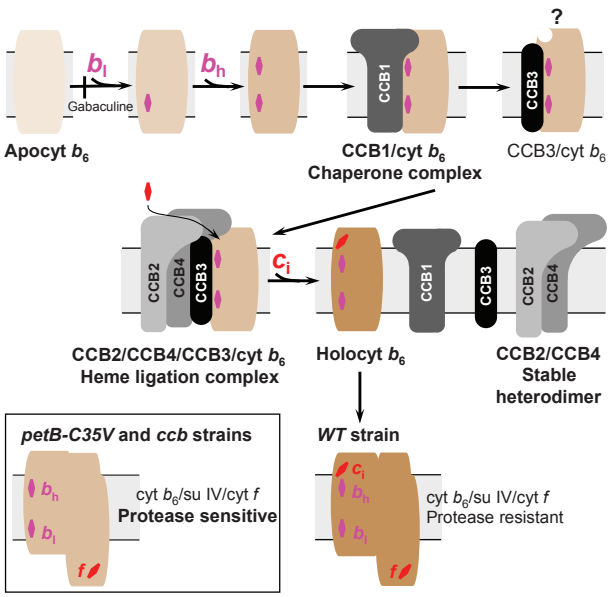

Fig. 4. Tentative model for CCB-mediated apo- to holocytochrome b_6 conversion. Model steps: membrane integration of apocytochrome b_6, formation of b_l heme-dependent intermediate that can be prevented by heme-depletion (gabaculine treatment); formation of b_l and b_h binding cytochrome b_6; formation of transient chaperone complex comprising CCB1/b_6; formation of transient complex comprising CCB3/b_6; association of CCB3/b_6 with stable heterodimer CCB2/CCB4 to form heme c_i ligation complex CCB2/CCB4/CCB3/b_6; holocytochrome b_6 associates with other b_6f subunits. A question mark indicates that CCB3/b_6 may arise before the formation or after dissociation of the heme–ligation complex. Without c_i-binding, the b_6f complex is assembled but highly protease sensitive (inset). WT, wild-type.

conserved. Once heme c_i is thioether bound to cytochrome b_6, the heme–ligation complex dissociates, freeing CCB3 and CCB2/CCB4 heterodimer, which are now available for a new round of catalysis.

Full maturation of cytochrome b_6 is not required for its assembly within b_6f complexes (Fig. 4, inset) because b_6f monomers and dimers are detected in absence of heme c_i binding [34,50]. Heme c_i lacking strains in *Chlamydomonas* retain some activity in quinol oxidation [34,60], although their b_6f complexes are highly protease sensitive. These features allowed us (via random mutagenesis of *ccb* mutants and selection for phototrophy restoration) to isolate protease mutants with stable b_6f complexes despite a lack of heme c_i [61]. Several of these mutants affected the ATP-dependent zinc metalloproteases FtsH of the photosynthetic membrane (A. Malnoë, J. Girard-Bascou, F. A. Wollman & C. de Vitry, unpublished results). Heme c_i lacking b_6f complexes showed an inactive quinone reduction Q_i site [61]. We exploited the possibilities of combining chloroplast and nuclear mutations in *Chlamydomonas* to allow an investigation of the photosynthetic activity *in vitro* by time-resolved absorption spectroscopy. For example, an engineered strain maintaining accumulation of b_6f complexes lacking hemes b_h and c_i rescued photosynthetic growth, indicating that the Q-cycle can be mechanistically bypassed; these short circuits prove to be vital because they restore, although imperfectly, the overall function of the impaired complex [61,62]. More generally, these mutants contribute toward the elucidation of how chloroplast proteases regulate photosynthetic membrane complexes.

Conclusions and perspectives

In recent years, our knowledge of System IV has progressed enormously. Several of its components are now defined and new ones are being investigated. Their further characterization in heme-delivery processes, including the roles of their conserved residues, as well as their regulation of expression, is the target for future studies. Suppressor studies are promising and should lead to important implications that help our understanding of protease and crippled complexes. In addition, the diversity of cytochrome *c* maturation processes on the *n*-side of the membrane remains to be explored.

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