Chapter 14

Biosynthesis of Chlorophylls *a* and *b*: The Last Steps

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Summary

The last steps of chlorophylls (Chls) *a* and *b* biosynthesis comprise the formation of chlorophyllide (Chlide) *a* from protochlorophyllide (PChlide) *a*, the oxygenation of Chlide *a* to Chlide *b*, the esterification of Chlides to the corresponding Chls, and the reduction of *b*-type pigments to *a*-type pigments. Two separate pathways exist for the biosynthesis of Chls, a light-dependent and a light-independent (dark) pathway. The decisive step is the hydrogenation of PChlide *a* to Chlide *a*, catalyzed either by a light-dependent PChlide oxidoreductase (POR) or by a light-independent PChlide oxidoreductase (DPOR, D for dark). The conversion of *a*- to *b*-type pigments and conversely of *b*- to *a*-type pigments, the 'chlorophyll cycle,' presumably allows the plants to adjust the Chl *a/b* ratio to the environment: the reduction of Chl *b* to Chl *a* precedes the degradation of the *b*-type pigment. This chapter describes the last steps of Chl biosynthesis with emphasis on the enzymes that catalyze the individual steps: included are discussions of the corresponding genes and recombinant enzymes.

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I. Introduction

Angiosperms synthesize chlorophylls (Chls) *a* and *b* only in the light. The enzymatic steps leading to the formation of protochlorophyllide (PChlide) *a* take place in darkness in all higher plants including angiosperms: the light-dependent step and all subsequent steps are the 'last steps' of Chl biosynthesis. The light-dependent step is the photoconversion of PChlide *a* to chlorophyllide (Chlide) *a* catalyzed by NADPH:protochlorophyllide oxidoreductase (POR). Phototrophic bacteria, cyanobacteria, algae, mosses, ferns and most gymnosperms synthesize Chl also in the absence of light. These organisms contain an alternative, light-independent enzyme (DPOR) for the conversion of PChlide *a* to Chlide *a*. The light-independent pathway is the sole pathway in anoxygenic photosynthetic bacteria, leading to the formation of bacteriochlorophylls (BChls), and the light-dependent pathway is the only pathway in angiosperms, leading to Chls *a* and *b*. Both pathways coexist in cyanobacteria, algae, mosses, ferns, and gymnosperms; these organisms have the advantage that after deletion of one pathway (e.g,. by mutation) the other can still maintain Chl synthesis. It is not yet known how much each pathway contributes to Chl formation under normal physiological conditions.

This chapter deals with the formation of Chls *a* and *b* from PChlide *a* with emphasis on the enzymes for the single steps. Fig. 1 gives an overview over these steps, cross-correlations to other biosynthetic routes, not discussed in this chapter, are indicated in brackets.

II. Protochlorophyllide Reduction

A. The Light-independent Reaction

Only some recent results are discussed here; for more details see the review by Armstrong (1998). The first indication that the DPOR consists of three subunits came from genetic studies which demonstrated that three genes, *bchL, bchN* and *bchB* in photosynthetic bacteria and *chlL, chlN* and *chlB* in cyanobacteria and algae, are involved in PChlide reduction. Surprisingly, the deduced amino acid sequences were found to possess a high degree of similarity to the sequences of NifH, NifD and NifK, the subunits of nitrogenase, leading to the speculation that DPOR, like nitrogenase, receives reduction equivalents from ferredoxin in an ATP-dependent reaction. Convincing proof of the composition of DPOR came from the reconstitution of the active enzyme from the isolated BchL, BchN and BchB subunits, purified after overexpression of their genes in *Rhodobacter* (*Rba.*) *capsulatus* (Fujita and Bauer, 2000). The in vitro reduction of PChlide *a* to Chlide *a* required all three subunits, ATP and an ATP-regenerating system but, instead of ferredoxin, dithionite was used as electron donor. Fujita and Bauer (2000) discuss the relationship of the BchB/BchN complex of DPOR to the NifE/NifN complex: the latter is presumed to provide a framework on which the MoFe-containing cofactor of nitrogenase is constructed prior to its transfer to the NifD/NifK nitrogen reductase protein. The number and position of cysteine residues indicate 4Fe-4S clusters in the DPOR-BchB/BchN complex as in the NifE/NifN complex while NifD/NifK contains 8Fe:7S P-clusters.

B. The Light-dependent Reaction

The observation that angiosperms fail to synthesize Chl when kept in complete darkness but become green when exposed to light prompted, over the years, many investigations of the light-dependent step of Chl biosynthesis catalyzed by POR. Recently, a resurgence of interest in this reaction occurred because it is a rare example where substrate binding can be separated from the commencement of the reaction: substrate and cosubstrate bind to the enzyme in darkness and subsequently the reaction is started by a suitable flash of light permitting even the fast initial phase of the reaction, in the ps or sub-ps range, to be investigated, which is not possible in most enzyme reactions.

While chloroplasts are formed from proplastids in the light, etioplasts develop in darkness. Etioplasts

Abbreviations: A. – Arabidopsis; BChl – bacteriochlorophyll; *C. – Chlamydomonas;* CAO – chlorophyllide *a* oxygenase; *Cfl. – Chloroflexus;* Chl – chlorophyll, the esterifying alcohol is indicated by subscripts, e.g. Chl_{GG} for Chl esterified with geranyl-geraniol; Chlide – chlorophyllide; DPOR – light-independent (= dark) protochlorophyllide reductase; *E. – Escherichia;* GG – geranyl-geraniol; GGPP – geranyl-geranyl-pyrophosphate; LHCB – light-harvesting chlorophyll protein of photosystem II, often also termed LHCII; PChlide – protochlorophyllide; Phe – pheophytin; Pheide – pheophorbide; PhyPP – phytyl-pyrophosphate; PLB – prolamellar body; POR – NADPH:protochlorophyllide oxidoreductase, light-dependent; PPheide – protopheophorbide; PS I – photosystem I; PT – prothylakoid; *Rba. – Rhodobacter*

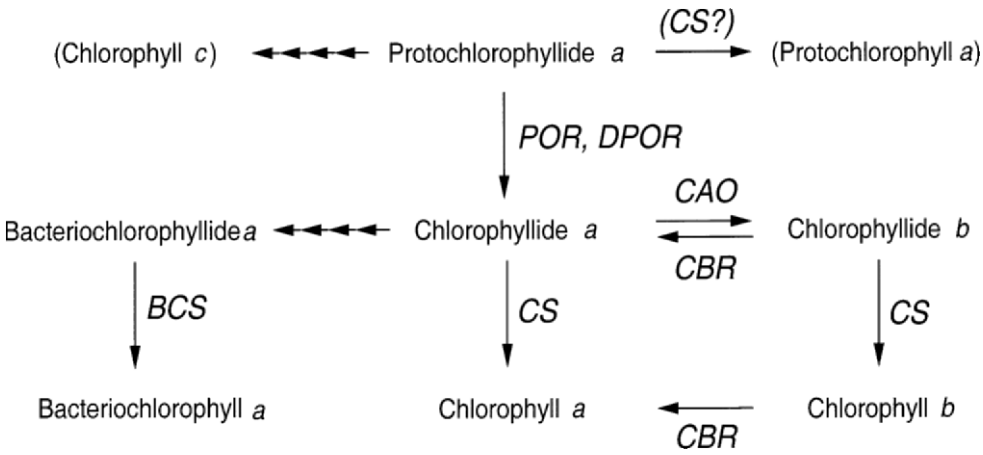

Fig. 1. Overview of the last steps of Chl biosynthesis. Two alternative pathways lead from protochlorophyllide *a* to chlorophyllide *a*: a light-dependent (catalyzed by LPOR) and a light-independent pathway (catalyzed by DPOR). The enzymes discussed in this chapter and indicated in the figure are: *POR*, NADPH:protochlorophyllide oxidoreductase; *CAO*, chlorophyllide *a* oxygenase, *CBR*, chlorophyll(ide) *b* reductase and 7^1-hydroxy-chlorophyll(ide) *a* reductase; *CS*, chlorophyll synthase; *BCS*, bacteriochlorophyll synthase. In brackets are the pathways to chlorophyll *c* and protochlorophyll *a*, which are not treated in this chapter.

contain typical inner membranes known as prolamellar bodies (PLBs) and prothylakoids (PTs). There is a close connection between the PLB structure and the accumulation of POR. The regular structure of PLBs depends on the presence of the pigment-POR complex and 98% of the protein of PLBs is POR (Ryberg and Sundqvist, 1991): in the *cop 1* photomorphogenic mutant which has nearly no POR and lacks PLBs, the formation of PLBs can be restored by overexpressing POR (Sperling et al., 1998).

When etiolated plants are transferred from darkness to light, etioplasts develop into chloroplasts. In the first step, PTs protrude out of the PLBs which can be observed only 2–5 min after a single flash of light (Domanskii et al., 2003). Interestingly, POR is translocated by illumination from PLBs into the growing PTs (Ryberg and Dehesh, 1986). The transformation of PLBs, a lipid tubular system with a bicontinuous cubic phase organization, into the lamellar lipid phase of PTs is generally believed to be triggered by the phototransformation of PChlide into Chlide indicating that the NADPH:PChlide-POR complex acts as photoreceptor for this process; however, the participation of other photoreceptors cannot be excluded at present.

C. POR Genes and Proteins

The first POR gene to be isolated, now called *PORA*, was obtained from barley (reviewed by Schulz and Senger, 1993). Its down-regulation by light, leading to nearly complete disappearance of the POR protein at the onset of maximum Chl synthesis, was considered a paradox until a second constitutively-expressed gene, *PORB*, was detected in barley (Holtorf et al., 1995) and in *Arabidopsis* (*A.*) *thaliana* (Armstrong et al., 1995). Even a third gene, *PORC*, which is up-regulated by light is present in *A. thaliana* (Oosawa et al., 2000). The different expression patterns indicate specific roles for the different POR enzymes during development in the dark and in the light, an assumption, which was verified by investigation of *Arabidopsis* mutants deficient in either one or other of the *POR* genes (Masuda et al., 2003). Attempts to demonstrate completely different functions for different POR proteins failed: PORA and PORB are redundant for PLB formation and for photoprotection of plants (Sperling et al., 1998), different mechanisms for the import of the precursor proteins pPORA and pPORB into the plastids (Reinbothe et al., 2000) were questioned (Aronsson et al., 2000; 2003a; 2003b), and the proposal of a light-harvesting role, specific for PORA (Reinbothe et al., 1999; 2003b) was rejected by others (Armstrong et al., 2000; Franck et al., 2000). The notion of such different functions can also be questioned because only one *POR* gene was detectable in pea (Sundqvist and Dahlin, 1997), cucumber (Fusada et al., 2000), *Chlamydomonas* (*C.*) *reinhardtii* (Li and Timko, 1996) and cyanobacteria (Rowe and Griffiths, 1995; Suzuki and Bauer, 1995).

Early in vitro investigations on the activity of POR were performed with etioplasts or etioplast membrane fractions (reviewed by Griffiths, 1991). POR occurs as a photoactive ternary complex together with NADPH and PChlide *a* in these preparations, and the availability of only the preformed enzyme-substrate complex sets certain limitations for the investigation of the enzyme reaction. The isolation of pigment-free POR from oat etioplasts was more-recently described (Klement et al., 1999). Alternatively, pigment-free POR has been obtained by heterologous expression of *POR* genes in *Escherichia* (*E.*) *coli*; for example, the *POR* gene from pea (Martin et al., 1997), *PORB* from barley (Lebedev and Timko, 1999) and *POR* from *Synechoystis* (Heyes et al., 2000, 2003a,b). Using the latter enzyme, Heyes et al. (2002, 2003a,b) identified intermediates of the reaction by time-resolved and low-temperature spectroscopy.

Several modified PChlide derivatives were tested in a substrate specificity study of POR (Griffiths, 1991; Klement et al., 1999; see also supplement). Various modifications of the side chains on rings A and B, even the introduction of bulky substituents, were tolerated without essential loss of activity; however, modification of ring E is not tolerated (for details see supplement). Of particular interest is the replacement of the C-7 methyl group of PChlide *a* by a formyl group to form PChlide *b*. The observation that PChlide *b* and its Zn analogue can be photoreduced (Schoch et al., 1995) prompted the proposal that PLBs contained mainly PChlide *b* (Reinbothe et al., 1999, 2003a); the authors postulated that PChlide *b*, bound to PORA, was not immediately photoreduced at the onset of illumination but served rather as a light-harvesting pigment. Scheumann et al. (1999a), however, found that exogenous PChlide *b* was readily photoreduced to Chlide *b* in the presence of etioplast membranes and extraction of etioplasts with 80% acetone yielded only PChlide *a* and no PChlide *b* was detected. Later, Reinbothe et al. (2003a) reported detection of PChlide *b* as the major tetrapyrrole of etioplasts after extraction with 99% acetone containing 0.1% diethyl pyrocarbonate and suggested that PChlide *b* was rapidly metabolized to PChlide *a* in the presence of 80% acetone (see also Section IIIB).

D Phototransformation of Protochlorophyllide in Vivo

The in vivo transformation of PChlide to Chlide is accompanied by characteristic spectral shifts of PChlide *a* and Chlide *a* (Fig. 2; reviewed by: Griffiths, 1991; Ryberg and Sundqvist, 1991). The situation is complex since there exist 4 or more spectral forms of PChlide *a* in dark-grown plants of which at least 2 are phototransformable (Böddi et al., 1992). One explanation for the different spectral forms is the assumption that POR aggregates are red-shifted with increasing aggregate size, the largest being present in PLBs and the smaller in PTs. Immediately after the phototransformation, Chlide *a*, still bound to POR, is translocated from PLBs to PTs, and the prominent shift from 684 nm to 672 nm (Shibata shift) has been

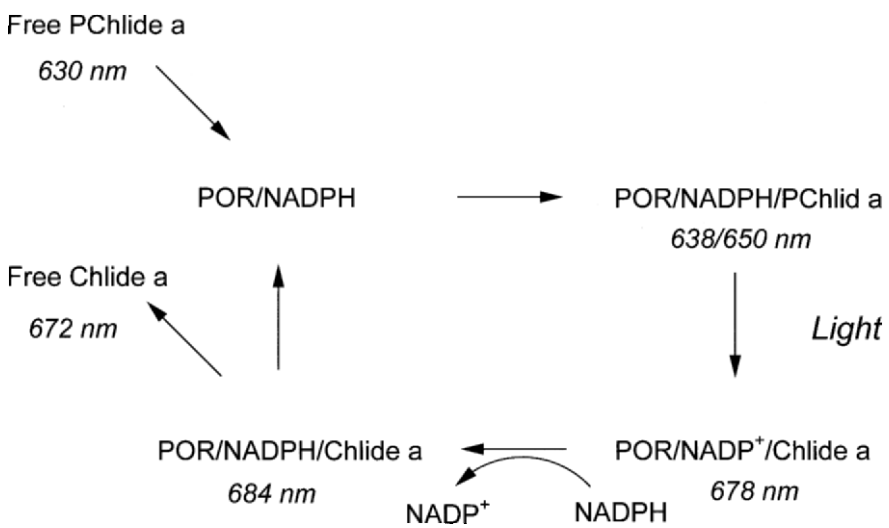

Fig. 2. Schematic representation of pigment-POR complex formation. Also shown are associated shifts of absorption maxima, observed when etioplast membranes are supplied with exogenous Pchlide *a* and NADPH (after Griffiths, 1991).

tentatively attributed to disaggregation of POR complexes during this translocation (Böddi et al., 1990); however, conformational changes of the Chlide-POR complex may also contribute to these shifts (Zhong et al., 1996). The Shibata shift is inhibited by fluoride, indicating the involvement of a phosphatase (Wiktorsson et al., 1996).

An alternative explanation for different spectral forms was based on in vitro experiments with etioplast membranes, consisting of PLBs and PTs in which the PLBs were probably disaggregated (Griffiths, 1991). The binding of PChlide *a* to POR in the membrane caused a red shift, and the addition of NADPH increased this red shift. After phototransformation, the exchange of $NADP^+$ by NADPH resulted in a red shift of Chlide *a*. The Shibata shift was then assumed to be connected with the release of Chlide *a* from POR. The in vitro shifts were reproduced with isolated, pigment-free POR supplied with PChlide *a* or Zn-PPheide *a* and NADPH; the typical red shift required monogalactosyldiacylglycerol and a reduction of the water potential, achieved by dialysis against 80% glycerol (Klement et al., 2000). Unfortunately, it is not yet known whether reduction of the water potential leads to increased aggregation, conformational changes of POR or altered interaction of PChlide with NADPH. Because these processes seem to be intimately interconnected, it will be difficult to recognize causal relationships from in vivo experiments.

III. Metabolism of Chlorophyll *b* and Chlorophyllide *b*

A. Synthesis of Chlorophyllide b

Indirect evidence led to the hypothesis of Chl *b* formation from Chl *a* or 'newly-synthesized Chl *a*' (Shlyk, 1971). The successful incorporation of $^{18}O_2$ into Chl *b* in *Chlorella vulgaris* (Schneegurt and Beale, 1992) and greening seedlings of *Zea mays* (Porra et al., 1994) indicated a dioxygenase or monooxygenase mechanism. The authors proposed a hydroxylation of Chl *a* at C-7^1 followed by dehydrogenation of the putative hydroxy intermediate to form the C-7 formyl group of Chl *b*. We now know that the reaction consists of two hydroxylation steps and takes place with Chlide *a* and not the (esterified) Chl *a* (see below).

A milestone for our understanding of Chl *b* formation was the detection of the Chlide *a* oxygenase (*CAO*) gene (Tanaka et al., 1998). The authors mutagenized *C. reinhardtii* and found deletions in the same region of the genomic DNA in all Chl *b*-less mutants; further, they were able to reinstate Chl *b* formation in the mutants by transformation with a genomic DNA fragment of this region containing a gene which they later named *CAO*. The encoded protein was thought to be an oxygenase because it contained consensus sequences for both a Rieske-type [2Fe-2S] cluster and for a mononuclear non-heme Fe-binding site. Using the *Chlamydomonas CAO* sequence, Espineda et al. (1999) cloned the homologous gene (*AtCAO*) from *A. thaliana* and showed that a Chl *b*-less *Arabidopsis* mutant (*ch1-3*) contained a lesion in the *CAO* sequence. The studies with both organisms indicated that this gene is essential for Chl *b* biosynthesis and that no alternative pathway for Chl *b* formation exists.

Oster et al. (2000) isolated the full-length cDNA from *A. thaliana* and showed its intactness and functionality by rescue of Chl *b*-free mutants. After expression of the *CAO* gene in *E. coli*, the cell extract was able to catalyze the formation of Chlide *b* from Chlide *a*; traces of 7^1-OH-Chlide *a* were likewise produced. Oster et al. then demonstrated that the incubation of the cell extract with Zn-7^1-OH-Pheide *a* (Pheide = pheophorbide) gave a high yield of Zn-Pheide *b*. This second reaction step was also catalyzed by CAO, no reaction of the hydroxy-compound was found in the extract of control cells which did not contain the *CAO* gene. On the other hand, CAO did not catalyze the oxygenation of esterified Chl *a* or of PChlide *a* in the in vitro test indicating that Chlide *a* is the sole substrate for formation of the formyl group of Chl *b*. This does not exclude side reactions with these substrates under special conditions (see the work of Xu et al., 2002, discussed below).

Interesting aspects of chloroplast evolution came from further investigations of the *CAO* gene. Chl *b* has been detected in a wide range of organisms extending from cyanobacteria ('prochlorophyta') to higher plants. The Chl *a/b*-binding proteins of the prokaryotes are unrelated to those of the eukaryotes indicating at least two independent evolutionary origins for these proteins (La Roche et al., 1996). The hypothesis that the synthesis of Chl *b* was also invented at least twice during evolution has been questioned. Tomitani et al. (1999) compared the *CAO* genes from cyanobacteria to higher plants and found at least 50% identity for any of two of the deduced amino acid sequences. This high degree of homology

excluded the possibility that Chl *b* biosynthesis has been re-invented several times, provided that the CAO reaction is the obligatory step for Chl *b* formation in all organisms. Tomitani et al. (1999) have proposed that the common ancestor of chloroplasts and recent cyanobacteria contained both antenna pigments, namely, biliproteins and Chl *b*, and that the loss of one of these pigments occurred several times, either before or after endosymbiosis: loss of biliproteins resulted in prochlorophyta and chlorophyta while loss of Chl *b* resulted in biliprotein-containing cyanobacteria and rhodophyta. Support for the existence of such a common ancestor came from the earlier detection of a *Prochlorococcus marinus* strain which contained both Chl *b* and biliprotein (Hess et al., 1996).

Very few investigations on the molecular basis of the regulation of Chl *b* biosynthesis have been reported. Espineda et al. (1999) found up- and down-regulation of the *AtCAO* mRNA level with the light intensity, possibly related to the well-known changes in the Chl *a/b* ratio during acclimation of plants to different light conditions. Overexpression of the *AtCAO* gene in *A. thaliana* resulted in an increase of about 20% of the light-harvesting chlorophyll protein of Photosystem II (LHCB) content together with an increase in Chl *b* content (Tanaka et al., 2001). When *Synechocystis* sp., a biliprotein-containing cyanobacterium, was transformed with the same gene, about 10% of the total Chl was Chl *b*, incorporated mainly into the P700 complex (Satoh et al., 2001). The same cyanobacterium strain, transformed with both the *CAO* and the *LHCB* genes, produced as much as 80% of its Chl as Chl *b* but did not accumulate any LHCB protein: Chl *b* had replaced Chl *a* in most of the Chl *a*-binding proteins under these conditions (Xu et al., 2001). When this strain, which was unable to synthesize Chl in darkness due to a deficiency in the *chlL* gene, was kept in darkness for several days, it first produced PChlide *a* and, after a delay of several more days, produced PChlide *b* (Xu et al., 2002). These surprising results have still to be explained.

B. Reduction of Chlorophyll b and the Chlorophyll Cycle

The chemical reduction of a formyl group to a hydroxy group under mild conditions is well-known. This reaction, performed with aqueous cyanoborohydride and pheophorbide *b* (Pheide *b*), readily yields 7^1-hydroxy-Pheide *a* but, in methanol, the 7^1-methoxy-derivative is formed. The 13^1-oxo group is only reduced by prolonged incubation with a large excess of the reductant. Surprisingly, the same incubation with Zn-Pheide *b* proceeded further to Zn-Pheide *a*: the 7^1-hydroxy- and 7^1-methoxy-compounds proved to be intermediates in this reaction (Scheumann et al., 1996). The electron-withdrawing effect of the central metal is apparently so strong at C-7 that the formyl group can be reduced to a methyl group while, again, the 13^1-oxo group is essentially unaffected under the same conditions.

The biochemical reduction of *b*-type to *a*-type pigments was demonstrated in vitro with etioplast membranes (Ito et al., 1996; Scheumann et al., 1998). The reduction was successful with Chlide *b*, Zn-Pheide *b* and the esterified Chl *b*; the 7^1-hydroxy-compounds were also intermediates in this biochemical reaction. Reductant for the first step was NADPH while the second step, the reduction of the hydroxymethyl to the methyl group, required reduced ferredoxin. The existence of two enzymes, provisionally named Chl *b* reductase and OH-Chl reductase, was supported by more observations on their substrate specificity: Chl *b* reductase also reduced PChlide *b* (but not the metal-free Pheide *b*), Zn-Pheide *b* and Zn-Pheide *d* to their respective hydroxy compounds while no further reduction of these hydroxy compounds was observed in etioplast membranes (Scheumann, 1999). Reinbothe et al. (2003a), however, described indirect evidence for the reduction of endogenous PChlide *b* to PChlide *a* in etioplast preparations while exogenous PChlide *b* was not reduced at all. The occurrence of endogenous PChlide *b* in etioplasts has not yet been confirmed in other laboratories (see also Section IIC).

The physiological significance of the Chl *b* to Chl *a* reduction is not entirely clear. The discussion concentrates on two situations where the reduction could play a role. (i) Many plants can acclimate to changing light condition by a change in the Chl *a*/*b* ratio (Tanaka et al., 1991). It is difficult to distinguish between a direct reduction of Chl *b* to Chl *a* and the alternative, which is Chl *b* degradation with new synthesis of Chl *a*; however, the formation of Chl *a* and Zn-pheophytin (Zn-Phe *a*) after infiltrating etiolated plants with Chlide *b* and Zn-Pheide *b*, respectively, proved that direct reduction occurred in the tissue (Scheumann et al., 1996; Vezitskii, 2000). (ii) Both Chls *a* and *b* disappear in senescing plants. While degradation products derived from both Chls *a* and *b* were found in algae (Gossauer and Engel, 1996), all the degradation products of angiosperms exclusively

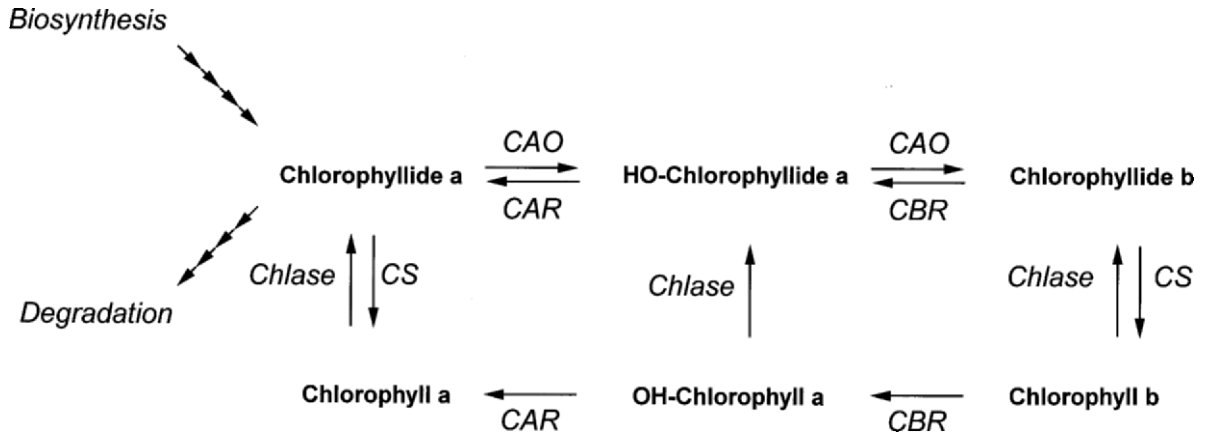

Fig. 3. The Chl cycle. The reversible transformation of Chl *a* into Chl *b* proceeds via Chlide *a* which is the key intermediate for biosynthesis and degradation of Chls. *CS* = chlorophyll synthase; *Chlase* = Chlorophyllase; *CAO* = chlorophyllide *a* oxygenase; *CBR* = chlorophyll(ide) *b* reductase; *CAR* = 7^1-hydroxy-chlorophyll(ide) *a* reductase.

contain the 7-methyl group characteristic for Chl *a* (Kräutler and Matile, 1999). Senescence of barley in the presence of D_2O resulted in a labeled methyl group, indicating reduction of the formyl group of Chl *b* before degradation (Folly and Engel, 1999). Consistent with this interpretation, the higher-plant degrading enzyme, Pheide oxygenase, accepts only Pheide *a* while Pheide *b* is an inhibitor (Hörtensteiner et al., 1995). Scheumann et al. (1999b) found a transient increase in the Chl *b* reductase activity corresponding to the onset of Chl degradation. Since the activity could be localized in the thylakoid membrane of gerontoplasts, in contrast to all other degrading enzymes which are located in the envelope (Matile and Schellenberg, 1996), the reductase can be assumed to be the first enzyme of Chl *b* degradation. It is tempting to assume a key role for Chl *b* reductase in LHCII degradation: the reaction product, 7^1-OH-Chl *a*, does not bind to LHCB (Ohtsuka et al., 1997), and Chl-binding proteins are generally degraded if not bound to Chl.

Ito et al. (1996) coined the name 'chlorophyll cycle' for the mutual conversion of Chl *a* into Chl *b* in angiosperms but they assumed a direct inter-conversion of the esterified pigments. In view of the properties of the enzymes involved which are discussed in this chapter, a more detailed description of the individual steps of the cycle is now possible (Fig. 3). The Chl cycle is not an obligatory metabolic cycle running always in one direction; rather, it consists of a number of enzymatic steps enabling the plants, when necessary, to adapt to ambient conditions by altering Chl *a/b* ratios. The key intermediate Chlide *a,* which is synthesized from PChlide *a,* can be considered as entry point into the cycle for the biosynthesis of Chls *a* and *b.* It is likewise an obligatory intermediate in the degradation of Chls and, therefore, can also be considered as the exit point from the cycle. Completion of Chl *a* biosynthesis is achieved by esterification of Chlide *a*, catalyzed by Chl synthase (Section IV), and Chl *b* biosynthesis requires the CAO reaction of Chlide *a* followed by esterification of Chlide *b*. In higher-plants, degradation of Chl *b* starts by reduction to the hydroxy intermediate which is a good candidate for the transport from the thylakoids to the envelope where chlorophyllase can hydrolyze the phytyl ester to be followed by reduction to Chlide *a*. Currently, it cannot be excluded, however, that Chl *b* and Chl *a* are also transported to the envelope to react with chlorophyllase.

IV. Esterification

A. Chlorophyll and Bacteriochlorophyll Synthases

Esterification of Chlides *a* and *b* is the last step of Chl *a* and *b* biosynthesis, and the substrate specificity of the respective enzymes, discussed below, indicates that esterification is also the last step of BChl synthesis. The reaction type is a prenylation, and the Chl and BChl synthases are prenyl transferases. The activity of Chl synthase was detected in etio-

plast membranes and most early investigations were performed with membrane fractions of etioplasts or chloroplasts (reviewed by Rüdiger and Schoch, 1991). A general disadvantage of this material was the uncertainty concerning the number of synthases present. More recent investigations were carried out with single recombinant enzymes that became available after isolation of the genes encoding Chl and BChl synthases.

B. Synthase Genes and Recombinant Enzymes

Disruption of the *bchG* gene of *Rhodobacter* (*Rba.*) *capsulatus* resulted in lack of BChl synthesis and accumulation of non-esterified BChlide indicating that this gene coded for BChl synthase (reviewed by Suzuki et al., 1997). Homologous *bchG* genes were isolated from different photosynthetic bacteria (*Chloroflexus* (*Cfl.*) *aurantiacus*: Niedermeier et al., 1994; Lopez et al., 1996; *Heliobacillus mobilis*: Xiong et al., 1998; *Rba. sphaeroides*: Naylor et al., 1999), and *chlG* genes encoding Chl synthases were described for *A. thaliana* (Gaubier et al., 1995), *Synechocystis* sp. (Oster et al., 1997), and *Avena sativa* (Schmid et al., 2001). Except for *Cfl. aurantiacus*, only a single Chl synthase gene was detected in these organisms. Nine membrane-spanning helices were predicted for the deduced amino acid sequences (Schmid et al., 2001): this prediction agreed with the membrane localization of Chl and BChl synthases and explained why all attempts to obtain a soluble, active enzyme failed.

Expression of the recombinant *ChlG* genes from *A. thaliana and Avena sativa* in *E. coli* resulted in active enzymes in the bacterial membrane fraction. The substrate specificity of the recombinant Chl synthase, derived from *Avena sativa,* was identical with that in the *Avena sativa* etioplast membranes (Schmid et al., 2002). Modification of the Chlide structure gave results with Chl synthase comparable to those with POR after modification of the PChlide structure: exchange of the substituents at rings A and B are tolerated including even the introduction of bulky side chains while steric hindrance exists around ring E: Chlide *a´*, the 13^2-epimer of Chlide *a,* was not accepted as a substrate (Helfrich et al., 1994; for details see supplement). If Chlide *a´* is also unacceptable to Chl synthase in vivo, then the esterified Chl *a´* that occurs naturally in PS I (Watanabe et al., 1985; Jordan et al., 2001; Chapter 4, Kobayashi et al.) must be produced by epimerization of Chl *a* after esterification.

Expression of *bchG* genes in *E. coli* yielded active BChl synthases. The gene product from *Rba. capsulatus* accepted BChlide *a* but not Chlide *a* which is a precursor in the biosynthesis of BChlide *a* (Oster et al., 1997). The two *bchG* genes from *Cfl. aurantiacus* yielded two BChl synthases with different substrate specificities (Schoch et al., 1999). The best substrate for bchG was BChlide *a* while BChlides *c, e* and *d* were not accepted: the bchG2 enzyme esterified BChlides *c, e* and *d* but not BChlide *a* (for details see supplement).

C. Biosynthesis of Phytol and Esterification of Chlorophyllide in Vivo

With only relatively few exceptions, natural Chls and BChls contain the isoprenoid alcohol phytol. The last step of phytol biosynthesis is the hydrogenation of geranylgeranyl diphosphate (GGPP): the earlier steps of isoprenoid synthesis in plants are discussed by Lichtenthaler (1999). The reductase, encoded by the *bchP* gene of BChl-producing organisms (Bollivar et al., 1994) and the *chlP* gene of Chl-forming organisms (Addlesee et al., 1996), can reduce geranylgeraniol both as the diphosphate and as the ester group of Chl (Keller et al., 1998). The main pathway for Chl biosynthesis is probably the reduction of Chl_{GG}, as outlined below, however, the reduction of GGPP also plays a role in vivo since tobacco plants that expressed antisense RNA for *ChlP* showed not only reduced levels of Chls *a* and *b*, some Chl_{GG}, but also reduced levels of tocopherol (Tanaka et al., 1999): it is known that tocopherol synthesis in tobacco depends on phytyl diphosphate.

The sequence Chlide $\rightarrow Chl_{GG} \rightarrow Chl_P$ (Chl containing phytol), at first described for the initiation of Chl synthesis in etioplasts, was also more recently reported to be true in tissues containing proplastids; since the hydrogenation of Chl_{GG} was more rapid in proplastids than in etioplasts, Chl_{GG} could only be detected as an intermediate at 0 °C (Schoefs and Bertrand, 2000). It is not unlikely that the same reaction sequence is true also for green tissue; the finding of Chl_P after 20 min (Rüdiger, 1987), when Chl_{GG} predominated in etioplast-containing tissue, may indicate a rapid hydrogenation of Chl_{GG} in green tissue.

A rapid phase of esterification was detected in flash-illuminated etiolated seedlings (Adra and Rebeiz, 1998; Domanskii and Rüdiger, 2001; Domanskii et

al., 2003) that was completed within about 15s and involved only 10–15% of the Chlide formed by full photoconversion. After a lag phase of 2–5 min, the residual 85–90% of the Chlide was esterified in the main phase within 30–40 min. The lag phase coincides with the beginning of PLB dispersal (Domanskii et al., 2003) and translocation of Chl synthase from PLBs, where it is inactive, to PTs (Lindsten et al., 1990; 1993). Since preloading of recombinant Chl synthase with phytyldiphosphate resulted in a similar rapid esterification (Schmid et al., 2002), preloading was also assumed in vivo. The reaction sequence via Chl_{GG} indicates that Chl synthase is preloaded with GGPP in vivo. Kinetics of the rapid phase are identical at 20 °C and 0 °C while esterification of the main phase is completely blocked at 0 °C. Domanskii et al. (2003) assumed a close connection of POR and Chl synthase in vivo, possibly as a complex of defined composition, to explain the rapid and direct transfer of Chlide, formed by phototransformation while bound to POR, to Chl synthase for esterification. Direct proof of the existence of such a complex is still missing.

Supplement

The supplement contains data on the substrate specificity of POR, Chl synthase and BChl synthase, determined with chemically modified substrates. PChlide *a*, the substrate of POR, was modified at rings A and B (Table S1) and at rings D and E (Table S2). Chlide *a*, the substrate of Chl synthase, was modified at C-7 and C-20 (Table S3) and at ring E (Table S4). The specificity of two BChl synthases, BChG and BChG2, was tested with Bchlid *a* modified at C-3, C-7, C-13^2 and C-20 (Table S5). This material is available at http://epub.ub.uni-muenchen.de/archive/00000776/.

Note Added in Proof

The nitrogenase model for DPOR was supported by more recent results using overexpression of BchL and BchN-BchB in photosynthetically competent strains of *Rhodobacter casulatus* (Nomata et al., 2005). Reduced ferredoxin served as electron donor in the assay system, which was stable for at least six months under anaerobic conditions. In analogy to nitrogenase subunits $(NifH)_2$ and $[(NifE)_2(NifN)_2]$, DPOR subunits form a homodimer $(BchL)_2$ and a heterotetramer $[(BchN)_2(BchB)_2]$, respectively. The presence of PChlide *b* in etiolated seedlings, reported by Reinbothe et al. (2003a) was later questioned: Kolossov and Rebeiz, repeating the extraction with 99% acetone containing 0.1% diethyl pyrocarbonate, were not able to detect any Pchlide *b* (Kolossov and Rebeiz, 2003).

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