Biochimie 82 (2000) 603–614

S0300908400006052/FLA

Assembly of chloroplast cytochromes *b* and *c*

Stacie S. Nakamoto, Patrice Hamel, Sabeeha Merchant*

Department of Chemistry and Biochemistry, University of California, Box 951569, Los Angeles, CA 90095-1569, USA

(Received 15 January 2000; accepted 18 April 2000)

Abstract — The synthesis of holocytochromes in plastids is a catalyzed process. Several proteins, including plastid CcsA, Ccs1, possibly CcdA and a thioredoxin, plus at least two additional Ccs factors, are required in sub-stoichiometric amounts for the conversion of apocytochromes *f* and c_6 to their respective holoforms. CcsA, proposed to be a heme delivery factor, and Ccs1, an apoprotein chaperone, are speculated to interact physically in vivo. The formation of holocytochrome b_6 is a multi-step pathway in which at least four, as yet unidentified, Ccb factors are required for association of the b_H heme. The specific requirement of reduced heme for in vitro synthesis of a cytochrome b_{559}-derived holo-β_2 suggests that cytochrome *b* synthesis in PSII might also be catalyzed in vivo. © 2000 Société française de biochimie et biologie moléculaire / Éditions scientifiques et médicales Elsevier SAS

heme / cytochrome *b* / cytochrome *c* / assembly / chloroplast

1. Introduction

Metalloproteins such as cytochromes are ubiquitous electron-transferring molecules fulfilling important and diverse functions in basic biological processes like oxidative phosphorylation, photosynthesis, and nitrogen fixation. They acquire their function by post-translational assembly of a metal-containing prosthetic group or a metallocluster. Besides their obvious and well-documented role in electron transfer, they can also be recruited for other purposes such as signaling, sensing, or regulatory functions (reviewed in [1–4]). The elucidation of metalloprotein assembly in vivo has become a topical area of research. It is well appreciated that the metabolism of metal cofactors (such as Fe) and metal containing prosthetic groups (such as heme) is biologically catalyzed – from delivery to distribution to association with specific apoforms of metalloproteins. However, much remains to be understood, especially with respect to the mechanistic details of specific cofactor-polypeptide association. Owing to the catalytic properties of their metal cofactors, numerous and abundant metalloproteins operating in reactions involving electron transfer, oxygen chemistry and photochemistry are found in the plastid (reviewed in [5]). Each metalloprotein has a particular sub-organellar location, specific interactions with partner subunits, and a unique cofactor binding site. Thus, the assembly of each protein must be studied separately. In this minireview, we aim to summarize our present understanding of these processes, with a focus on cytochromes as rather simple models of metalloprotein biogenesis in the chloroplast.

* Correspondence and reprints: merchant@chem.ucla.edu
Abbreviations: PS, photosystem; cyt, cytochrome.

2. Cytochromes

2.1. Definitions and general features

Cytochromes are defined as heme-proteins in which the heme prosthetic group undergoes oxidation/reduction as part of the function of the protein. Cytochromes can be classified according to their heme iron coordination, by heme type, and further by sequence similarity. By definition, *b*-type cytochromes contain non-covalently bound heme while the *c*-type cytochromes harbor the same heme group attached covalently via one or, in most cases, two thioether linkages between conserved cysteines of a CxxCH motif and the vinyl side chains on the porphyrin ring *(figure 1)*. For both *b*-type and *c*-type cytochromes, the protein also provides axial ligands to the heme iron. One ligand is usually a histidine residue. In the *c*-type cytochromes, this histidine residue is adjacent to the cysteine involved in the C8 thioether linkage.

Cytochromes b_{559}, b_6, *f* and c_6 are the only four cytochromes characterized at the biochemical and molecular levels so far in chloroplast thylakoid membranes, the two former being of the *b*-type and the two last being of the *c*-type. Cytochrome b_{559} is a membrane-spanning protein in PSII (reviewed in [6]). Cytochromes b_6 and *f* are catalytic subunits of the b_6f complex, which transfers electrons from plastoquinol to plastocyanin. Cyt b_6 and cyt *f*, encoded by the chloroplast *petB* and *petA* genes, respectively, are found in plastids and cyanobacteria. Cyt c_6, encoded by a nuclear gene in *Chlamydomonas reinhardtii*, is transcribed in copper deficient and hypoxic conditions [7–9]. It is a soluble cytochrome found in cyanobacteria and certain algal species and it serves as a functional replacement for plastocyanin.

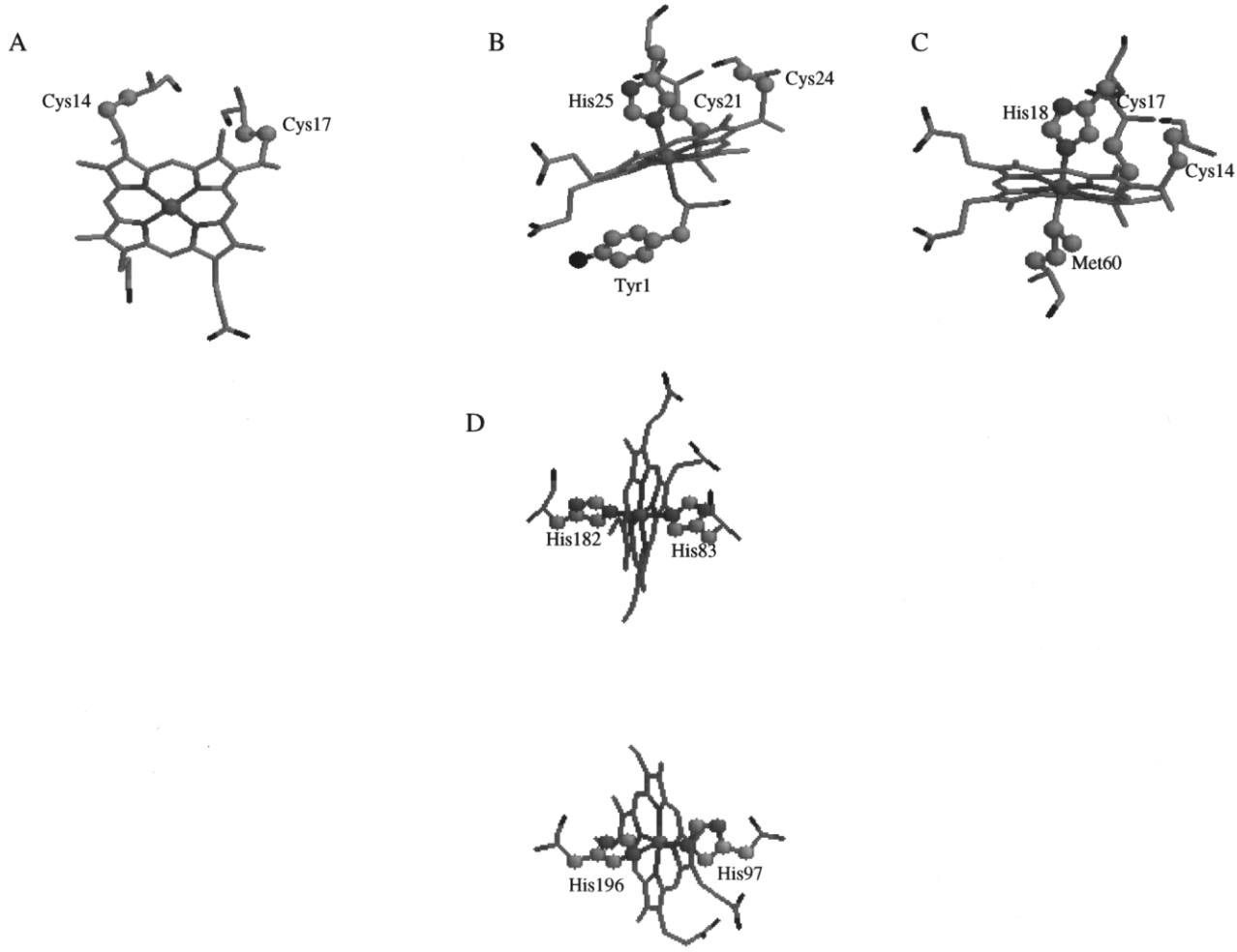

Figure 1. Cytochrome ligands involved in cofactor binding are illustrated using Rasmol V2.6. Sidechains are shown with spheres whereas heme atoms are joined by distance. The heme Fe is depicted as a sphere for emphasis. **A.** Coordination of heme and residues Cys14 and Cys17 are from the *C. reinhardtii* cyt c_6 structure [96] (PDB file 1cyi). **B.** Cyt *f* structure from cyanobacteria [97] (PDB file 1ci3); thioether linkages by Cys21 and Cys24; His25 and Tyr1 are axial ligands. **C.** Cyt c_6 thioether linkages between heme and Cys14, Cys14; axial ligands Met60, His18 from *C. reinhardtii* structure as in **A. D.** Example of axial ligands in *b*-type cytochromes. His87 and His182 binding b_L heme and His97 and His308 binding b_H heme in the bovine cytochrome bc_1 complex structure [98] (PDB file 1be3).

3. Heme

3.1. Heme metabolism

Synthesis of the heme cofactor requires the operation of the tetrapyrrole pathway for synthesis of protoporphyrin IX and mobilization of iron from a mineralized ferritin-containing storage form. During plastid development, a dramatic decrease in ferritin accumulation parallels a high demand for iron in heme biosynthesis and iron-sulfur cluster formation, suggesting that ferritin acts as an iron-storage molecule [10]. The mechanism of iron mobilization in the plastid is not known. In *Saccharomyces cerevisiae*, a role of the mitochondria in regulating iron uptake and distribution in relation to the making of heme and Fe/S containing proteins is evident [11–14]. In a plant, one might imagine that the plastid might play an equivalent role, especially considering the localization of plant ferritin to the chloroplast, but this aspect of Fe metabolism has not been studied yet.

The pathways leading to the synthesis of heme are well characterized in photosynthetic organisms [15]. The tet-

rapyrrole biosynthetic pathway leading to heme from precursor 5-aminolevulinate is highly conserved in all living organisms. In addition to heme synthesis, this pathway branches to produce chlorophyll or bacteriochlorophyll in photosynthetic organisms. The final steps of heme synthesis, catalyzed by protoporphyrinogen oxidase (EC 1.3.3.4) and ferrochelatase (EC 4.99.1.1), occur in both the mitochondria and plastid [16] with distinct isoforms of the enzymes located in each organelle [17, 18]. It is assumed that the heme for plastid cytochromes is the product of plastid ferrochelatase activity. The plastid enzyme is thylakoid membrane bound. Since heme attachment into apocytochrome *c* occurs in the lumen after processing to the mature form [19], the heme must be delivered after it is synthesized from the membrane into an aqueous compartment. It has been commonly assumed that heme diffuses freely through the membranes but this assumption is probably not correct in cell sublocations such as energy-transducing membranes where heme can generate damaging reactive oxygen species.

3.2. Heme-dependent synthesis of cytochromes

The availability of cofactors can affect both the synthesis and accumulation of the polypeptide component of heme- and chlorophyll-containing proteins. In *Chlamydomonas*, it is possible to examine rates of protein synthesis through pulse-labeling experiments (e.g., [20–22]), and this approach was used to look at the effect of heme availability on *b*- and *c*-type cytochrome synthesis [22, 23]. In these studies, gabaculine, which inhibits the enzyme catalyzing the synthesis of 5-aminolevulinate, was used to decrease heme production. These experiments suggest that a tetrapyrrole pathway intermediate is required for increased translation of Cyc6 mRNA. Although gabaculine treatment affects the synthesis of many plastid-resident proteins, the inhibition of Cyc6 mRNA translation is more severe and suggests a more specific requirement for tetrapyrroles for the synthesis of heme proteins in addition to a perhaps more general effect on gene expression as a result of tetrapyrrole-dependent signaling between the plastid and nucleus [24–27]. A similar approach with wheat seedlings indicates that cyt *f* can be synthesized even when heme synthesis is inhibited [28] but whether or not the rate of synthesis of pre-apocytochrome *f* is affected in gabaculine treated plants is not known. Nevertheless, an important conclusion from this work, which is supported by later work in *Chlamydomonas* [22, 23, 29], is that heme synthesis is not required for translocation of pre-apocytochrome *f* nor for its processing. In the etiolated wheat system the apoprotein accumulates, but in *Chlamydomonas* the apoprotein is degraded. Whether this difference is attributable to a fundamental difference between *Chlamydomonas* versus plant plastid degradation mechanisms or whether the use of etiolated versus green cells constitutes an important variable is open for speculation. The pattern of labeling of cyt b_6 in gabaculine treated wild type *Chlamydomonas* cells mimics that of untreated cyt *b* site-directed mutants defective in heme binding [30]. These mutants synthesize a membrane-associated apocytochrome b_6 but accumulate very low amounts of apocytochrome *b* suggesting that the apoprotein is degraded.

4. The *c*-type cytochromes

Because holo *c*-type cytochromes reside in a different location than the site of apoprotein and heme synthesis, assembly of cofactor with polypeptide requires consideration of the temporal order of synthesis, polypeptide translocation, and holoprotein formation.

4.1. Pre-cytochrome processing and translocation

Cytochrome *f* is synthesized on thylakoid membrane bound ribosomes [31, 32] with an N-terminal extension [33]. This extension resembles bacterial signal sequences [34] and is required for insertion into the thylakoid membrane [35]. The presequence from pea cyt *f* can target β-galactosidase to the bacterial cytoplasmic membrane via the SecA-dependent transport system [36]. Although unprocessed full-length precursors of many plastid proteins have been detected in vivo, despite their very short half-life and low abundance, pre-cytochrome *f* is not detectable unless translocation is blocked. While this does not exclude post-translational import, the co-translational model is preferred because it is compatible also with translation of *petA* mRNA on thylakoid bound ribosomes (reviewed in [37–39]). A conserved AXA sequence in the pre-protein is required for processing that occurs on the lumen side [29]. This exposes the N-terminal tyrosine that serves as an axial ligand to the heme iron [40]. Site-directed mutation of the processing site from Ala-Gln-Ala to Leu-Gln-Leu, or mutation of the N-terminal Tyr to Pro resulted in slower processing of the precursor in *Chlamydomonas* [29, 41], which emphasizes the importance of the conserved processing site. When processing is slowed down by mutation, the precursor can bind heme. The pre-holo form of the polypeptide is also assembled with the other subunits into a complex, illustrating that processing does not have to precede heme binding nor complex assembly. The temporal order of processing, heme attachment, and complex assembly is probably determined kinetically in the chloroplast system. Interestingly, in *Synechocystis*, when heme attachment is blocked, processing of cyt *f* is also inhibited [42], suggesting that the two steps are either coupled or that heme attachment must precede processing as is the case for cyt c_1 maturation in *Saccharomyces* mitochondria (reviewed in [43]). Whether this reflects a difference in the substrate specificity of the plastid versus cyanobacterial protease or

whether it reflects a difference in the accessibility of the cleavage site in the plastid versus cyanobacterial pre-apocytochrome needs to be tested.

Cytochrome c_6 is nucleus-encoded and translated as a precursor protein with a bipartite signal sequence. The first signal targets the protein to the chloroplast envelope membrane import apparatus and is removed upon translocation to generate the intermediate form, which is translocated across the thylakoid membrane into the lumen [19] (reviewed in [44]). Because pre-cyt c_6 lacks the twin arginine motif found in the signal sequences of proteins utilizing the pH-dependent pathway, we assume that it uses the SecA-dependent pathway but this has not been verified experimentally since the preprotein is not imported into pea plastids, the usual system for in vitro import assays (N.S. Pergam and S.M. Theg, unpublished data). Processing and translocation of the pre-protein occurs independent of heme attachment [45] but the apoprotein is degraded rapidly if it is not converted to the holoform [23].

4.2. Holocytochrome formation

4.2.1. Mitochondria and bacteria

What can we deduce about plastid apocytochromes *c* maturation from our knowledge of this process in bacteria and mitochondria?

4.2.1.1. Heme attachment

In *S. cerevisiae* mitochondria (and perhaps in animal mitochondria) two cyt *c*/heme lyases operate in the mitochondrial inter-membrane space [43]. The two cytochrome heme lyases are specific for their respective apoprotein substrates, namely apocytochromes *c* and c_1 [46–48]. These appear to be the only factors required for the biogenesis of mitochondrial *c*-type cytochromes and it is assumed that they catalyze thioether bond formation, although the chemistry has not been detailed yet. The lyase in the bacterial periplasm has not been identified. Genome sequence analysis suggests that it is a different/unrelated protein. It is likely that the mechanism is different because the bacterial periplasm provides a different chemical environment than does the mitochondrial inter-membrane space. Indeed, the bacterial system of cytochrome *c* assembly is different and requires several ccm (cytochrome *c* maturation) genes [49–51].

Candidate Ccm homologues of vascular plants (*Arabidopsis*, *Oenothera*, wheat) are found in mitochondrial but not plastid genomes, where it is assumed that they function in a cytochrome maturation pathway more closely resembling the bacterial system. Because homologues of the cytochrome heme lyases are not found in plant genomes (1/00) and the candidate Ccm homologues appear to be localized in mitochondria rather than plastids, it is reasonable to imagine that the plant Ccm homologues function in the mitochondria. Therefore, the mechanism of cytochrome *c* assembly in the chloroplast could require distinct factors from either the bacterial Ccm system or the fungal/mammalian cyt *c*/heme lyase system [52] (reviewed in [53]).

4.2.1.2. Heme transport and delivery

Heme needs to be transported, in a conventional sense, from one side of a membrane to another. It also needs to be delivered properly and targeted specifically from a membrane location to the cytochrome assembly complex in a usable form. What can we learn from what has been resolved in the bacterial system? The ccm genes encode components of an ABC-type transporter, which are known to be required for cyt *c* assembly in the bacterial periplasm, but the substrate transported is not known. Although it has been assumed that heme is the substrate [54], recent work [55] shows that a periplasmic heme chaperone (CcmE), which delivers heme to the apocytochrome in the *Escherichia coli* periplasm, can still be loaded in the absence of CcmA and CcmB. A transmembrane protein, CcmC, containing a signature 'WWD' motif [52–54] is, nevertheless, required for CcmE loading, suggesting that CcmC might function in heme transport. One tryptophan in this motif [56] and two conserved histidines [54, 55] in CcmC are required for *c*-type cytochrome biogenesis.

In *E. coli*, CcmE functions as an intermediate in heme delivery to apocytochromes [57]. CcmE is membrane associated with the C-terminal bulk of the protein lying in the periplasm. CcmE binds heme covalently through a functionally essential histidine residue within the conserved sequence VLAKHDE [57]. CcmC is required for loading CcmE with heme, while a presumptive lyase, perhaps CcmF, delivers heme from CcmE to the apocytochromes [55]. In plants, a CcmE-homologue resides in the mitochondria (G. Bonnard, personal communication) and presumably functions in cytochrome c/c_1 assembly, rather than in plastid cyt *f* assembly.

4.2.1.3. Thioreduction

It is evident that a thioreduction and oxidation pathway is necessary to maintain the cysteines (in the CxxCH motif) of the apocytochrome in an appropriate form for heme attachment to occur in the bacterial periplasm. CcmG (also called HelX, CycY, or DsbE) is a membrane anchored thiol:disulfide oxidoreductase required for cytochrome *c* maturation in the bacteria periplasm [58–61]. In *E. coli*, deletion of ccmG results in the complete absence of cytochrome *c* while mutating either cysteine in the active site of CcmG dramatically reduces the accumulation of cytochrome *c* [61]. This suggests that CcmG has an auxiliary function besides its role in thiol metabolism. In the active site mutants, cytochrome *c* accumulation can be restored upon addition of certain thiol compounds into the medium [61] illustrating the importance of the cysteines in the reducing function for CcmG and supporting the role of CcmG in thiol maintenance. In addition to CcmG, DipZ

(also called DsbD) is required for cytochrome maturation in *E. coli* [62]. *dipZ* null mutants have defects in disulfide bond formation [61, 63–65]. In the absence of DipZ, CcmG remains oxidized [66], and as with the CcmG active site mutants, *c*-type cytochrome synthesis is restored by the addition of certain thiol compounds [67] supporting its role in a reductant transfer pathway.

Interestingly, disruption of the *dipZ* gene in *Pseudomonas aeruginosa* results in only a partial defect of cytochrome *c* synthesis [64]. It is proposed that, in *P. aeruginosa*, a CcdA-like protein could substitute for part of the DipZ function [64]. Since CcdA does not have a thioredoxin motif, it seems unlikely that it could replace the thioreduction function of the C-terminus of DipZ. Instead CcdA might replace the membrane domain, which functions to transfer electrons across the membrane. The C-terminal thioredoxin domain might be replaced by a periplasmic thioredoxin like molecule. CcdA was discovered originally as being required for cytochrome *c* biogenesis in *Bacillus subtilis* [68, 69] and will be discussed in more detail in section 4.2.2.3. The conflict in cytochrome accumulation of the *dipZ* disruption mutants from *E. coli* and *P. aeruginosa* might be due to the fact that *E. coli* has only *dipZ* and not *ccdA*, while *P. aeruginosa* has both [64], possibly allowing for partial overlap in function. On the other hand, *R. capsulatus* also has both CcdA and DipZ; yet *ccdA* mutants do show a cytochrome assembly defect, suggesting that the two proteins are not functionally redundant in this organism. Perhaps, in *R. capsulatus*, the two proteins may have evolved to have specific interactions for cytochrome *c* maturation versus general periplasmic disulfide metabolism.

In *E. coli*, DsbA and DsbB, which are responsible for disulphide bond formation, are required also for cytochrome maturation [70, 71]. *E. coli* expresses multi-heme cytochromes which are not present in mitochondria and chloroplast. The DsbA/B oxidation pathway might be important only in the *E. coli* situation where multiple thiol groups occur in a protein. Oxidation of the right pair ensures that the lyase reaction proceeds with the correct dithiol substrate [72]. The DsbA/B requirement may not apply to systems such as chloroplasts where the apopolypeptides contain a single CxxCH heme binding motif.

4.2.2. Plastid holocytochrome c assembly

To elucidate the factors required for cytochrome *c* assembly in the plastid, non-photosynthetic/acetate-requiring mutants of *C. reinhardtii* were screened for a specific loss of *c*-type cytochromes [45]. Such mutants, called ccs strains (for cytochrome *c* synthesis) were pleiotropically deficient in cytochromes *f* and c_6, but contained normal amounts of mitochondrial cyt *c* and plastid cyt b_{559} [45]. The defect was localized to the step of heme attachment because the apoproteins were synthesized normally, targeted and processed [22, 45], but not converted to the respective holoforms. A total of 20 ccs strains were analyzed and shown genetically to represent a minimum of six loci, nucleus-encoded Ccs1 to Ccs5 and plastid-encoded ccsA [45, 73, 74]. There might be more genes involved since three of the loci are represented by only one mutant, suggesting that the screen is not saturated. The large number of genes is not a surprise since the process in bacteria also requires numerous ccm genes. The ccsA [75] and Ccs1 [76] genes have been cloned.

4.2.2.1. *ccsA*

The plastid encoded *ccsA* gene, formerly known as *ycf5* [75], encodes a novel hydrophobic protein. The *ccsA* gene was identified on the basis of its ability to complement strain B6 and is now known to rescue all *ccsA* mutant alleles including ct34, ct59, and FUD8. The sequence of CcsA does not speak to its specific biochemical function. The protein has a molecular mass of approximately 3.5×10^4 and spans the membrane several times. Approximately only 80 residues (out of > 300) are absolutely conserved in a multiple alignment of 12 plastid and cyanobacterial homologues [52]. Less related proteins are found in the genomes of many bacteria including cyanobacteria, *B. subtilis*, *Mycobacterium* spp., *Neisseria* spp., and *Helicobacter pylori* [52]. But again, their specific biochemical functions are not known. In *Synechocystis*, where cyt b_6f function is essential, *ccsA* cannot be 'knocked out' [77], which is not inconsistent with a role for cyanobacterial CcsA in cyt *f* assembly. The C-terminal portion of CcsA is very highly conserved, especially the region containing the 'WWD motif'. This motif, consisting of WGxxWx-WDxE, was noted originally in CcmF and CcmC [50, 54, 78, 79]. Since it is found only in cytochrome biosynthesis components, it was suggested that it is involved in heme binding. Some of the tryptophans and the aspartic acid of the WWD motif in CcsA are essential for its function in cytochrome assembly along with conserved histidine residues in putative trans-membrane domains (B.W. Dreyfuss et al., unpublished data). The tryptophan-rich motif is predicted, by PhoA fusion analysis in *E. coli*, to lie in the periplasm [54] (B.W. Dreyfuss et al., unpublished data), which corresponds to the lumen, and this is consistent with a possible role in heme delivery or heme binding during holoprotein assembly. Based on the role of CcmC in *E. coli* and the similar functional importance of tryptophan and histidine residues in the bacterial proteins [55, 80], it is likely that CcsA functions in a heme delivery pathway.

4.2.2.2. *Ccs1*

Six *ccs1* alleles have been isolated and characterized [73] (B.W. Dreyfuss, S. Merchant, unpublished data). Loss of function alleles and point mutations resulting from insertional and UV mutagenesis, respectively, can be isolated at high frequency. Pulse-chase analysis of *ccs1* strains shows that the mutants are blocked in heme attachment; specifically, they synthesize the apocyto-

chromes but can not convert the apoprotein to its holoform [45, 73]. The cloned *Ccs1* gene complements all *C. reinhardtii ccs*1 mutant alleles. The nucleus-encoded *Ccs1* gene of *Chlamydomonas* also encodes a novel protein with three potential transmembrane domains in the N-terminal half of the protein and a hydrophilic C-terminal half predicted to lie in the lumen. It has a molecular mass of approximately 6.0×10^4. Ccs1 is less conserved than CcsA. Candidate homologues occur in virtually all organisms that encode CcsA-like proteins, suggesting that they might function together. Indeed, in some organisms they are physically linked [52]. The protein sequence does not speak to any obvious function in the maturation of *c*-type cytochromes. A Ccs1-homologue, called CcsB, in *Synechocystis* sp. PCC 6803, appears to be essential like CcsA of *Synechocystis* sp. PCC 6803 [42]. Nevertheless, a partially functional version was created by deleting the first 24 codons. In this Δ(M1-A24) mutant, pre-apocytochrome *f* accumulates and holocytochrome *f* is found at levels corresponding to about 20% of wild type. This suggests that processing is coupled to heme attachment in cyanobacteria in contrast to what was noted for cytochrome *f* synthesis in chloroplasts (see above, section 3.2). Interestingly, the cyanobacterial mutant could not be generated in a Δ*petE* strain (lacking plastocyanin) where cyt c_6 function becomes essential, demonstrating that CcsB is required for cyt c_6 maturation in *Synechocystis* as well. The impairment of now two essential proteins, cyt *f* and cyt c_6, might well result in a synthetic lethal phenotype for the strain carrying the *ccsB* Δ(1–24) mutation.

Since pre-apsocytochrome *f* accumulates in a *Synechocystis* Δ(M1-A24) mutant, Tichy and Vermaas have proposed that CcsB is an apocytochrome chaperone. Nevertheless, the accumulation of a truncated CcsB in this strain remains to be verified. The accumulation of stoichiometric amounts of CcsB as proposed by these authors, may open the door to biochemical characterization of the protein in this system. By contrast, Ccs1 appears to be a very low abundance protein in *C. reinhardtii*, as is expected for an assembly factor. In greening *C. reinhardtii y-1* cells, the accumulation of Ccs1 parallels that of holocytochrome *f* (B.W. Dreyfuss et al., unpublished data), an observation which is compatible with a role of Ccs1 as an apoprotein chaperone.

Ccs1 does not accumulate to wild-type levels in the ccsA mutants (B.W. Dreyfuss et al., unpublished data) suggesting that the two proteins may interact in a complex such that the stability of one affects the other. As noted already, the genes encoding the two proteins are often found in operon-like arrangements with other potential cytochrome assembly factors in some bacterial and plastid genomes [52]. One working model is that the CcsA and Ccs1 proteins form a 'CCS complex' involved in heme transport and attachment as has been demonstrated for the bacterial *Ccm* system [54, 80] *(figure 2)*.

4.2.2.3. CcdA and thioredoxin

What could the unidentified *Ccs* genes encode? The CCS pathway of plastids is one of three different processes for cytochrome *c* maturation [53]. Other organisms that contain CcsA- and Ccs1-like components include *B. subtilis* and *Mycobacterium* spp. In these bacteria, a thioredoxin-like protein is linked to the *ccs* genes [52, 81]. Based on what we know about the ccm pathway in *E. coli* (see above, section 4.2.1.3), it is reasonable to imagine that a thioredoxin-like component might be required for maintenance of the cysteinyl thiols of plastid apocytochromes in a state suitable for the lyase reaction. Thus, one of the unknown *Ccs* genes of *C. reinhardtii* may well encode such a thioredoxin-like protein. A search of the EST database reveals four different thioredoxin-like sequences: three of them correspond to known thioredoxins [82], the fourth is probably lumen-targeted and hence not likely to be a *Ccs* factor. This is not surprising, since *Ccs* encoding RNAs are expected to be low abundance species. For instance only one *C. reinhardtii Ccs1*-encoding EST was found (4/00) versus 200 for *Pcy1* (4/00).

Genetic analysis of cytochrome *c* formation in *B. subtilis* led to the identification of the *ccdA* gene [68, 69]. Mutations in *ccdA* result in a pleiotropic cytochrome *c*-deficiency. CcdA displays low but significant sequence similarity to the DipZ/DsbD family of thiol-disulfide oxidoreductases. Two cysteines are conserved in the CcdA-like sequences found in the databases (12/99), but these are not arranged in a thioredoxin-type motif. Recent work with *R. capsulatus* has shown that both conserved cysteines in CcdA are in a transmembrane domain and both are required for its function [83]. CcdA possibly functions in concert with a thioredoxin in apocytochrome thiol metabolism (reviewed in [84]). In *Porphyra purpurea*, CcdA is encoded in the plastid genome, suggesting that it functions in this organelle. Interestingly, it is physically linked to a Ccs1-like protein (as is also the case in *Mycobacterium* spp., Genbank Z74024 and several cyanobacteria, Genbank AF052290, AF079137, D90916, D64001). Again, this argument suggests a function for CcdA in the plastid cytochrome *c* biogenesis pathway. Nevertheless, neither biochemical nor genetic evidence is available at present to assign a function to the plant or cyanobacterial CcdA-like proteins, nor even to present a sub-organellar location or topology. At least one CcdA-encoding gene (Genbank AF225913) is found in *Arabidopsis* (Chromosome 5; 31% sequence identity in a 239 amino acid overlap with *P. purpurea* CcdA *(figure 3)*. Also, many ESTs encoding CcdA-like proteins are found in the genomes of rice, tomato, and soybean. They are found with BLAST [85] scores ranging from $\approx 10^{-17}$ to $\approx 10^{-55}$, suggesting that they probably are homologues of *Arabidopsis* CcdA. The gene product from *Arabidopsis* has an N-terminal extension relative to the plastid and bacterial proteins, suggesting that the *Arabidopsis* protein could be targeted to an organelle (U. Boronowsky et al.,

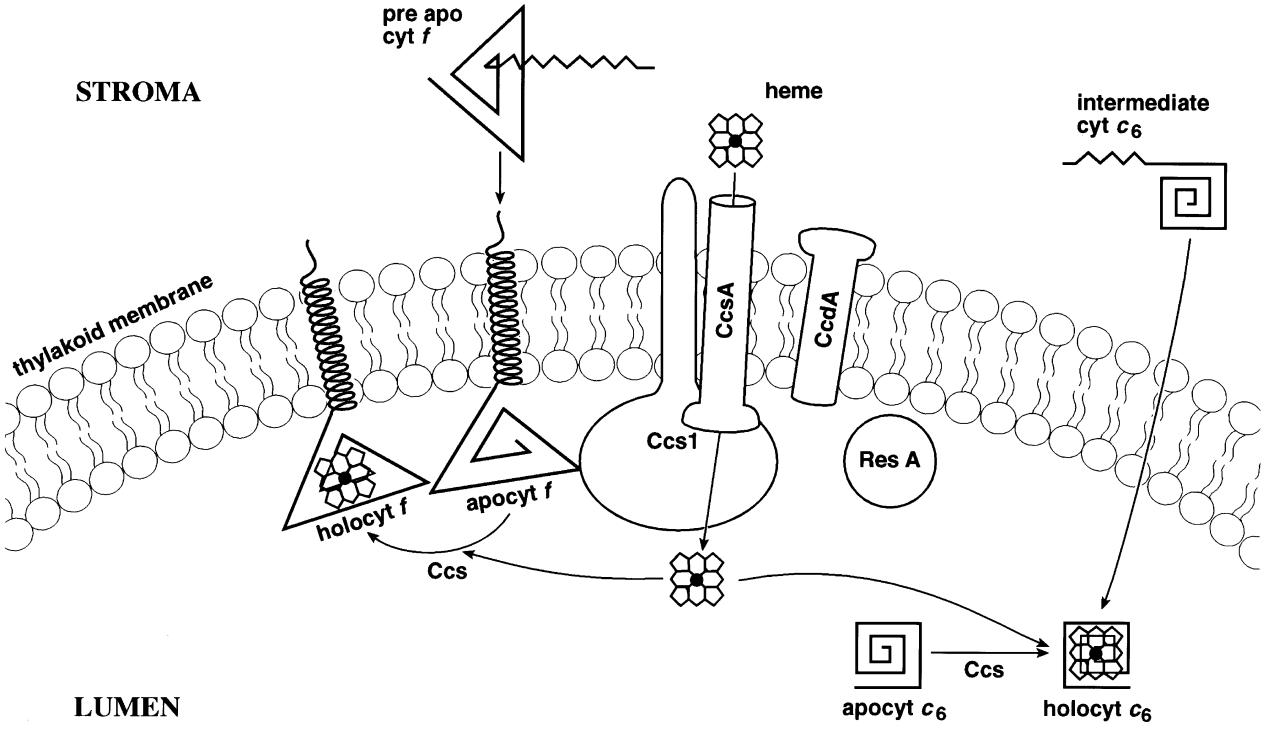

Figure 2. Summary of the proposed cytochrome *c* synthesis pathway. Ccs1 and CcsA are drawn as part of the CCS complex with CcdA and ResA as potential additional players. Ccs1 is proposed to interact with the apo-cytochrome, illustrated here as a chaperone for apo-cyt *f*. CcsA is a potential heme delivery factor. Although ResA has not been identified in the plastid system, it is expected that thioredoxin-like proteins, such as ResA and CcdA, will be required for cysteinyl thiol maintenance.

work in progress). Since a CcdA-encoding gene has been identified in *Arabidopsis* and since *Chlamydomonas* chloroplast components are extremely similar to those of vascular plants, we suspect that one of the uncharacterized CCS loci in *Chlamydomonas* could encode CcdA.

5. *b*-type cytochromes

While the requirement of catalytic factors for heme/apoprotein association has long been acknowledged in the case of holocytochrome *c*, the need for cytochrome *b* specific assembly factors was not considered until recently. Because the cofactor is retained by non-covalent interactions in *b*-type cytochromes, it has been assumed that, in vivo, the heme moiety is captured spontaneously into the apoprotein. Indeed, heme is able to bind in vitro, without catalysis, to synthetic peptides mimicking the helices of mitochondrial cytochrome *b* [86]. This view has long been uncontested by virtue of the fact that no specific factor involved in heme binding to *b*-type cytochrome had been identified. However, in many systems, the fact that heme association defects cannot be distinguished from other defects related to *b*-type cytochrome assembly has rendered difficult any investigation of holoform maturation.

5.1. Cytochrome b_6

Cytochrome b_6 spans the membrane four times and binds two hemes, b_L and b_H, on the lumen and stromal side, respectively, by interhelix bis-histidyl ligation *(figure 1D)* [87, 88]. However, in the absence of assembly partners, apocytochrome b_6 is synthesized normally, incorporates heme but undergoes rapid degradation [30]. Interestingly, unlike most *b*-type cytochromes, the heme/cytochrome b_6 association is resistant to denaturing treatments [30, 89, 90], which suggests that the interaction might be covalent. Mutation of any of the four histidine ligands that coordinate the b_H and b_L hemes led to the appearance of distinct immunoreactive species of cytochrome b_6 corresponding to apocytochrome b_6, holocytochrome b_6, and a b_L-heme dependent intermediate. The identification of these species has been exploited to define a pathway for apo to holocytochrome b_6 conversion [30]. Heme-depleted cells or cells carrying altered b_L sites

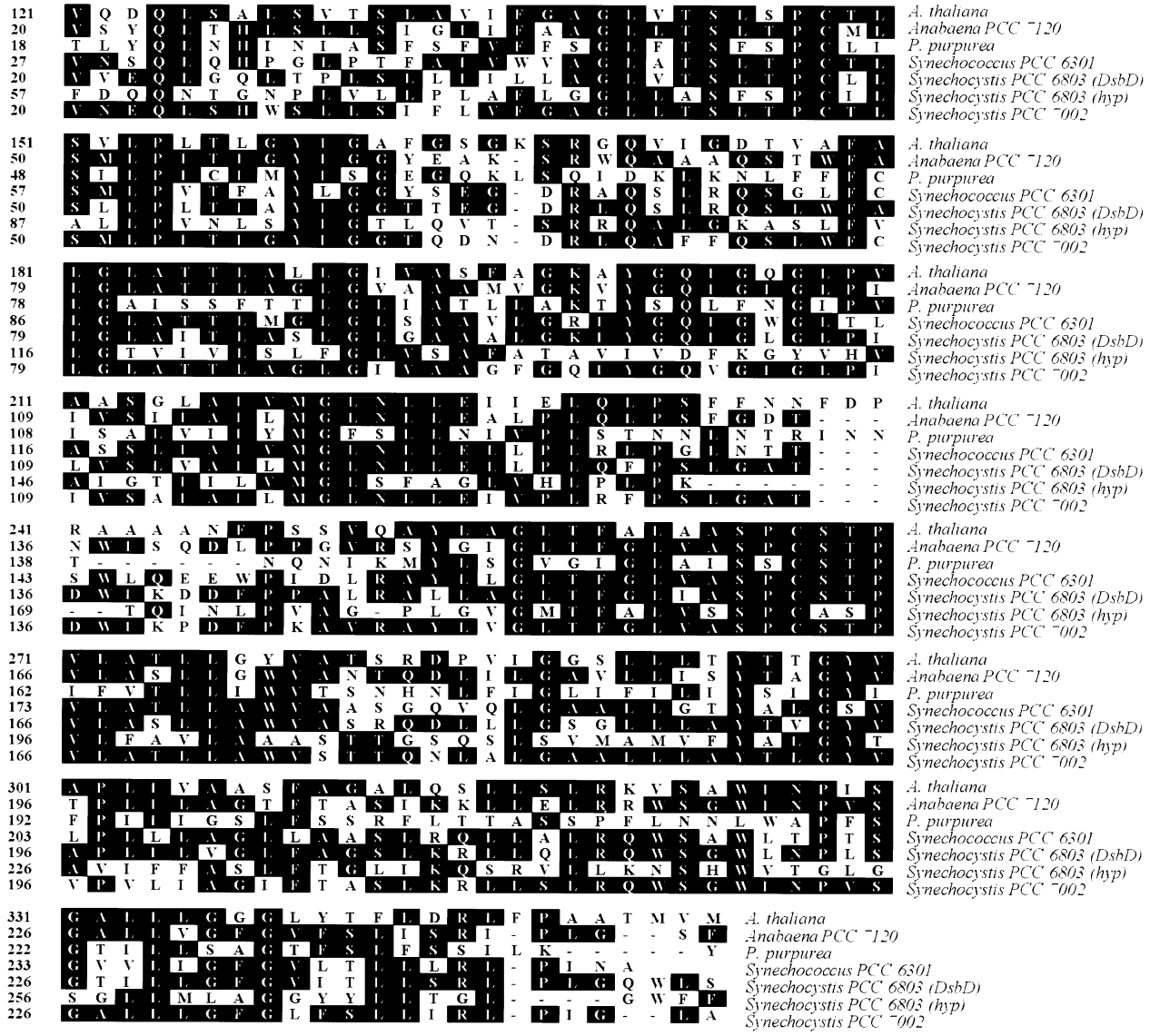

Figure 3. CcdA sequence alignment of plastid organisms: *Arabidopsis thaliana* (Genbank AF225913), *Anabaena* PCC7120 (Genbank AF136760), *Porphyra purpurea* (*ycf*44) (Genbank U38804), *Synechococcus* PCC 6301 (Genbank AF079137), *Synechocystis* PCC 6803 (DsbD and hypothetical protein) (Genbank D90916 and D64001), and *Synechococcus* PCC 7002 (Genbank AF052290). Alignment was done using the CLUSTAL method. Residues that match the consensus sequence determined by the CLUSTAL program are shaded in black.

accumulate a single immunoreactive band corresponding to apocytochrome b_6. Cells harboring b_H site-directed mutations accumulate an additional species identified as a b_L-intermediate in the formation of holocytochrome b_6. Thus, the assembly pathway proceeds in the following order *(figure 4)*: apocytochrome $\rightarrow b_L$ heme dependent intermediate $\rightarrow$ holocytochrome.

Among a collection of nuclear photosynthetic mutants displaying fluorescence characteristics typical of mutants impaired in b_6f activity, a subset exhibits a specific block in the insertion of b_H revealed by the co-occurrence of apocytochrome b_6 and b_L-intermediate species but not holocytochrome b_6. Genetic analysis concluded that these mutants defined four unlinked *CCB* loci, for conversion of

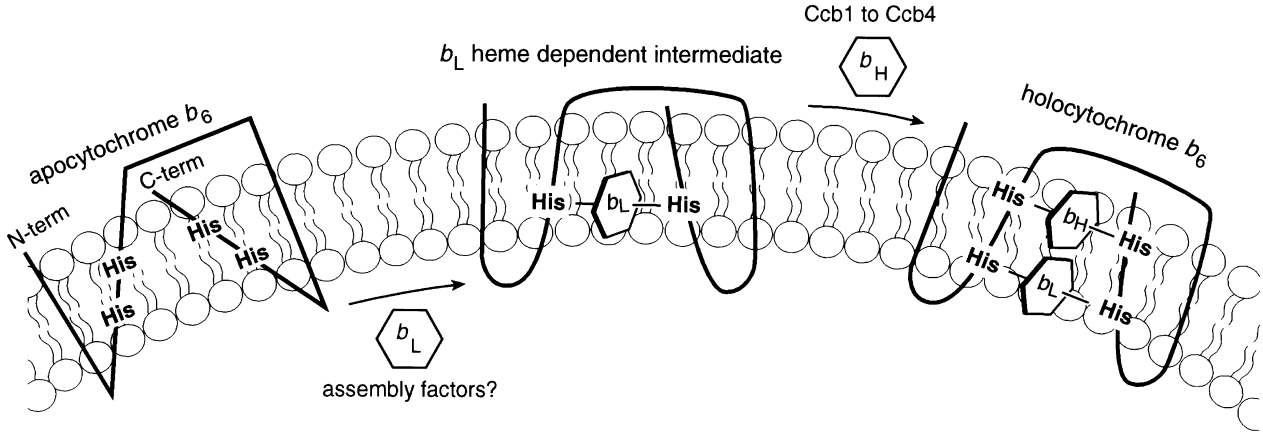

Figure 4. Cytochrome b_6 assembly pathway. The apoprotein is integrated into the membrane even when heme is not present. No factors have been found that are required for the assembly of the b_L heme-dependent intermediate. The formation of this intermediate can be prevented by gabaculine treatment (section 3.2). Nuclear encoded factors, Ccb1 to Ccb4, are required for holocytochrome formation.

cytochrome *b*, whose products are required specifically for the formation of holocytochrome b_6 [30]. The identification of *trans*-acting mutants, which are blocked at one step of this pathway, gives credence to the fact that conversion of apo to holocytochrome b_6 is catalyzed. However, b_L-heme association mutants could not be identified and it is quite possible that b_L insertion is indeed an uncatalyzed step or that mutations resulting in a block in this step lead to a pleiotropic cytochrome deficiency phenotype. For instance, a defect in cyt b_{559} would cause an inability to assemble PSII. And therefore such mutants would have escaped the screen, which relied on a b_6f-specific defect [30].

Since the *Ccb* genes are not cloned, their putative functions can only be speculated upon. Candidate functions for the *CCB* loci include cofactor chaperoning/delivery/transport, apocytochrome processing and catalysis of heme/apoprotein association. One might expect the *Ccb* factors to be conserved in other organisms such as *Synechocystis* spp. and *Bacillus* spp., which contain a b_6f or b_6f-related complex showing an unusually tight binding of heme to cytochrome *b* [90]. It will be very interesting to see whether chloroplast and bacteria show diversity in the mode of assembly of b_6-related cytochromes *b* as they do for the formation of *c*-type cytochromes [53].

5.2. Cytochrome b_{559}

Cytochrome b_{559} consists of two small polypeptides, the α (9 kDa) and β (4 kDa) subunits encoded by plastid genes *psbE* and *psbF*, respectively. Although the precise structure of the cytochrome is not known, it has been determined that each subunit contains a single histidine involved in the coordination of the heme cofactor. Mutation of this histidine residue from either subunit results in destabilization of PSII and loss of assembly [91]. The two subunits have a 1:1 stoichiometry [92] and may form a heterodimer α/β with one bis-histidinyl coordinated heme molecule [93]. In *Synechocystis* sp. PCC 6803, however, a genetic fusion of the amino terminus of the β subunit with the carboxy terminus of the α subunit enables photoautotrophic growth [94] and supports an α_2/β_2 homodimer model with one heme molecule in each α_2 or β_2 homodimer. The stoichiometry between b_{559} and PSII subunits has yet to be agreed upon. Several groups report one heme in PSII, supporting one α/β heterodimer in the complex; while other groups report two hemes, which could correspond to either two heterodimers or one α_2/β_2 homodimer in PSII (reviewed in [6]). In vitro assembly of a β_2 holocytochrome b_{559}-like complex, with spectral and redox properties similar to the native cyt b_{559}, from a synthetic peptide corresponding to the *Synechocystis psbF* gene product was accomplished recently [95]. In this work, assembly of the β_2 dimer was successful only when ferro-heme was supplied. The assembled protein contained a single heme per dimer. This work supports an α_2/β_2 model and suggests that specific factors may well be required for cyt b_{559} assembly in vivo, at least for maintenance of the heme iron in the reduced state.

6. Conclusion

The assembly of cofactor-containing proteins requires several catalyzed steps. For simple proteins, like the cytochromes, these include cofactor delivery to the correct

compartment, maintenance of the apoprotein in a structure/conformation amenable for holoprotein assembly, and specific ligation of the cofactor to the functional groups on the polypeptide. The process is difficult to study by traditional biochemical methods, since the specific reactions are not yet known. Further, as judged from the low abundance of Ccs transcripts in *Chlamydomonas* and the rather meager representation of cDNAs for Ccs1 and CcdA in the EST databases (4/00), the factors are likely to be low abundance proteins. The discovery of such factors therefore relies heavily on genetic approaches. Indeed, this has proved useful for the discovery of similar factors required for mitochondrial and bacterial cytochrome assembly. Based on the specificity of the six Ccs factors for cytochromes c_6 and *f*, and the four Ccb factors for only one of the two heme binding sites of cytochrome b_6, we predict that a substantial number of assembly factors remain to be discovered.

Acknowledgments

We thank Dr. Beth Dreyfuss and Professor Dominique Missiakas for their helpful comments on the manuscript. Our work is supported by the National Institutes of Health (GM48350). S.M. was supported by a Guggenheim Foundation fellowship.

References

[1] Rouault T.A., Klausner R.D., Iron-sulfur clusters as biosensors of oxidants and iron, Trends Biochem. Sci. 21 (1996) 174–177.

[2] Beinert H., Kiley P.J., Fe-S proteins in sensing and regulatory functions, Curr. Opin. Chem. Biol. 3 (1999) 152–157.

[3] Bernardi P., Scorrano L., Colonna R., Petronilli V., Di Lisa F., Mitochondria and cell death. Mechanistic aspects and methodological issues, Eur. J. Biochem. 264 (1999) 687–701.

[4] Kroemer G., Cytochrome c, Curr. Biol. 9 (1999) R468.

[5] Merchant S., Dreyfuss B.W., Posttranslational assembly of photosynthetic metalloproteins, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49 (1998) 25–51.

[6] Stewart D.H., Brudvig G.W., Cytochrome b_{559} of photosystem II, Biochim. Biophys. Acta 1367 (1998) 63–87.

[7] Merchant S., Bogorad L., Metal ion regulated gene expression: use of a plastocyanin-less mutant of *Chlamydomonas reinhardtii* to study the Cu(II)-dependent expression of cytochrome *c*-552, EMBO J. 6 (1987) 2531–2535.

[8] Merchant S., Hill K., Howe G., Dynamic interplay between two copper-titrating components in the transcriptional regulation of cyt c_6, EMBO J. 10 (1991) 1383–1389.

[9] Quinn J.M., Barraco P., Eriksson M., Merchant S., Coordinate copper- and oxygen-responsive *Cyc6* and *Cpx1* expression in *Chlamydomonas* is mediated by the same element, J. Biol. Chem. 275 (2000) 6080–6089.

[10] Waldo G.S., Wright E., Whang Z.H., Briat J.F., Theil E.C., Sayers D.E., Formation of the ferritin iron mineral occurs in plastids, Plant Physiol. 109 (1995) 797–802.

[11] Craig E.A., Voisine C., Schilke B., Mitochondrial iron metabolism in the yeast *Saccharomyces cerevisiae*, J. Biol. Chem. 380 (1999) 1167–1173.

[12] Li J., Kogan M., Knight S.A., Pain D., Dancis A., Yeast mitochondrial protein, Nfs1p, coordinately regulates iron-sulfur cluster proteins, cellular iron uptake, and iron distribution, J. Biol. Chem. 274 (1999) 33025–33034.

[13] Lill R., Diekert K., Kaut A., Lange H., Pelzer W., Prohl C., Kispal G., The essential role of mitochondria in the biogenesis of cellular iron-sulfur proteins, J. Biol. Chem. 380 (1999) 1157–1166.

[14] Lange H., Kaut A., Kispal G., Lill R., A mitochondrial ferredoxin is essential for biogenesis of cellular iron-sulfur proteins, Proc. Natl. Acad. Sci. USA 97 (2000) 1050–1055.

[15] Jordon P.E., Biosynthesis of Tetrapyrroles, in: Neuberger A., van Deenen L. (Eds.), New Comprehensive Biochemistry, Elsevier Science Publishers, Amsterdam, 1991.

[16] Smith A., Marsh O., Elder G., Investigation of the subcellular location of the tetrapyrrole-biosynthesis enzyme coproporphyrinogen oxidase in higher plants, Biochem. J. 292 (1993) 503–508.

[17] Lermontova I., Kruse E., Mock H.P., Grimm B., Cloning and characterization of a plastidal and a mitochondrial isoform of tobacco protoporphyrinogen IX oxidase, Proc. Natl. Acad. Sci. USA 94 (1997) 8895–8900.

[18] Chow K.S., Singh D.P., Roper J.M., Smith A.G., Lermontova I., Kruse E., Mock H.P., Grimm B., A single precursor protein for ferrochelatase-I from Arabidopsis is imported in vitro into both chloroplasts and mitochondria. Cloning and characterization of a plastidal and a mitochondrial isoform of tobacco protoporphyrinogen IX oxidase, J. Biol. Chem. 272 (1997) 27565–27571.

[19] Howe G., Merchant S., Maturation of thylakoid lumen proteins proceeds post-translationally through an intermediate in vivo, Proc. Natl. Acad. Sci. USA 90 (1993) 1862–1866.

[20] Mishkind M., Schmidt G., Posttranscriptional regulation of ribulose 1, 5-bisphosphate carboxylase small subunit accumulation in *Chlamydomonas reinhardtii*, Plant Physiol. 72 (1997) 847–854.

[21] Schmidt R., Richardson C., Gillham N., Boynton J., Sites of synthesis of chloroplast ribosomal proteins in *Chlamydomonas*, J. Cell Biol. 96 (1983) 1451–1463.

[22] Howe G., Mets L., Merchant S., Biosynthesis of cytochrome *f* in *Chlamydomonas reinhardtii*: analysis of the pathway in gabaculine-treated cells and in the heme attachment mutant B6, Mol. Gen. Genet. 246 (1995) 156–165.

[23] Howe G., Merchant S., Role of heme in the biosynthesis of cytochrome c_6, J. Biol. Chem. 269 (1994) 5824–5832.

[24] Oelmuller R., Levitan I., Bergfeld R., Rajasekhar V.K., Mohr H., Expression of nuclear genes as affected by treatments acting on the plastids, Planta 168 (1986) 482–492.

[25] Mochizuike N., Susek R., Chory J., An intracellular signal transduction pathway between the chloroplast and nucleus is involved in de-etiolation, Plant Physiol. 112 (1996) 1465–1469.

[26] Kropat J., Oster U., Rudiger W., Beck C.F., Chlorophyll precursors are signals of chloroplast origin involved in light induction of nuclear heat-shock genes, Proc. Natl. Acad. Sci. USA 94 (1997) 14168–14172.

[27] Lopez-Juarez E., Jarvis R.P., Takeuchi A., Page A.M., Chory J., New Arabidopsis cue mutants suggest a close connection between plastid- and phytochrome regulation of nuclear gene expression, Plant Physiol. 118 (1998) 803–815.

[28] Anderson C., Gray J., Effect of gabaculine on the synthesis of heme and cyt *f* in etiolated wheat seedlings, Plant Physiol. 96 (1991) 584–587.

[29] Kuras R., Buschlen S., Wollman F., Maturation of pre-apocytochrome *f* in vivo. A site-directed mutagenesis study in *Chlamydomonas reinhardtii*, J. Biol. Chem. 270 (1995) 27797–27803.

[30] Kuras R., De Vitry C., Choquet Y., Girard-Bascou J., Culler D., Buschlen S., Merchant S., Wollman F.A., Molecular genetic identification of a pathway for heme binding to cytochrome b_6, J. Biol. Chem. 272 (1997) 32427–32435.

[31] Doherty A., Gray J., Synthesis of cytochrome *f* by isolated pea chloroplasts, Eur. J. Biochem. 98 (1979) 87–92.

[32] Phillips A., Gray J., Synthesis of components of the cytochrome *b-f* complex by isolated pea chloroplasts, Eur. J. Biochem. 138 (1984) 591–595.
[33] Alt J., Westhoff P., Sears B.B., Nelson N., Hurt E., Hauska G., Herrmann R.G., Genes and transcripts for the polypeptides of the cytochrome b_6/f complex form spinach thylakoid membranes, EMBO J. 2 (1983) 979–986.
[34] Willey D.L., Auffret A.D., Gray J.C., Structure and topology of cytochrome *f* in pea chloroplast membranes, Cell 36 (1984) 555–562.
[35] Smith T.A., Kohorn B.D., Mutations in a signal sequence for the thylakoid membrane identify multiple protein transport pathways and nuclear suppressors, J. Cell. Biol. 126 (1994) 365–374.
[36] Rothstein S., Gatenby A., Willey D., Gray J., Binding of pea cytochrome *f* to the inner membrane of *Escherichia coli* requires the bacterial *secA* gene product, Proc. Natl. Acad. Sci. USA 82 (1985) 7955–7959.
[37] Willey D.L., Gray J.C., Synthesis and assembly of the cytochrome *b-f* complex, Photosyn. Res. 17 (1988) 125–144.
[38] Howe G., Merchant S., The biosynthesis of bacterial and plastidic *c*-type cytochromes, Photosyn. Res. 40 (1994) 147–165.
[39] Perret M., Bernd K., Kohorn B., Chloroplast protein translocation, in: Rochaix J.-D., Goldschmidt-Clermont M., Merchant S. (Eds.), The Molecular Biology of Chloroplasts and Mitochondria in *Chlamydomonas*, Kluwer Academic Publishers, the Netherlands, 1998.
[40] Martinez S.E., Huang D., Szczepaniak A., Cramer W.A., Smith J.L., Crystal structure of chloroplast cytochrome *f* reveals a novel cytochrome fold and unexpected heme ligation, Structure 2 (1994) 95–105.
[41] Baymann F., Zito F., Kuras R., Minai L., Nitschke W., Wollman F.A., Functional characterization of *Chlamydomonas* mutants defective in cytochrome *f* maturation, J. Biol. Chem. 274 (1999) 22957–22967.
[42] Tichy M., Vermaas W., Accumulation of pre-apocytochrome *f* in a *Synechocystis* sp. PCC 6803 mutant impaired in cytochrome *c* maturation, J. Biol. Chem. 274 (1999) 32396–32401.
[43] Gonzales D.H., Neupert W., Biogenesis of mitochondrial *c*-type cytochromes, J. Bioenerg. Biomembr. 22 (1990) 753–768.
[44] Cline K., Henry R., Import and routing of nucleus-encoded chloroplast proteins, Annu. Rev. Cell Dev. Biol. 12 (1996) 1–26.
[45] Howe G., Merchant S., The biosynthesis of membrane and soluble plastidic c-type cytochromes of *Chlamydomonas reinhardtii* is dependent on multiple common gene products, EMBO J. 11 (1992) 2789–2801.
[46] Dumont M.E., Ernst J.F., Hampsey D.M., Sherman F., Identification and sequence of the gene encoding cytochrome *c* heme lyase in the yeast *Saccharomyces cerevisiae*, EMBO J. 6 (1987) 235–241.
[47] Drygas M.E., Lambowitz A.M., Nargang F.E., Cloning and analysis of the *Neurospora crassa* gene for cytochrome *c* heme lyase, J. Biol. Chem. 264 (1989) 17897–17906.
[48] Steiner H., Zollner A., Haid A., Neupert W., Lill R., Biogenesis of mitochondrial heme lyases in yeast. Import and folding in the intermembrane space, J. Biol. Chem. 270 (1995) 22842–22849.
[49] Thony-Meyer L., Loferer H., Ritz D., Hennecke H., Bacterial genes and proteins involved in the biogenesis of c-type cytochromes and terminal oxidases, Biochim. Biophys. Acta 1187 (1994) 260–263.
[50] Thony-Meyer L., Fischer F., Kunzler P., Ritz D., Hennecke H., *Escherichia coli* genes required for cytochrome *c* maturation, J. Bacteriol. 177 (1995) 4321–4326.
[51] Grove J., Tanapongpipat S., Thomas G., Griffiths L., Crooke H., Cole J., *Escherichia coli* K-12 genes essential for the synthesis of *c*-type cytochromes and a third nitrate reductase located in the periplasm, Mol. Microbiol. 19 (1996) 467–481.
[52] Xie Z., Merchant S., A novel pathway for cytochromes *c* biogenesis in chloroplasts, Biochim. Biophys. Acta 1365 (1998) 309–318.
[53] Kranz R., Lill R., Goldman B., Bonnard G., Merchant S., Molecular mechanisms of cytochrome *c* biogenesis: three distinct systems, Mol. Microbiol. 29 (1998) 383–396.
[54] Goldman B.S., Beckman D.L., Monika E.M., Kranz R.G., Transmembrane heme delivery systems, Proc. Natl. Acad. Sci. USA 95 (1998) 5003–5008.
[55] Schulz H., Fabianek R.A., Pellicioli E.C., Hennecke H., Thony-Meyer L., Heme transfer to the heme chaperone CcmE during cytochrome *c* maturation requires the CcmC protein, which may function independently of the ABC-transporter CcmAB, Proc. Natl. Acad. Sci. USA 96 (1999) 6462–6467.
[56] Gaballa A., Baysse C., Koedam N., Muyldermans S., Cornelis P., Different residues in periplasmic domains of the CcmC inner membrane protein of *Pseudomonas fluorescens* ATCC 17400 are critical for cytochrome *c* biogenesis and pyoverdine-mediated iron uptake, Mol. Microbiol. 30 (1998) 547–555.
[57] Schulz H., Hennecke H., Thony-Meyer L., Prototype of a heme chaperone essential for cytochrome *c* maturation, Science 281 (1998) 1197–1200.
[58] Fabianek R.A., Huber-Wunderlich M., Glockshuber R., Kunzler P., Hennecke H., Thony-Meyer L., Characterization of the *Bradyrhizobium japonicum* CycY protein, a membrane-anchored periplasmic thioredoxin that may play a role as a reductant in the biogenesis of *c*-type cytochromes, J. Biol. Chem. 272 (1997) 4467–4473.
[59] Monika E.M., Goldman B.S., Beckman D.L., Kranz R.G., A thioreduction pathway tethered to the membrane for periplasmic cytochromes *c* biogenesis; in vitro and in vivo studies, J. Mol. Biol. 271 (1997) 679–692.
[60] Page M.D., Ferguson S.J., *Paracoccus denitrificans* CcmG is a periplasmic protein-disulphide oxidoreductase required for *c*- and aa_3-type cytochrome biogenesis; evidence for a reductase role in vivo, Mol. Microbiol. 24 (1997) 977–990.
[61] Fabianek R.A., Hennecke H., Thony-Meyer L., The active-site cysteines of the periplasmic thioredoxin-like protein CcmG of *Escherichia coli* are important but not essential for cytochrome *c* maturation in vivo, J. Bacteriol. 180 (1998) 1947–1950.
[62] Crooke H., Cole J., The biogenesis of *c*-type cytochromes in *Escherichia coli* requires a membrane-bound protein, DipZ, with a protein disulphide isomerase-like domain, Mol. Microbiol. 15 (1995) 1139–1150.
[63] Missiakas D., Schwager F., Raina S., Identification and characterization of a new disulfide isomerase-like protein (DsbD) in *Escherichia coli*, EMBO J. 14 (1995) 3415–3424.
[64] Page M.D., Saunders N.F., Ferguson S.J., Disruption of the *Pseudomonas aeruginosa dipZ* gene, encoding a putative protein-disulfide reductase, leads to partial pleiotropic deficiency in c-type cytochrome biogenesis, Microbiology 143 (1997) 3111–3122.
[65] Stewart E.J., Katzen F., Beckwith J., Six conserved cysteines of the membrane protein DsbD are required for the transfer of electrons from the cytoplasm to the periplasm of *Escherichia coli*, EMBO J. 18 (1999) 5963–5971.
[66] Chung J., Chen T., Missiakas D., Transfer of electrons across the cytoplasmic membrane by DsbD, a membrane protein involved in thiol-disulphide exchange and protein folding in the bacterial periplasm, Mol. Microbiol. 00 (2000) in press.
[67] Sambongi Y., Ferguson S.J., Specific thiol compounds complement deficiency in c-type cytochrome biogenesis in *Escherichia coli* carrying a mutation in a membrane-bound disulphide isomerase-like protein, FEBS Lett. 353 (1994) 235–238.
[68] Schiott T., Von Wachenfeldt C., Hederstedt L., Identification and characterization of the *ccdA* gene, required for cytochrome *c* synthesis in *Bacillus subtilis*, J. Bacteriol. 179 (1997) 1962–1973.
[69] Schiott T., Throne-Holst M., Hederstedt L., *Bacillus subtilis* CcdA-defective mutants are blocked in a late step of cytochrome *c* biogenesis, J. Bacteriol. 179 (1997) 4523–4529.
[70] Metheringham R., Griffiths L., Crooke H., Forsythe S., Cole J., An essential role for DsbA in cytochrome *c* synthesis and formate-dependent nitrite reduction by *Escherichia coli* K-12, Arch. Microbiol. 164 (1995) 301–307.

[71] Metheringham R., Tyson K.L., Crooke H., Missiakas D., Raina S., Cole J.A., Effects of mutations in genes for proteins involved in disulphide bond formation in the periplasm on the activities of anaerobically induced electron transfer chains in *Escherichia coli* K12, Mol. Gen. Genet. 253 (1996) 95–102.

[72] Rietsch A., Belin D., Martin N., Beckwith J., An in vivo pathway for disulfide bond isomerization in *Escherichia coli*, Proc. Natl. Acad. Sci. USA 93 (1996) 13048–13053.

[73] Xie Z., Culler D., Dreyfuss B.W., Kuras R., Wollman F.A., Girard-Bascou J., Merchant S., Genetic analysis of chloroplast c-type cytochrome assembly in *Chlamydomonas reinhardtii*: One chloroplast locus and at least four nuclear loci are required for heme attachment, Genetics 148 (1998) 681–692.

[74] Dreyfuss B., Merchant S., CCS5, a new locus required for chloroplast c-type synthesis, in: Pusztai J., Garab G. (Eds.), Proceedings of the XIth International Congress on Photosynthesis, Kluwer Academic Publishers, the Netherlands, 1998.

[75] Xie Z., Merchant S., The plastid-encoded *ccsA* gene is required for heme attachment to chloroplast c-type cytochromes, J. Biol. Chem. 271 (1996) 4632–4639.

[76] Inoue K., Dreyfuss B.W., Kindle K.L., Stern D.B., Merchant S., Sodeinde O.A., Ccs1, a nuclear gene required for the post-translational assembly of chloroplast c-type cytochromes, J. Biol. Chem. 272 (1997) 31747–31754.

[77] Hubschmann T., Wilde A., Elanskaya I., Shestakov S.V., Borner T., A putative cytochrome *c* biogenesis gene in *Synechocystis* sp. PCC 6803, FEBS Lett. 408 (1997) 201–205.

[78] Beckman D.L., Trawick D.R., Kranz R.G., Bacterial cytochromes *c* biogenesis, Genes Dev. 6 (1992) 268–283.

[79] Ritz D., Thony-Meyer L., Hennecke H., The *cycHJKL* gene cluster plays an essential role in the biogenesis of c-type cytochromes in *Bradyrhizobium japonicum*, Mol. Gen. Genet. 247 (1995) 27–38.

[80] Goldman B.S., Beckman D.L., Bali A., Monika E.M., Gabbert K.K., Kranz R.G., Molecular and immunological analysis of an ABC transporter complex required for cytochrome *c* biogenesis, J. Mol. Biol. 268 (1997) 724–738.

[81] Sun G., Sharkova E., Chesnut R., Birkey S., Duggan M.F., Sorokin A., Pujic P., Ehrlich S.D., Hulett F.M., Regulators of aerobic and anaerobic respiration in *Bacillus subtilis*, J. Bacteriol. 178 (1996) 1374–1385.

[82] Jacquot J.P., Stein M., Lemaire S., Decottignies P., Le Marechal P., Lancelin J.M., Molecular aspects of components of the ferredoxin/thioredoxin systems, in: Rochaix J.-D., Goldschmidt-Clermont M., Merchant S. (Eds.), The Molecular Biology of Chloroplasts and Mitochondria in *Chlamydomonas*, Kluwer Academic Publishers, Netherlands, 1998.

[83] Deshmukh M., Brasseur G., Daldal F., Novel *Rhodobacter capsulatus* genes required for the biogenesis of various c-type cytochromes, Mol. Microbiol. 35 (2000) 123–138.

[84] Page M.D., Sambongi Y., Ferguson S.J., Contrasting routes of c-type cytochrome assembly in mitochondria, chloroplasts, and bacteria, Trends Biochem. Sci. 23 (1998) 103–108.

[85] Altschul S.F., Madden T.L., Schaffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J., Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.

[86] Robertson D.E., Farid R.S., Moser C.C., Urbauer J.L., Mulholland S.E., Pidikiti R., Lear J.D., Wand A.J., DeGrado W.F., Dutton P.L., Design and synthesis of multi-haem proteins, Nature 368 (1994) 425–432.

[87] Szczepaniak A., Cramer W., Thylakoid membrane protein topography. Location of the termini of the chloroplast cytochrome b6 on the stromal side of the membrane, J. Biol. Chem. 29 (1990) 17720–17726.

[88] Cramer W.A., Martinez S.E., Huang D., Tae G.S., Everly R.M., Heymann J.B., Cheng R.H., Baker T.S., Smith J.L., Structural aspects of the cytochrome b_6f complex; structure of the lumen-side domain of cytochrome *f*, J. Bioenerg. Biomembr. 26 (1994) 31–47.

[89] Salerno J.C., Yoshida S., King T.E., Effects of protein-protein and protein-lipid interactions on heme site conformation in the mitochondrial *b* cytochromes, J. Biol. Chem. 261 (1986) 5480–5486.

[90] Yu J., Le Brun N.E., Studies of the cytochrome subunits of menaquinone:cytochrome *c* reductase (bc complex) of *Bacillus subtilis*. Evidence for the covalent attachment of heme to the cytochrome *b* subunit, J. Biol. Chem. 273 (1998) 8860–8866.

[91] Pakrasi H.B., De Ciechi P., Whitmarsh J., Site directed mutagenesis of the heme axial ligands of cytochrome b_{559} affects the stability of the photosystem II complex, EMBO J. 10 (1991) 1619–1627.

[92] Widger W., Cramer W., Hermodson R., Herrmann R., Evidence for a hetero-oligomeric structure of the chloroplast cytochrome *b*-559, FEBS Lett. 191 (1985) 186–190.

[93] Babcock G.T., Widger W.R., Cramer W.A., Oertling W.A., Metz J.G., Axial ligands of chloroplast cytochrome b-559: identification and requirement for a heme-cross-linked polypeptide structure, Biochemistry 24 (1985) 3638–3645.

[94] Mc Namara V.P., Sutterwala F.S., Pakrasi H.B., Whitmarsh J., Structural model of cytochrome b_{559} in photosystem II based on a mutant with genetically fused subunits, Proc. Natl. Acad. Sci. USA 94 (1997) 14173–14178.

[95] Francke C., Loyal R., Ohad I., Haehnel W., In vitro assembly of a b_2 cytochrome *b*like complex from the chemically synthesised beta-subunit encoded by the *Synechocystis* sp. 6803 *psbF* gene, FEBS Lett. 442 (1999) 75–78.

[96] Kerfeld C.A., Anwar H.P., Interrante R., Merchant S., Yeates T.O., The structure of chloroplast cytochrome c6 at 1.9 A resolution: evidence for functional oligomerization, J. Mol. Biol. 250 (1995) 627–647.

[97] Carrell C.J., Schlarb B.G., Bendall D.S., Howe C.J., Cramer W.A., Smith J.L., Structure of the soluble domain of cytochrome *f* from the cyanobacterium *Phormidium laminosum*, Biochemistry 38 (1999) 9590–9599.

[98] Iwata S., Lee J.W., Okada K., Lee J.K., Iwata M., Rasmussen B., Link T.A., Ramaswamy S., Jap B.K., Complete structure of the 11-subunit bovine mitochondrial cytochrome bc1 complex, Science 281 (1998) 64–71.