

# CHLOROPHYLL SYNTHESIS IN RELATION TO THE ASSEMBLY OF PHOTOSYSTEMS

Fabrice FRANCK and Benoît SCHOEFS

## *Chloroplast differentiation.*

Most information available on the biochemistry of chlorophyll synthesis and on the formation of the photosynthetic apparatus during chloroplast biogenesis in higher plants is derived from experiments on dark-grown plant material. Etiolated angiosperms do not synthesize chlorophyll. Rapid chlorophyll accumulation can be initiated upon transfer from darkness to light (Barry et al., 1991). In etiolated plants an unusual plastid differentiation takes place during growth in darkness. The proplastids differentiate to etioplasts, which are characterized by the presence of prolamellar bodies. The prolamellar body is a network of tubular membranes that show a crystalline appearance when examined by electron microscopy. It is a part of the inner membrane system of the etioplasts, which also show thylakoid-like membrane structures called prothylakoids (Virgin, 1963; Henningsen and Boynton, 1969). Prolamellar bodies contain lipids and proteins, the most abundant of which is the photoenzyme which, in the light, reduces Pchl<sub>id</sub> to Chl<sub>id</sub>: the light-dependent NADPH-Pchl<sub>id</sub>-oxidoreductase (POR). After several hours of irradiation by white light, etioplasts will transform to normal chloroplasts (Robertson and Laetsch, 1974).

Etioplasts with large prolamellar bodies are not encountered in nature since prolonged darkness does not occur normally. In natural conditions, another pattern of plastid differentiation occurs, where proplastids slowly differentiate to chloroplasts. Small prolamellar bodies in developing chloroplasts have however been reported at early stages of development, for instance at the end of the night at early stages of growth in cucumber (Rebeiz and Rebeiz, 1986). In such cases partly developed chloroplasts show small prolamellar bodies which appear in continuity with the thylakoids. Therefore the natural differentiation of chloroplasts is rather complex and most researchers have preferred to use the etioplast-chloroplast differentiation system in dark-grown plant material to study chlorophyll biosynthesis and the assembly of the photosynthetic apparatus. This experimental system has its advantages and disadvantages. The advantages are:

- the possibility of having leaves of a size which allows easy manipulation;
- the greening process upon transfer to light is fast (in barley:  $\pm$  one day);
- during greening the assembly of the photosynthetic apparatus shows some relatively well-defined steps, which can be separated by choosing the duration and the conditions of irradiation.

The disadvantages are:

- as stated above, the non-natural character of the etioplast-chloroplast differentiation system;
- the presence of prolamellar bodies at the beginning of greening, which makes it difficult to analyse the very first steps of the formation of photosystems (however procedures have been designed to separate prolamellar bodies from prothylakoids (Wellburn and Hampp, 1979; Lindsten et al., 1988));
- the difficulty in obtaining good preparations of etiochloroplasts during greening due to frequent contamination with nuclear components. This can however be solved by further purifying the etiochloroplasts by Percoll density gradient or sucrose gradient centrifugation (Høyer-Hanson and Simpson, 1977).

Other systems than dark-grown leaves of angiosperms exist with which Chl accumulation and formation of thylakoids can be conveniently studied without the peculiarity of starting with an already differentiated etioplast. Among them:

- Some yellow-in-the-dark mutants of *Chlamydomonas* (y1 mutant) which only synthesize Chl in the light (Hooper et al., 1994). In such mutants degreened cells are obtained after cultivating the cells in darkness for some times. Their plastids have completely lost their inner membranes, but no prolamellar bodies are formed. Upon irradiation rapid Chl accumulation and reformation of thylakoid membranes takes place.
- The aquatic higher plant *Spirodela* (McCormac et al., 1996). When grown in darkness, it accumulates the Chl precursor protochlorophyllide but does not differentiate any prolamellar bodies. Greening in the light is very slow. This system can serve as a model-system for the proplastid to chloroplast differentiation.

### *Chlorophyll biosynthesis*

A detailed account of the biochemical steps leading to Chl biosynthesis can be found in a recent review by von Wettstein et al. (1995). The Chl biosynthetic pathway starts with the synthesis of  $\delta$ -aminolevulinic acid from glutamate or  $\alpha$ -ketoglutarate along the Beale pathway. This series of biochemical reactions involves the activation of a glutamate molecule by a tRNA<sup>Glu</sup> (Weinstein et al., 1986). The glutamyl-tRNA is then enzymatically transformed into glutamate-1-semialdehyde. The molecular form of glutamate-1-semialdehyde occurring *in vivo* (*i.e.*, linear versus cyclic) remains a debated point. The addition of a second amino group onto glutamate-1-semialdehyde by an aminotransferase yields to the diaminovalerate formation and not to dioxoalverate as claimed before (Smith et al. 1991). Diaminovalerate is then deaminated to yield  $\delta$ -aminolevulinic acid (for a review, see Schoefs and Bertrand, 1996).

Two molecules of  $\delta$ -aminolevulinic acid are condensed to form a molecule of porphobilinogen (for a review, see Jordan 1991). Four molecules of porphobilinogen are assembled to yield, first, a linear tetrapyrrole, namely preuroporphyrinogen, which is enzymatically cyclized to uroporphyrinogen III. The random decarboxylations of acetate side chains of uroporphyrinogen III yields coproporphyrinogen III (for a review, see Jordan 1991). The oxidative decarboxylation of the two propionic side chains of the A and B coproporphyrinogen III rings leads to the formation of divinyl-protoporphyrinogen IX. It

was suggested that this decarboxylation step yields actually two products *i.e.* monovinyl- and divinyl-protoporphyrinogen IX (Rebeiz & Mattheis 1981). Both monovinyl- and divinyl-protoporphyrinogen IX are then transformed to monovinyl- and divinyl-protoporphyrin IX respectively (for reviews, see Jordan 1991, Schoefs and Bertrand 1996). These compounds are at the biochemical crossroad of the chlorophyll and heme pathways. In the former, a chelatase inserts  $Mg^{2+}$  into protoporphyrin IX whereas in the later, another chelatase inserts  $Fe^{2+}$  (for a review, see Castelfranco et al. 1994, Schoefs & Bertrand 1996). Mg-protoporphyrin IX is then esterified with a monomethyl ester which is used to form the 5th ring characteristic of the chlorophyll related pigments whose the first member is protochlorophyllide *a* (for a review, see Castelfranco et al. 1994)

On the basis of their content in monovinyl- or divinyl-protochlorophyllide *a* in darkness or in the light, higher plants have been classified into four groups (Rebeiz et al. 1986). They are: 1) dark divinyl/light divinyl (*e.g.*, cucumber, douglas fir), 2) dark monovinyl/light divinyl (*e.g.*, monocotyledons), 3) dark divinyl/light monovinyl (*e.g.*, *Ginkgo biloba*, *Viola* spp.), and 4) dark monovinyl/light monovinyl (*e.g.*, apple tree).

All enzymes involved in Chl biosynthesis are active in darkness, except for the light-dependent POR. In gymnosperms, most green algae and cyanobacteria, another enzyme catalyses Pchlde reduction in addition to POR: it is the light-independent Pchlde reductase. In angiosperms, because the activity of POR has a strict requirement for light and the light-independent Pchlde-reductase is absent, Chl biosynthesis is blocked at the level of Pchlde in the absence of light. Pchlde (and to some extent Pchl) is the only pigment which accumulates in significant amounts in dark-grown leaves of angiosperms. Other intermediates are only present in trace amounts.

When dark-grown plants are suddenly transferred to light, Chl accumulation starts. The first step is the photoreduction of accumulated Pchlde. The whole chain of intermediate reactions from glutamate to Pchlde is then activated by a phytochrome-dependent process and rapid greening is then observed after a 2-4 hours lag phase during which this activation takes place.

#### *Protochlorophyllide photoreduction and the assembly of the first photosynthetic units.*

The enzyme NADPH-protochlorophyllide-oxidoreductase (POR) belongs to a special class of enzymes called photoenzymes. Absorption of light by the substrate protochlorophyllide is absolutely required for the reduction to occur. For the photoreduction to proceed efficiently, it is therefore necessary that stable enzyme-substrates complexes are synthesized in darkness. To elucidate the structure of these complexes and the molecular mechanism of light-induced electron and proton transfer is a challenging task for future research.

POR is the best characterized enzyme involved in Chl biosynthesis (reviewed in Griffiths, 1992; Fujita, 1996). This is mainly due to the fact that it can be easily isolated from etioplast inner membranes where its concentration is high. It is synthesized in the cytoplasm as a precursor of 44 kDa which is further processed to a mature form of 36 kDa (probably during transport across the etioplast envelope). Since early studies two POR isoenzymes were often described with close apparent molecular weight. Recently two closely related POR (A and B) genes were found in barley and *Arabidopsis*. The PORA and PORB

polypeptides have high homology (more than 70 %). They differ mainly in their transit peptide and are differentially regulated by light (Reinbothe et al., 1996).

In dark-grown plants, POR is associated to the inner membrane system of the etioplast. Immunogold experiments have shown it to be localized mainly in prolamellar bodies. However hydrophathy plots did not reveal membrane-spanning regions and POR should rather be considered as belonging to the category of extrinsic membrane proteins.

*In vivo* spectroscopy has been used extensively to characterize various forms of Pchlde occurring in etioplasts and to monitor changes in pigment-protein relationships during and after photoreduction. In dark-grown leaves of higher plants, the concentration of the pigment as well as the absence of other pigments absorbing light significantly in the red region of the spectrum make it an ideal case both for absorbance and fluorescence spectroscopy studies. Three spectral Pchlde forms are usually encountered in dark-grown leaf tissue. Pchlde650-655 is the main one. This photoactive Pchlde form consists of a stable, aggregated state of the POR-substrates complex found in prolamellar bodies (Böddi, 1994). In this complex Pchlde is reduced to Chlide within microseconds at room temperature (Franck and Mathis, 1980). Pchlde638-645 is a minor photoactive Pchlde form. It most probably represent a monomeric state of the POR-substrates complex. Besides photoactive Pchlde forms, photoinactive Pchlde628-632 occurs in minor amount. Pchlde628-632 is actually a mixture of Pchlde and Pchl and has a complex spectral fine composition (Schoefs and Franck, 1995). Energy migration between different Pchlde forms as well as from Pchlde to Chlide during photoreduction has been demonstrated (Kahn et al., 1970; Sironval and Brouers, 1980). Fluorescence spectra should be therefore interpreted with care when quantitative estimates of different pigment forms is wanted.

Since the etioplast is a non-natural state of the plastid, the occurrence of these Pchlde forms (and in particular of the photoactive Pchlde650-655) in other circumstances has been questioned. Recent studies have shown that Pchlde650-655 is synthesized very early after seed germination at the stage of proplastid (Schoefs and Franck, 1993). Photoactive Pchldes with similar spectroscopic properties have also been detected in the light during the phase of fast chlorophyll accumulation in greening leaves of dark-grown barley (Franck and Strzalka, 1992; Schoefs and Bertrand, 1996). An example of the changes in 77 K emission spectra during transformation and regeneration of photoactive Pchldes in greening barley is shown in Fig. 1. Similar Pchlde spectral forms were found also during temperature-induced greening in light-grown maize leaves (Franck, Schoefs and Mauro, unpublished). It seems therefore that the formation and phototransformation of aggregated forms of POR-substrates complexes is a normal step in the process of Chl accumulation in chloroplasts. Massive accumulation of PORA enzyme is however restricted to etioplasts due to a Pchlde-dependent import mechanism (Reinbothe et al., 1996). One expects therefore that photoactive Pchlde complexes observed in the above conditions contain mainly PORB. It has been suggested that, besides its role in Chl formation, PORA is specifically involved in the protection of Pchlde (Runge et al., 1996) and Chlide (Franck and Schmid, 1985) against photodegradation.

When Pchlde is photoreduced to Chlide by a short light pulse, Chlide-POR-NADP<sup>+</sup> complexes (Chlide678-688) are produced within microseconds from the photoactive Pchldes. In a minor amount of these complexes, Chlide is released within some seconds, a process which yields short-wavelength Chlide670-675 and is paralleled by the regeneration of some photoactive Pchlde by rapid rebinding of Pchlde and NADPH (Schoefs and

Franck, 1993). However most Chlide-complexes dissociate much more slowly, in a time-scale of several mins. In this slow pathway, exchange of NADP+ for NADPH first occurs within some seconds after irradiation, yielding Chlide684-696 (El Hamouri et al., 1981). Slow dissociation of these complexes and esterification by Chl-synthetase then takes place and is manifested by a slow (c.a. 20 min) blue shift known as Shibata shift, of which the end product is Chl672-682 (Shibata, 1957; Sironval et al., 1965). In the same period the dispersal of prolamellar bodies is observed (Henningsen and Boynton, 1969). Part of Chl *a* resulting from this process then bounds to Chl-apoproteins of photosystem I and II cores while photoactive Pchlides regenerate from *de novo* synthesized Pchlides pigments (Eichacker et al., 1990; Franck, 1993). Whether PORA is used in the regeneration process or is replaced by PORB is a matter of debate (see Reinbothe et al., 1996).

The above described events form a cycle which produces Chl *a* and regenerates photoactive Pchlides. Under repetitive flash irradiation small size photosynthetic units will accumulate from Chl *a* produced this way and from plastid-encoded Chl-apoproteins. The synthesis of Chl *b* in such conditions will not proceed significantly. The mechanism of regulation of the synthesis of Chl *b* from Chl *a* is not well understood. It appears that some threshold in the rate of Chl *a* synthesis must be reached before Chl *b* accumulation starts.

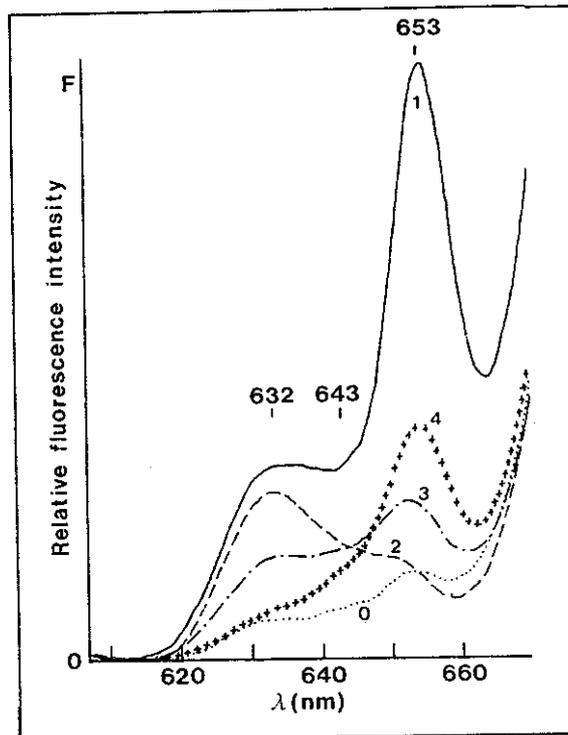


Figure 1. 77 K emission spectra of dark-grown barley leaves after a 3-hours greening under continuous polychromatic light ( $15 \text{ W}\cdot\text{m}^{-2}$ ). Pre-treatment as follows: none (frozen in the light) (0), 5 min of darkness (1) followed by a 1 millisecond flash (2) and a further 10 s (3) or 2 min (4) dark period. Excitation wavelength: 450 nm.

### *Assembly of photosystems.*

Each photosystem is composed of a number of chlorophyll-protein complexes which belong either to the reaction center cores or to the antenna system. The picture that emerged in the last 10 years of the photosystem I and II structure became more and more complex, as new genes and new polypeptides were discovered (for a recent review, see Jansson, 1994).

Studies on the changes in polypeptide composition and functional properties of the two photosystems during chloroplast biogenesis can be very useful to establish structure-function relationships and to understand some aspects of the assembly of photosynthetic units. In early studies on development of electron transport functions some general features of the development of photosystems I and II were established (for a review, see Baker, 1984). Electron transport studies clearly showed that reaction centers were synthesized in the very early stages of greening of angiosperms and that antenna only slowly increased in size thereafter (Kyle and Zalik, 1982). The drop in the Chl *a* *b* ratio during the first hours of greening parallels the process of antenna development. As a general rule, it was also found that photosystem I developed ahead of photosystem II.

In a broad sense two distinct phases in the assembly of photosynthetic apparatus must be distinguished:

- in the first phase naked reaction center cores accumulate; it corresponds to the accumulation of plastid-coded Chl *a* apoproteins.
- in the second phase Chl *a* *b* binding light-harvesting complexes, made of nucleus-encoded apoproteins, bind to the reaction center cores of both photosystems.

In barley greened in continuous light, the first phase extends during the first 3-4 hours of greening. It is possible to better separate the two phases by irradiating dark-grown leaves by repetitive short flashes of light (see above). In that case only Chl *a* and reaction center cores will accumulate during flash treatment. Subsequent irradiation by continuous light will induce the synthesis of additional chlorophyll including Chl *b* that will bind to light-harvesting complexes. In that period, as the concentration of photosynthetic units increases in the thylakoid membranes, energy migration between neighbour photosystem II units will appear as well spill-over.

Recent developments in the fine analysis of Chl-proteins, for instance by non-denaturing green gels or by isoelectric focusing, led to better insight into the details of photosystem assembly. This is particularly true for photosystem II, for which progressive formation of light-harvesting oligomers was demonstrated upon transfer from intermittent to continuous light (Dreyfuss and Thornber, 1994). A detailed analysis of the sub-populations of photosystem II light-harvesting complexes did not show significantly different developmental pattern (Sigrist and Staehelin, 1994). The greening approach has also been used to better understand the differences in protein stoichiometry between heterogenous photosystem II units differing in antenna size (Morrissey et al., 1989).

Studies on the regulation of nucleus-encoded Chl-apoprotein synthesis have demonstrated that phytochrome-dependent activation of transcription is involved. The ability of the plastids to import precursor forms of the proteins increases during chloroplast differentiation. Stabilization of light-harvesting proteins is achieved by binding of Chl *a* and

*b* (for reviews see Mullet, 1986; Jenkins, 1988). In the case of plastid-encoded Chl-apoproteins the same stabilization mechanism may be involved, but the hypothesis of regulation by Chl at the level of elongation has also been proposed (Eichacker et al., 1990). The accumulation and organization of Chl-proteins depends on the rates of both synthesis and degradation processes, which are sensitive to both endogenous (circadian rhythms, physiological state,...) and external (temperature, light intensity,...) factors.

#### Perspectives

The possibility of producing well-defined stages of differentiation of the photosynthetic apparatus through the control of chlorophyll synthesis has been and will continue to be a useful tool in photosynthesis research. It represents an interesting alternative to the use of photosynthetic mutants. As the knowledge of the structure of photosystems develops, assembly studies will help to understand structure-function relationships. While past studies have focussed mainly on biogenesis of the photosynthetic apparatus during extensive Chl accumulation, it will be interesting in future research to investigate on the coupling of chlorophyll metabolism and the maintenance of photosynthetic functions in mature chloroplasts. This coupling, suggested already long ago, has gained renewed interest in the context of the turn-over of important Chl-proteins such as those of photosystem II reaction center (Feierabend and Dehne, 1996).

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Laboratory of Photobiology, Department of Botany B22, University of Liège,  
B-4000 Liège / Sart Tilman, Belgium.