The Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas

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Chapter 31

Synthesis of Metalloproteins Involved in Photosynthesis: Plastocyanin and Cytochromes

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Summary

The introduction of a metal cofactor broadens the catalytic repertoire of a protein catalyst by facilitating certain types of chemical reactions. The chemistry of the cofactor depends upon the type of coordinating ligands, and also upon the geometry of the metal-containing active site. Metalloprotein biosynthesis requires 1) mechanisms of metal acquisition and transport to the sub-cellular compartment where metal delivery to the active site of the metalloprotein can occur, and 2) mechanisms of coordinate regulation of polypeptide synthesis with cofactor synthesis (for organic cofactors like heme) or assimilation (for inorganic cofactors like the Mn_4 cluster). The copper-responsive accumulation of copper-containing plastocyanin and heme-containing cytochrome c_6 in *Chlamydomonas reinhardtii* has served as a model for studies of regulation of metalloproteins by metals because the simple growth requirements of Chlamydomonas facilitate studies of metal metabolism. In a fully copper-supplemented medium, plastocyanin accumulates in *C. reinhardtii* but Cyt c_6 does not. As the medium is depleted of copper ($< 8 \times 10^6$ Cu/cell), mechanisms for adaptation to copper-deficiency are induced. These include degradation of plastocyanin, transcriptional activation of *Cyc6* and *Cpx1* genes (encoding Cyt c_6 and coprogen oxidase, respectively), and induction of copper transport and cupric reductase activity. Studies of chloroplast metalloprotein assembly in Chlamydomonas have focused primarily on the heme proteins, with the *c*-type cytochromes being the best studied examples. The occurrence and distribution in the genome databases of *Ccs* genes required for chloroplast *c*-type cytochrome maturation suggests that they represent a third family of cytochrome maturation components, distinct from the Cyt c/c_1 heme lyases of mammalian and fungal mitochondria and also distinct from the components identified in most proteobacteria and plant mitochondria. Approaches for the analysis of the assembly of other cofactor-containing chloroplast proteins are discussed.

I. Introduction

A. Metalloproteins in the Photosynthetic Apparatus

Metalloproteins are defined broadly as proteins with metal-containing cofactors. The metal cofactor may be liganded only by functional groups provided by amino acid side chains from the polypeptide, as in plastocyanin, carbonic anhydrase or the Zn-containing transcription factors; the metal cofactor may be a component of an organic prosthetic group, as in the chlorophyll and heme proteins; or the metal may associate with inorganic ions to form a stable cluster, as in the ferredoxins. Each of these types of metalloproteins is found in the photosynthetic apparatus (Table 1). Metalloproteins are abundant in photosynthetic membranes as they are in all energy transducing membranes owing to the catalytic properties of metal cofactors in reactions involving electron transfer, oxygen chemistry and photochemistry. Since the function of the metalloprotein is critically dependent upon the cofactor, consideration of the biosynthetic pathway of the protein must include the process of cofactor metabolism, cofactor-protein assembly and cofactor-dependent gene expression. While these aspects have been emphasized for the study of the biosynthesis of chlorophyll-binding proteins, there are very few studies of the cofactor-dependent synthesis of other types of metalloproteins in photosynthetic organisms.

Abbreviations: δ-ALA – δ-aminolevulinic acid; b_l – *b*-heme of cytochrome b_6 on the lumen side; b_h – *b*-heme of cytochrome b_6 on the stromal side; *Ccs* genes – cytochrome *c* synthesis genes; coprogen – coproporphyrinogen; –Cu – copper-deficient; +Cu – copper-sufficient; CuREs – copper-responsive elements; Cyt – cytochrome

B. Biosynthesis of Metalloproteins

1. Metal-Responsive Gene Expression in Cyanobacteria and Green Algae

Two systems for the study of metal-dependent synthesis of metalloproteins are provided by the reciprocal iron-dependent synthesis of ferredoxin vs. flavodoxin in cyanobacteria and the reciprocal copper-dependent synthesis of plastocyanin vs. cytochrome c_6 in some cyanobacteria and green algae. Ferredoxin and flavodoxin are functionally interchangeable soluble electron transfer proteins. Ferredoxin is the preferred catalyst in iron-replete media; in iron-deficient media flavodoxin synthesis is induced (Hutber et al., 1977; Sandmann and Malkin, 1983). The importance of this adaptation is underscored by the observation that the growth of

Table 1. Examples of some abundant metalloproteins which function in photosynthesis

Cofactor	Protein
Cu	plastocyanin
Mn_4	PS II reaction center/water oxidation
Fe	PS II reaction center
Zn	carbonic anhydrase
heme	cytochrome b_{559}
	cytochrome b_6
	cytochrome *f*
	cytochrome c_6
Fe_2S_2	Rieske iron-sulfur protein
	ferredoxin
Fe_4S_4	PS I (F_X)
	PS I (F_A)
	PS I (F_B)

The chlorophyll-binding light-harvesting and reaction center proteins are not included in this tabulation, since they are considered separately as a special sub-class (see Chapters 15, Erickson; 19, Hoober et al.; 20, Timko; 17, Webber and Bingham).

photosynthetic organisms in nature can be limited by iron availability (Martin and Fitzwater, 1988), and its relevance in natural populations is apparent from the demonstration that flavodoxin abundance in marine diatoms is determined by iron availability in the ocean (LaRoche et al., 1996). The interested reader is referred to a previous volume in this series for molecular details of the iron-responsive pathway in cyanobacteria (Straus, 1994).

Plastocyanin and cytochrome c_6 form another pair of interchangeable photosynthetic electron transfer catalysts in organisms that have genetic information for both proteins—in copper-supplemented (+Cu) media, plastocyanin is abundant while in copper-deficient (–Cu) media, cytochrome c_6 is abundant (Wood, 1978; Sandmann et al., 1983; Sandmann, 1986). Krogmann and coworkers have suggested that this switch is important in natural populations of photosynthetic microorganisms where copper becomes a limiting trace element during an algal bloom. Indeed, cytochrome c_6 is abundant in such populations but when the organism is cultured in the laboratory in supplemented medium, the capacity for plastocyanin synthesis is evident (Ho et al., 1979). A discussion of the copper-responsive pathway in cyanobacteria and other algae can be found in recent reviews of plastocyanin and cytochrome c_6 function and biosynthesis (Morand et al., 1994; Merchant, 1997). The focus of this chapter is on the copper-dependent synthesis of plastocyanin and cytochrome c_6 in Chlamydomonas.

2. Post-Translational Assembly of Metallocofactor-Containing Proteins

For most proteins of the photosynthetic apparatus, assembly of the apoprotein and cofactor is just one of many events that must occur to generate a functional electron transfer catalyst from a newly-translated polypeptide. In general, holoprotein formation tends to be a near-terminal step in the biosynthetic pathway of most metalloproteins. The in vivo process occurs rapidly and shows high substrate specificity, both for the polypeptide and the cofactor—features which are indicative of biologically catalyzed reactions. The distinctive cell biology and metabolism of the chloroplast suggests that metalloprotein assembly pathways in this organelle might exhibit some unique characteristics compared to analogous ones in other organisms. Since genetic approaches have been particularly useful in illuminating metalloprotein assembly in bacteria and mitochondria, it is expected that Chlamydomonas will be ideal for the dissection of such pathways in the chloroplast. This chapter will detail the present state of knowledge of plastocyanin, *b*- and *c*-type cytochrome assembly as deduced from studies with Chlamydomonas.

3. Metal Metabolism

A third topic of relevance to metalloprotein biosynthesis in the chloroplast is metal uptake, sequestration and delivery to the site of metalloprotein assembly, and also the pathway and mechanism of metallocofactor biosynthesis. With the exception of the tetrapyrrole pathway, which is well-studied (Chapter 20, Timko), metal and metallocofactor metabolism in photosynthetic organisms has not been as intensively studied as it has in other organisms (e.g. Hausinger et al., 1990; Dean et al., 1993; de Silva et al., 1996). The mechanisms involved in the synthesis of iron-sulfur centers in chloroplasts and cyanobacteria is just beginning to be unraveled (reviewed in Merchant and Dreyfuss, 1998), but while Chlamydomonas has proven to be an excellent model for the delineation of molybdocofactor biosynthesis (Chapter 33, Fernandez et al.), it has not yet been exploited for studies of metallocofactor metabolism in the context of chloroplast protein assembly.

II. Copper-Responsive Synthesis of Plastocyanin and Cytochrome c_6

A. Plastocyanin

1. Properties and Function

Plastocyanin is a small (98 amino acids in *C. reinhardtii*) copper protein, Em ~ 370 mV, which functions in photosynthesis to transfer electrons from cytochrome *f* to PSI (reviewed by Boulter et al., 1977; Redinbo et al., 1994; Gross, 1996). It is referred to as a 'blue' copper protein owing to the spectroscopic properties of its copper-binding site, viz. a high extinction coefficient around 600 nm for the oxidized protein (ϵ_{597} ~ 4.5 mM^{-1} cm^{-1}). The protein was isolated originally from *Chlorella ellipsoidea*, and subsequently from a number of other photosynthetic organisms including Chlamydomonas (Gorman and Levine, 1966a). Several algal plastocyanins have been purified and the structures of three of these have been determined by application of X-ray crystallographic or NMR methods (Kunert et al., 1976; Yoshizaki et al., 1981, 1989; Moore et al., 1988; Collyer et al., 1990; Nakamura et al., 1992; Redinbo et al., 1993). The role of plastocyanin in photosynthesis was deduced by measurements of photosynthetic activity (whole cell CO_2 fixation, Hill reaction, light-induced absorbance changes) in wild-type vs. mutant *ac208* (re-named *pcy1-ac208*; Table 2) which carries a frame-shift mutation in the *Pcy1* gene encoding pre-apoplastocyanin (Gorman and Levine, 1965; 1966c; Levine and Gorman, 1966; Quinn et al., 1993); this role was subsequently supported by demonstration of plastocyanin function in in vitro electron transfer assays with sonicated thylakoid membrane preparations (Hauska et al., 1971).

2. Genetic Information and Biosynthesis in Algae

The protein is encoded in the nuclear genome as a precursor (Merchant and Bogorad, 1986; Li and Merchant, 1992; Nakamura et al. 1992; Quinn et al., 1993) and transported post-translationally (Howe and Merchant, 1993; Lawrence and Kindle, 1997) to the thylakoid lumen where it functions (Hauska et al., 1971; Haehnel et al., 1981). Although the vascular plant genes (*PetE*) are intron-less, the algal *Pcy1* genes are interrupted (Quinn et al., 1993; Nakamura et al., 1997; J. Quinn and S. Merchant, manuscript in preparation). An intron in the *Scenedesmus obliquus* and *Pediastrum boryanum* sequences occurs at exactly the same position as does the single intron in the Chlamydomonas *Pcy1* gene; *S. obliquus* has a second intron which is unique to that organism.

Plastocyanin is the most abundant copper protein in green algae because these organisms do not contain a Cu-Zn superoxide dismutase as do plants (Hewitt, 1983; Sakurai et al., 1993). The copper at the active site of plastocyanin is, naturally, essential for the redox function of the protein in photosynthesis and the protein is not expected to be functional in copper-deficient photosynthetic cells. Many green algae, including *C. reinhardtii*, remain photosynthetically competent nevertheless because they substitute a soluble, heme-containing cytochrome under these conditions (see below).

B. Regulation of Plastocyanin Accumulation

1. mRNA Accumulation in Algae and Cyanobacteria

Plastocyanin does not accumulate in copper-deficient cultures of many green algae. The mechanisms of regulation are quite varied. In *Scenedesmus obliquus*, the abundance of mRNA encoding plastocyanin is reduced in copper-deficient cultures (Li and Merchant, 1992). The reduction in template is assumed to result in reduced synthesis, and hence, reduced accumulation of the protein in copper-deficient cells. The mechanisms contributing to differential copper-responsive accumulation of *Pcy1* mRNA in *S. obliquus* is not yet known. In another alga, *Pediastrum boryanum*, *Pcy1* mRNA accumulates in both copper-supplemented and copper-deficient cells but is not translatable in copper-deficient cells (Nakamura et al., 1997). The mRNA isolated from copper-deficient cells was noted to be shorter than that from copper-supplemented cells. The difference in size was attributed to the absence of the 5′ region (including part of the protein coding sequence) in the RNA from copper-deficient cells, which accounts for lack of synthesis of plastocyanin under these conditions. The mechanism by which the two transcripts are generated is not yet known, but the phenomenon certainly represents a novel example of metal-responsive gene expression (see also Section II.E.2).

Table 2. Mutant strains discussed in this chapter

Mutant	Gene[1]	Inheritance	Gene Product	Function	Ref.[2]
ac208 or *pcy1-ac208*, *pcy1-2*, *-3*, *-4* and *-5*	*Pcy1* or *PetE* (plants)	Mendelian	plastocyanin	electron transfer component	1 2
pcy2-1		Mendelian		copper binding to plastocyanin or stabilization of holoplastocyanin	2
ac206 or *ccs1-ac206*, *ccs1-2*, *-3*, *-4*, *ccs1-5::Arg7* and *ccs1-abf3::Nit1*	*Ccs1*, formerly *ycf44*	Mendelian	Ccs1	holocytochrome *c* assembly	3 4
ccsA-B6, *-ct59*, *-ct34*	*ccsA*, formerly *ycf5*	Uniparental	CcsA	holocytochrome *c* assembly	5,4
ccs2-1, *-2*, *-3*, *-4* and *-5*		Mendelian		holocytochrome *c* assembly	4
ccs3-F18		Mendelian		holocytochrome *c* assembly	4
ccs4-F2D8		Mendelian		holocytochrome *c* assembly	4
petB-H86A, *-H86S*, *-H187G*, *-H197S*, *-H100D*, *-H100L* *-H202D*, *-H202Q*	*petB*	Uniparental	Cyt b_6	*b*-heme binding	6
ccb1-1		Mendelian		holocytochrome b_6 assembly	6
ccb2-1		Mendelian		holocytochrome b_6 assembly	
ccb3-1		Mendelian		holocytochrome b_6 assembly	6
ccb4-1, *-2*, -MΦ35		Mendelian		holocytochrome b_6 assembly	6

[1]Only genes which have been cloned are named.
[2]1 = Quinn et al., 1993; 2 = Li et al., 1996; 3 = Inoue et al., 1997; 4 = Xie et al., 1998; 5 = Xie and Merchant, 1996; 6 = Kuras et al., 1997.

2. Degradation in C. reinhardtii

In Chlamydomonas, the *Pcy1* mRNA is abundant in phototrophically- or heterotrophically-grown cells. It is likely that the amount of mRNA template is in excess of that required for producing plastocyanin at the level required for photosynthesis. For instance, when strain *pcy1-ac208* is rescued by transformation with the wild-type *Pcy1* gene, transformants that accumulate only low levels of the *Pcy1* mRNA (few percent relative to wild-type) can accumulate plastocyanin to levels similar to that found in the wild-type strains (e.g. Quinn et al., 1993). Thus, template abundance is not a major factor in determining the amount of plastocyanin in *C. reinhardtii*.

The abundance of the mRNA is independent of the copper concentration of the medium; the pre-protein is synthesized and processed to the mature form at the same rate in copper-deficient and copper-sufficient cells (Merchant and Bogorad, 1986a,b). However, in copper-deficient cells, the protein is degraded in the thylakoid lumen whereas in copper-supplemented cells, it assembles with copper and accumulates (Fig. 1). Differential accumulation of plastocyanin in *C. reinhardtii* in response to copper is a consequence of the different half-life of the protein in copper-deficient vs. copper-replete cells. The apoprotein is thermodynamically de-stabilized and more protease-susceptible relative to the holoprotein, and this is one feature which contributes to the rapid degradation of plastocyanin in copper-deficient cells (Li and Merchant, 1995). The protease responsible for plastocyanin degradation has not been identified; it is likely to be lumen-localized and its activity is proposed to be increased in copper-deficient cells. By removing a major copper-binding protein, the cell would be ensuring copper availability for the biosynthesis and function of other essential copper enzymes, such as cytochrome oxidase.

C. Cytochrome c_6

1. Properties and Function

Cytochrome c_6 is also a small (90 amino acids in

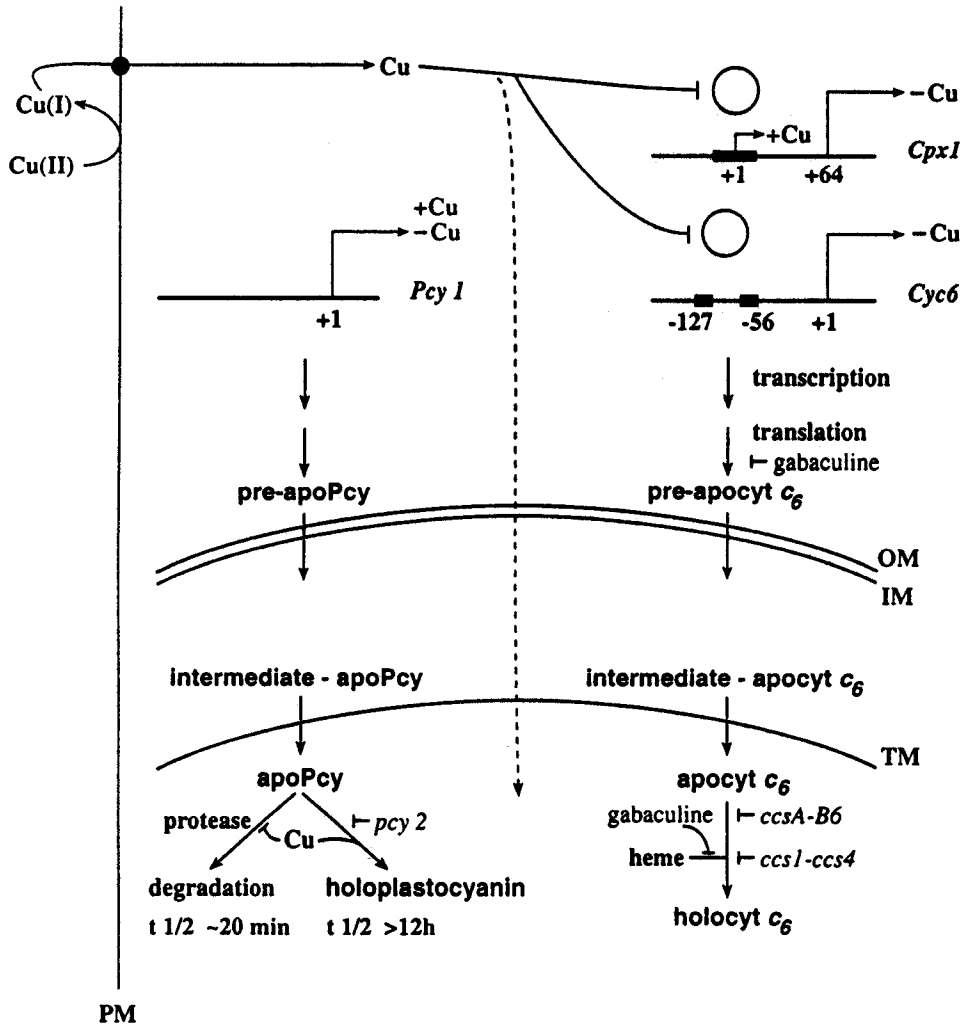

Fig. 1. Copper-responsive accumulation of plastocyanin and cytochrome c_6 in *Chlamydomonas reinhardtii.* Copper is taken up into the cell via an inducible copper transporter (Km ~ 200 nM), which may be associated with a cupric reductase (Hill et al., 1996). The copper is available for plastocyanin synthesis in the thylakoid lumen where it assembles with the apoprotein and also prevents degradation of the apoprotein (Merchant and Bogorad, 1986a; Li and Merchant, 1995). Assembly requires *PCY2* function, either for copper delivery to the lumen or for apoprotein metabolism (Li et al., 1996). The same copper pool serves as an effector of *Cyc6* and *Cpx1* transcription in the nucleus (Merchant et al., 1991; Hill and Merchant, 1992, 1995). In the presence of copper, transcription from these genes is de-activated. Translation of *Cyc6* mRNA is inhibited in gabaculine-treated cells, perhaps owing to depletion of a tetrapyrrole pathway intermediate. As for plastocyanin, cytochrome c_6 is synthesized in precursor form and imported post-translationally to the chloroplast and processed in two sequential steps to yield the mature apoprotein in the thylakoid lumen (Howe and Merchant, 1993). Assembly with heme requires the function of the Ccs factors and occurs in the thylakoid lumen, and this can be inhibited in gabaculine-treated cells owing to depletion of heme (Howe and Merchant, 1994a; Xie and Merchant, 1996; Xie et al., 1998). The large open circles indicate the copper-responsive signal transduction system, which includes minimally a copper sensor, a CuRE-binding protein and a transcription activator. These activities might be found in a single protein or they may be represented by separate proteins. OM = outer envelope membrane, IM = inner envelope membrane, TM = thylakoid membrane and PM = plasma membrane.

C. reinhardtii), lumen-localized redox-active (Em ~ 370 mV for *C. reinhardtii*) protein. In earlier work, the protein had been named according to the absorption maximum of the α band of the reduced protein. Thus, it has been called cytochrome c_{552} or c_{553} or c_{554} depending on the spectroscopic properties of the particular preparation (Gorman and Levine, 1966b; Wood, 1977). However, the proteins from various algae and cyanobacteria are functionally and structurally related; the name cytochrome c_6 now defines this class of soluble *c*-type cytochromes (Pettigrew and Moore, 1987).

Cytochrome c_6 was originally not distinguished from cytochrome *f* owing to the very similar spectroscopic properties of the two proteins (Gorman and Levine, 1966b; Kunert et al., 1976). The fact that the mutant strain *ac206* (now named *ccs1-ac206*) lacked both proteins supported the argument that the two cytochromes were one and the same, although it is now known that the strain exhibits a pleiotropic cytochrome assembly defect (Section III.A.1). Wood (1977) purified both proteins from Chlamydomonas and demonstrated that cytochrome c_6 and cytochrome *f* were distinctly different proteins. He discovered that cytochrome c_6 was synthesized only under certain conditions in *C. reinhardtii*—either in stationary phase cultures that were not well-aerated or in copper-deficient cultures when plastocyanin function was compromised. On the basis of the reciprocal pattern of accumulation of cytochrome c_6 vs. plastocyanin as a function of copper availability in the medium, a number of researchers suggested that cytochrome c_6 served as a back-up for plastocyanin (Wood, 1978; Bohner et al., 1980) and this was supported by several lines of evidence, including: 1) its location in the thylakoid lumen (Wildner and Hauska, 1974); 2) its ability to substitute for plastocyanin in vitro in plastocyanin-depleted thylakoid membrane preparations (Hauska et al., 1971); 3) its similar physical properties—Em and pI—compared to plastocyanin (Ho and Krogmann, 1984); and 4) its occurrence in all photosynthetic algae and cyanobacteria that lack plastocyanin (Sandmann et al., 1983).

The protein has been purified from several genera of algae and is biochemically well-characterized (Gorman and Levine, 1966b; Yakushiji, 1971; Bohme and Pelzer, 1982; Campos et al., 1993). The structure of Chlamydomonas Cyt c_6 reveals that it is a typical member of the soluble *c*-type cytochrome family; the protein is predominantly α-helical with a series of α-helices and tight turns enveloping the heme (Kerfeld et al., 1995).

2. Genetic Information and Biosynthesis

In *C. reinhardtii*, cytochrome c_6 is encoded by a single nuclear gene (Merchant and Bogorad, 1987; Hill et al., 1991). As for plastocyanin, the protein is synthesized in precursor form and transported post-translationally to the chloroplast via a lumen-targeting pathway (Howe and Merchant, 1993; see chapter 13, Perret et al.). In organisms that contain genetic information for both plastocyanin and cytochrome c_6, the latter protein does not accumulate except under copper-deficient conditions, or occasionally, under microaerobic or anaerobic culture conditions (Wood, 1978; Bohner et al., 1980).

D. Regulation of Cytochrome c_6 Synthesis

1. Copper as the Signal

Cytochrome c_6 functions only as a replacement for plastocyanin—it is not synthesized unless plastocyanin function is compromised by copper-deficiency (Hill and Merchant, 1992). To test whether cytochrome c_6 synthesis occurs in response to plastocyanin deficiency (perceived, perhaps, via the redox state of the electron transfer chain) or directly in response to copper ion deprivation, Merchant and Bogorad (1987b) examined Cyt c_6 accumulation in a *C. reinhardtii* mutant that could not synthesize the plastocyanin polypeptide (strain *pcy1-ac208*). Accumulation of cytochrome c_6 remained copper-responsive in the mutant despite the absence of plastocyanin—*pcy1* mutants are, therefore, acetate-requiring in copper-supplemented medium but not in copper-deficient medium.

2. Transcriptional Regulation

The copper-responsive regulation of the algal *Cyc6* gene has been studied only for *C. reinhardtii* where it is under transcriptional regulation by copper (Hill et al., 1991; Merchant et al., 1991; Fig. 1). *Cyc6* is not expressed in copper-replete cells (<1 mRNA per cell); in copper-deficient cells, the abundance of the *Cyc6* mRNA is proportional to the plastocyanin deficiency resulting from lack of copper (Hill and Merchant, 1992). The *Cyc6* mRNA accumulates to an abundance of several hundred molecules per cell when the gene is maximally induced. Copper-responsive elements (CuREs) associated with the *Cyc6* gene are localized to a 90 nucleotide sequence in close proximity to the start site of transcription (Quinn and Merchant, 1995). These sites appear to be targets for transcriptional activators which must function downstream of the putative copper sensor in the signal transduction pathway. Site-directed mutagenesis indicates that GTAC forms the core of a CuRE, but the element is not characterized beyond that (J. Quinn, unpublished). Analysis of the CuREs in the context of various reporter gene constructs indicates that they account for all the copper-

responsive properties of the *Cyc6* gene. The rapid and metal-selective response of the *Cyc6* gene suggests that it may be useful for the design of chemically-regulated promoters for algal cells.

The synthesis of plastocyanin and cytochrome c_6 has been examined also in *C. smithii* which has genetic information for both proteins, and the same pattern of reciprocal expression is observed (J. Quinn and S. Merchant, unpublished). On the other hand, *C. mundana*, which was isolated from sewage lagoons, lacks the capacity to synthesize plastocyanin (Wood, 1978). Accordingly, cytochrome c_6 accumulates in *C. mundana* independently of copper availability. This is true also for other algae and cyanobacteria which lack the genetic information for plastocyanin synthesis.

3. Response to Iron and Heme

The *Cyc6* gene does not respond at the transcriptional level to cellular iron status (K. Hill, unpublished). On the other hand, Cyt c_6 accumulation does depend on heme availability, since heme is a substrate for holoprotein formation (see below). In addition, heme (or a tetrapyrrole pathway intermediate) might serve also to regulate translation of the *Cyc6* mRNA (Howe and Merchant, 1994a). This suggestion is based on the reduced synthesis of Cyt c_6 in cells depleted of heme by treatment with gabaculine (an inhibitor of δ-ALA synthesis), but the identity of the tetrapyrrole regulator and the mechanisms involved have not been studied. The absence of *hem* mutants of Chlamydomonas makes it difficult to distinguish between heme vs. tetrapyrrole pathway intermediates as regulators. The effect may not be specific to the translation of heme-containing proteins, since some reduction of plastocyanin and Rubisco small subunit synthesis was also noted. The synthesis of cytochrome *f* is also reduced in gabaculine-treated cells (e.g. Howe et al., 1995; Kuras et al., 1997), but the mechanisms involved in the chloroplast are expected to be different from those operating outside the organelle.

E. Other Responses to Copper-Deficiency

1. Copper Transport

Chlamydomonas cells exhibit an extraordinary capacity for copper uptake. For instance, changes in gene expression are evident in response to provision of copper at concentrations as low as a few nanomolar (Merchant et al., 1991). Also, copper-deficient cells can deplete all measurable copper from their growth medium even when it is provided in complex with a chelator (Hill et al., 1996). Measurement of copper uptake by Chlamydomonas indicates the presence of a high affinity (Km ~2×10^2 nM) system in both −Cu and +Cu cells; however, −Cu cells have a ten- to twenty-fold increased capacity for copper uptake relative to +Cu cells, perhaps owing to increased expression of genes encoding transporter components. The copper transporter has not yet been identified, but it is possible that it may be associated with a cupric reductase, as has been proposed for other organisms. Indeed, copper-deficient cells exhibit up to two-fold increased cupric reductase activity relative to copper-sufficient cells (Hill et al., 1996).

2. Coproporphyrinogen Oxidase

Comparison of proteins synthesized by +Cu vs. −Cu cells revealed a 39 kD soluble protein, the synthesis of which was increased in copper-deficient relative to copper-sufficient cells (Merchant and Bogorad, 1986a). This protein was subsequently identified as the enzyme coproporphyrinogen (coprogen) oxidase and its activity was shown to be several-fold greater in copper-deficient vs. copper-sufficient cells (Hill and Merchant, 1995). Northern analysis indicated a corresponding increase in the abundance of *Cpx1* transcripts (encoding coprogen oxidase). Coprogen oxidase functions in tetrapyrrole biosynthesis and its increased activity in −Cu cells can be rationalized on the basis of an increased demand for heme in cells that are synthesizing cytochrome c_6. Coprogen oxidase is also induced in other organisms when there is an increased demand for heme. The induction cannot be mimicked by treatment with inhibitors of the tetrapyrrole pathway (e.g. gabaculine), suggesting that the increase in coprogen oxidase occurs in response to copper as the signal rather than to feedback from the tetrapyrrole pathway. The expression of genes encoding early enzymes of the tetrapyrrole pathway (such as δ-aminolevulenic acid dehydratase and glutamate semi-aldehyde aminotransferase) are not induced in −Cu cells—the response of the *Cpx1* gene may well be unique.

Coprogen oxidase is encoded by a single nuclear gene, *Cpx1*, in Chlamydomonas. The gene produces

two transcripts, a longer form synthesized in both −Cu and +Cu cells, and a shorter form which is synthesized only in −Cu cells. The size difference is attributed to a difference in the length of the 5′ untranslated region. Therefore, the reading frame remains intact in both forms, unlike the situation for *Pcy1* of *P. boryanum* (Section II.B.1). The abundance of the short form of *Cpx1* is up to 35-fold higher under copper-deficient conditions relative to the long form, and this is attributed to transcriptional activation via CuREs (J. Quinn, unpublished). The significance of the two forms of *Cpx1* mRNA is not yet known, but it makes one wonder whether the generation of alternate mRNA species with distinct 5′ ends may be a common regulatory mechanism in the copper-responsive signal transduction pathway of green algae.

Analysis of the time-course, metal selectivity and metal sensitivity of the various responses to copper-deficiency suggest that a common regulatory pathway controls each of the above copper-responsive processes in *C. reinhardtii* including activation of *Cyc6* and *Cpx1* gene transcription, increased cupric reductase and copper transport, and increased plastocyanin degradation.

F. Adaptation to Copper-Deficiency

The function of the regulatory pathway is viewed as an adaptation to copper-deficiency which allows the organism to survive in face of a changing supply of an essential, yet potentially toxic, micronutrient in its growth environment. While the stimulation of copper uptake is an immediate and predictable response, the substitution of a heme protein for a copper protein is a more long term and novel adaptation. Besides plastocyanin, Chlamydomonas contains at least two other copper proteins, cytochrome oxidase and urate oxidase. A substitute for cytochrome oxidase is not evident because severely copper-deficient cells are unable to grow heterotrophically in the dark—conditions where cytochrome oxidase function is essential (J. Quinn and S. Merchant, unpublished). The replacement of plastocyanin with cytochrome c_6 can be viewed therefore as a mechanism for ensuring re-distribution of copper from the photosynthetic apparatus to the essential respiratory complex.

III. Genetic Analysis of Chloroplast Metalloprotein Assembly

A. The c-type Cytochromes

In the *c*-type cytochromes, the heme group is covalently attached to the polypeptide by thioether linkage between cysteinyl residues of a CxxCH sequence on the apoprotein and the vinyl side chains at C3 and C8 of the porphyrin ring. Consequently, the possibility that cytochrome assembly might be catalyzed has long been appreciated and the pathway of *c*-type cytochrome maturation has been studied in various organisms, including Chlamydomonas (reviewed by Gonzales and Neupert, 1990; Howe and Merchant, 1994b; Kranz and Beckman, 1995; Thony-Meyer, 1997).

1. The ccs Mutants

Chlamydomonas contains two *c*-type cytochromes: Cyt *f* of the b_6f complex (Chapter 14, Olive and Wollman) and soluble cytochrome c_6 (discussed above). The post-translational pathway for maturation of cytochrome c_6 was determined by in vivo radiolabeling experiments which established the following sequence in vivo—pre-apocyt c_6 → intermediate-apocyt c_6 → mature apocyt c_6 → holocyt c_6—and placed the heme attachment reaction in the thylakoid lumen (Howe and Merchant, 1993; 1994a). Thus, as for other *c*-type cytochromes, assembly occurs on the *p*-side of the energy transducing membrane, and as for most metalloproteins, holoprotein formation is a near terminal step in the biosynthetic pathway. The ability to monitor the biosynthetic pathway in vivo by application of radiolabeling techniques facilitated the definition of heme attachment mutants from a collection of acetate-requiring strains (Howe and Merchant, 1992; Xie et al., 1998). The pathway of cytochrome c_6 maturation in these strains, called *ccs* for *c*-type cytochrome synthesis, is normal with respect to the rate of synthesis and processing of the pre-apoprotein, but the apoprotein is not converted to the holoform to any appreciable extent.

The *ccs* strains are pleiotropically deficient in both chloroplast *c*-type cytochromes. Since cytochrome *f* is encoded in the plastid genome (*petA*) and cytochrome c_6 in the nuclear genome (*Cyc6*), the post-translational steps involving translocation across

the thylakoid membrane and heme attachment are the only steps that could be common in their biosynthesis. While translocation across the thylakoid membrane is a process that is shared with other lumen proteins (Chapter 13, Perret et al.), heme attachment is required only for the *c*-type cytochromes. The recognition of the pleiotropic Cyt c_6-minus/Cyt *f*-minus phenotype therefore simplified the screen for additional candidate *ccs* strains (Table 2). Sixteen such strains have been placed into five complementation groups—*ccsA* corresponding to the plastid *ccsA* gene, and *CCS1* through *CCS4* corresponding to four nuclear genes (Xie et al., 1998). Since some of the loci are represented by only one mutant strain, it is possible that the pathway has not yet been saturated by mutagenesis and additional loci might yet be discovered.

2. The Ccs Factors

a. CcsA

The plastid *ccsA* gene, which rescues all three uniparental mutants ct34, ct59 and B6, corresponds to an open reading frame (formerly called *ycf5*) found in all plastid genomes characterized to date (Reith, 1995). CcsA had been proposed to be a cytochrome biogenesis protein on the basis of limited sequence similarity with Ccl1, a *Rhodobacter capsulatus* protein required for the synthesis of all *c*-type cytochromes in the bacterial periplasm (Beckman et al., 1992). Indeed, inactivation of the *C. reinhardtii* open reading frame led to a non-photosynthetic strain whose phenotype was attributed to a pleiotropic cytochrome deficiency (Xie and Merchant, 1996). The C-terminal region of the protein is the most highly conserved and includes a functionally important sequence motif, WGxxWxWDxxE, found originally in Ccl1 of *Rb. capsulatus*. CcsA is highly hydrophobic and probably spans the membrane multiple times. The WGxxWxWDxxE motif is predicted to lie on the lumen-side of the membrane which is consistent with a role for this sequence in some part of the heme attachment reaction..

b. Ccs1

The *Ccs1* gene was identified on the basis that it was disrupted in a cytochrome b_6f-deficient strain—abf3 (Inoue et al., 1997). The authors recognized a pleiotropic *c*-type cytochrome deficiency in this strain and determined on the basis of further biochemical characterization that abf3 was blocked at the step of heme attachment. Although abf3 was not analyzed genetically, the fact that the cloned gene rescued all *ccs1* mutants (B. Dreyfuss and S. Merchant, unpublished) but not other *ccs* strains indicated that the cloned gene corresponded to the *CCS1* locus. Candidate homologues of Ccs1, encoded by *ycf44* (for hypothetical chloroplast frame) occur in *Synechocystis* sp. 6803 and the plastid genomes of several algae, which suggests that, like CcsA, Ccs1 functions in all plastids. Analysis of the sequence indicates that Ccs1 is a membrane protein—but its specific biochemical function has not yet been deduced. The availability of *ccs1* mutants which can be rescued by the cloned gene opens the door to functional analysis of this novel protein. Ccs1 is absent in *ccsA* mutants, suggesting that CcsA may form a complex with Ccs1 in vivo (B. Dreyfuss, unpublished)

The abundance of *ccsA* and *Ccs1* mRNAs is very low compared to the abundance of the *petA* or *Cyc6* mRNAs, which suggests that the gene products are low abundance proteins: this is consistent with a catalytic role of Ccs factors in the assembly of the *petA* and *Cyc6* gene products. A number of functions may be envisioned for the Ccs factors. One possibility is that the membrane-associated components may serve as subunits of a heme transporter which would deliver heme from its site of synthesis to the lumen side of the thylakoid membrane. Some of the other subunits of the putative Ccs complex might serve as substrate (apoprotein/heme) chaperones or as cytochrome *c*/heme lyases. By analogy to the thioredoxin-like proteins which are required for sequential oxidation and reduction of apocytochromes in the bacterial periplasm, one might propose that similar reactions might be catalyzed by the chloroplast Ccs factors. Alternatively, an oxidoreductase might be required to maintain the heme iron in the reduced state.

3. A Novel Pathway in the Chloroplast

Previously, two pathways for cytochrome biogenesis were known. One pathway, discovered originally by genetic analysis of cytochrome *c* and c_1 biogenesis in *Saccharomyces cerevisiae* and *Neurospora crassa*, operates in fungal, mammalian, Drosophila and *C. elegans* mitochondria and consists of the Cyt *c*-

and c_1-heme lyases (reviewed by Gonzales and Neupert, 1990). Another, discovered originally by analysis of bacterial cytochrome *c*-deficient mutants, operates in many proteobacteria—including rhizobia, *Rhodobacter* spp., *E. coli*, *Pseudomonas* spp.—and plant mitochondria, and consists of a multi-component cytochrome assembly complex in the energy transducing membrane (reviewed by Kranz and Beckman, 1995; Thony-Meyer, 1997). Analysis of the distribution of CcsA and Ccs1 homologues in the genome databases indicates that they define a third, novel pathway for *c*-type cytochrome biogenesis which operates not only in plastids and cyanobacteria but also in a subset of bacterial species including *Bacillus subtilis*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Neisseria* spp. and *Helicobacter pylori* (discussed in Xie et al., 1998). Candidate homologues of CcsA and Ccs1 are encoded in an operon-like arrangement in some of these bacteria, suggesting that the other open reading frames in the operon may correspond to products encoded at the remaining *CCS* loci. The putative Ccs factors appear to be essential in bacteria and they have not been subject to functional analyses in these organisms. The possibility of combining genetic and biochemical methodologies makes Chlamydomonas an ideal experimental system for the study of the Ccs pathway of cytochrome biogenesis.

B. The b-Heme(s) of the b_6f Complex

The definition of other assembly defective mutants has not been as straightforward because it can be difficult to distinguish the phenotype of a cofactor-insertion defect from a defect associated with a cofactor-independent step (such as subunit-subunit association). In general, unassembled photosynthetic complexes are degraded, especially in Chlamydomonas chloroplasts, regardless of the nature of the defect. Thus, a *c*-heme insertion mutant does not accumulate any of the subunits of the cytochrome b_6f complex, and this phenotype is not significantly different from a *b*-heme insertion mutant (Kuras et al., 1997; discussed below). Nevertheless, specific defects can be distinguished if the assembly pathways can be monitored in vivo in wild-type cells so that intermediates are visualized. For cytochrome b_6 maturation, Wollman and co-workers exploited pulse-radiolabeling techniques, in combination with site-directed mutagenesis of cofactor binding ligands, to identify biosynthetic intermediates and to deduce the following pathway for sequential heme insertion, which occurs post-translationally and independently of inter-subunit associations: apocyt b_6 + heme → b_l-heme-dependent intermediate + heme → holocyt b_6 (Kuras et al., 1997; see also chapter 24, Wollman).

The elucidation of this pathway allowed the identification of *ccb* strains among a collection of cytochrome b_6f-deficient mutants (Kuras et al., 1997). The phenotype of the *ccb* strains mimicked exactly the phenotype of strains carrying site-directed mutations at the b_h-heme ligands, and the block could therefore be assigned to the step where the b_l-heme-dependent intermediate assembles with heme to yield holocytochrome b_6. Genetic analysis classified the mutants to four nuclear loci (Table 2), but the genes have not yet been cloned and the functions of the gene products can only be speculated upon. These include cofactor processing (delivery, chaperoning), apoprotein processing or catalysis of heme insertion. The catalyzed assembly of *b*-type cytochromes has not been studied in any system; thus, the further characterization of the Chlamydomonas pathway will undoubtedly lead to novel insights into cofactor-protein assembly. Since the association of the *b*-hemes with the protein was thought to be non-covalent, the need for catalysis had not been appreciated until the identification of the *ccb* mutants.

C. Plastocyanin

A similar approach, namely screening non-photosynthetic strains for specific assembly defects, led to the identification of two loci required for plastocyanin biosynthesis—*PCY1*, corresponding to the *Pcy1* gene encoding pre-apoplastocyanin and *PCY2* (Li et al., 1996). In the *pcy2-1* mutant, plastocyanin is synthesized and processed as in wild-type cells, but the apoprotein accumulates at the expense of the holoprotein (Fig. 1). The accumulation of apoplastocyanin is consistent with in organello studies using plant chloroplasts, which established that copper assembles with apoplastocyanin in the thylakoid lumen after processing by the thylakoid peptidase (Li et al., 1990). Studies of copper-protein assembly in other organisms indicates that the *pcy2* phenotype might be attributable to a defect in copper delivery to the lumen or copper delivery to the active site of plastocyanin (Zumft et al., 1990; Chen et al., 1993; Glerum et al., 1996). Nevertheless, the possibility that the phenotype results from post-

assembly de-stabilization of holoplastocyanin cannot be ruled out at present.

D. Approaches for the Dissection of Other Assembly Pathways

Several strategies are being employed for the study of metalloprotein assembly in photosynthetic organisms (reviewed by Merchant and Dreyfuss, 1998). One approach exploits genome sequence information in the databases to identify candidate genes (and hence gene products) involved in the pathway of interest on the basis of relationships to well-characterized proteins of known function. In this situation, the gene products can be tested for function in the pathway of interest if a biochemical assay for metalloprotein assembly is available, and reverse genetic approaches can be applied to test for relevance of that function to the in vivo biosynthetic pathway. Assembly factors that function in the chloroplast can also be cloned on the basis of their ability to complement mutations in homologous genes in other organisms. This approach has been applied to the study of molybdenum cofactor biosynthesis (e.g. Stallmeyer et al., 1995) and has also been particularly useful for the cloning of various metal transporters of plants (e.g. Eide et al., 1996). The third approach, involving screening for mutants with assembly defects followed by the identification of the wild-type loci and assembly factors, has enormous potential because it can lead to the discovery of novel factors (such as the Ccs proteins) or reveal the existence of previously-undefined pathways (such as for holocyt b_6 synthesis). Furthermore, the availability of mutant strains can simplify functional studies by site-directed mutagenesis.

Chlamydomonas is an excellent model system for such an approach. The key to identifying mutants of interest is the recognition of a cofactor assembly defect: when apoproteins accumulate as in strain *pcy2-1* (Li et al., 1996) or the *nia* mutants of tobacco (Gabard et al., 1987; Chapter 33, Fernandez et al.), the assignment is simple, but this is generally not the case for proteins of the photosynthetic apparatus. Many of the cofactors in the photosynthetic complexes are integral to their structure, and cofactor-binding mutants often do not accumulate cofactor-depleted versions of the complex of interest (e.g. Whitmarsh et al., 1991, and *ccb* or *ccs* mutants discussed above). In this case, assembly defects are deduced when the mutation: 1) is not in the genes encoding the various polypeptide constituents of the complex; and 2) does not affect the expression of individual subunits of a complex (e.g. Voelker and Barkan, 1995). In vitro systems for the assay of assembly, or the ability to monitor assembly in vivo, are pre-requisites for the more precise localization of the defect to a particular assembly step involving cofactor insertion. The Chlamydomonas system is particularly suited for the development of in vivo assays owing to the facility with which radiolabeling experiments can be executed.

IV. Conclusions

Chlamydomonas has served as a useful model for studying metal-responsive gene expression and metalloprotein assembly in the context of assembly of the photosynthetic apparatus. The copper-dependent regulation of plastocyanin and cytochrome c_6 remains one of the best characterized adaptations to a trace element deficiency. However, the regulatory molecules involved in sensing cellular copper status and responding to it have not been identified. The use of genetic approaches for dissecting this regulatory pathway holds great promise and needs to be exploited. Iron metabolism is another topic of great interest. *C. reinhardtii* cells display dramatic responses to iron starvation, including de-greening and changes in gene expression; however, little is known about the molecular basis of adaptation. The recent discovery of an iron-regulated transferrin-like protein in *Dunaliella salina* suggests that studies of iron metabolism in green algae is bound to lead to new findings (Fisher et al., 1997). Likewise, the rich repertoire of non-photosynthetic mutants and the facility with which they can be generated and analyzed ensures that this experimental system will continue to contribute to our understanding of metalloprotein assembly processes.

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