The formation of chlorophyll from chlorophyllide in leaves containing proplastids is a four-step process

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Abstract The time course of the different esters of chlorophyllide (Chlide) during the formation of chlorophyll *a* (Chl) in embryonic bean leaves containing proplastids was investigated by HPLC. After the reduction of photoactive Pchlide (Pchlide) to Chlide, three intermediates, i.e. Chlide geranylgeraniol, Chlide dihydrogeranylgeraniol and Chlide tetrahydrogeranylgeraniol were detected before the formation of Chlide phytol, i.e. authentic Chl. The transformation of Chlide to Chl was found to be much faster in leaves containing proplastids than in etiolated leaves with etioplasts.

Key words: Pigment biosynthesis; HPLC; Greening; Chlorophyll; Esterification; Last step of the chlorophyll biosynthetic pathway

1. Introduction

Reconstitution experiments [1] as well as in vivo studies (*Chlamydomonas* [2], higher plants [3]) have shown that correct assembly of pigment–protein complexes of the photosynthetic apparatus requires esterified chlorophyllide (Chlide), in addition to xanthophylls. Chlide esterification occurs after the photoreduction of photoactive protochlorophyllide (Pchlide) by NADPH:Pchlide oxidoreductase (EC 1.6.1.33) since it is generally accepted that Pchlide esters are not substrates for this enzyme [4].

Chlide esterification has been mainly studied with etiolated material, i.e. containing etioplasts. With this type of plant material it was established that Chlide resulting from photoactive Pchlide photoreduction (reviewed in [5]) is first esterified with geranylgeraniol pyrophosphate. The reaction is catalyzed by chlorophyll synthase [6]. Then the Chlide geranylgeraniol moiety is hydrogenated to successively yield Chlide dihydrogeranylgeraniol, Chlide tetrahydrogeranylgeraniol and finally Chlide phytol, i.e. authentic chlorophyll (Chl) which constitutes the final product of the reaction [7]. Since the esterification and the hydrogenations have different sensitivity to low temperature and anaerobiosis, it was proposed that the enzyme(s) catalyzing the hydrogenations is (are) different from Chl synthase (reviewed in [8]). More recently, the *chlP* gene from *Synechocystis* sp. PCC 6803 was found to restore the hydrogenation capacity of geranylgeraniol in a mutant of *Rhodobacter sphaeroides* accumulating bacteriochlorophyll geranylgeraniol [9]. This experimental result is a strong evidence in favor of this hypothesis. Great differences in the esterification process were however found in intact plants. The Chl formed within the first 15–20 min of the esterification is mainly Chlide geranylgeraniol from etiolated seedlings [7,10] but exclusively Chlide phytol in green barley seedlings [11,12]. On the basis of this difference it was concluded that in green plants Chlide is directly esterified with phytyl pyrophosphate (reviewed in [13]). Since investigations on plastid ultrastructure have shown that the etioplast stage is usually not reached by plant tissues developing in natural light/dark cycle (reviewed in [14]), one can ask whether in these plants Chlide is esterified directly with phytol pyrophosphate or with geranylgeraniol pyrophosphate at the beginning of greening. Studying Chl formation in embryonic bean leaves with proplastids under a natural light/dark regime, Schoefs et al. [15] never observed intermediates between Chlide and Chlide phytol. The authors concluded that either the esterification with phytyl pyrophosphate is also direct or that the intermediates have a very short live time making them not distinguishable in the time scale used in their study. This contribution aims to solve this question.

2. Materials and methods

2.1. Plant growth

Seeds of *Phaseolus vulgaris* cv. Red Kidney were grown in a dark room on water according to Schoefs et al. [16]. After 2 days of growth, the seedlings were dissected under a dim green light and the embryonic leaves were used for experiments. For each analysis, 50 leaf pairs were used.

2.2. Leaf illumination and pigment extraction

The leaves were illuminated with a commercial electronic photographic flash (2 ms duration). Then the pigments were extracted in methanol (Merck, Darmstadt, Germany) using a mortar and a pestle either immediately after the flash or after a period of darkness, up to 30 min. The extract was centrifugated at $15\,000 \times g$ during 3 min. The supernatant was collected and filtered through a 0.45 μm PTFE filter (Millipore, Millex) and dried under a nitrogen stream. To avoid pigment degradation, all the extraction steps were performed as rapidly as possible, under a dim green light and at 273 K, as recommended by Bertrand and Schoefs [17]. We found this last condition important since chlorophyllase, the enzyme which catalyzes phytol removal remains active in organic solvents. However, the optimum temperature of the reaction is rather high (approximately 300 K, reviewed in [18]). Therefore, in our experimental conditions, artefact due to the activity of this enzyme is rather limited.

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Abbreviations: Chl, chlorophyll *a*; Chlide, chlorophyllide *a*; Pchlide, protochlorophyllide *a*

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For the experiments at 273 K, the leaves were incubated on melting ice during 30 min before being flashed. The temperature at the leaf level was checked using contact digital thermometer.

2.3. HPLC analysis

The dried samples were dissolved in 250 μl of methanol and directly used for HPLC measurements. A 200 μl aliquot of the pigment solution was injected into a column packed with original Zorbax phase (4.6 mm×150 mm; Hewlett Packard). The pigments were eluted according to Schoefs et al. [19] at a flow rate of 1 ml min^{-1} and were monitored using a Waters 996 photodiode array detector. All the solvents used were of HPLC quality.

3. Results and discussion

Fig. 1 compares the actual HPLC chromatograms recorded at 434 nm (Chl(ide) and Pchl(ide) *a*) and 662 nm (Chl(ide) *a*) with 2 days old leaves flashed and then placed in the dark for 4 min before pigment extraction. The first peak is due to Pchlide (for the identification of the peaks corresponding to carotenoids, see Schoefs et al. [19,20]). The inset presents an enlarged view of the region where the Chlide esters were eluted. Because Pchlide esters are also eluted in this region, the chromatogram recorded at 623 nm is also presented. Six peaks were detected in this region. On the basis of the absorption spectra and the comparison of the capacity factor values with those of literature [19], the different peaks were identified as Chlide (peak 1), Pchlide (peak 2), Chlide geranylgeraniol (peak 3), Chlide dihydrogeranylgeraniol (peak 4), Chlide tetrahydrogeranylgeraniol (peak 5), Chlide phytol (peak 6) and Pchlide phytol (peak 7). The presence of this last peak was not expected since it was not reported previously in our [15]. No other esters of Pchlide were detected. This result strongly suggests that the formation of Chlide phytol from Chlide is a four-step process as it is in the plants with etioplasts.

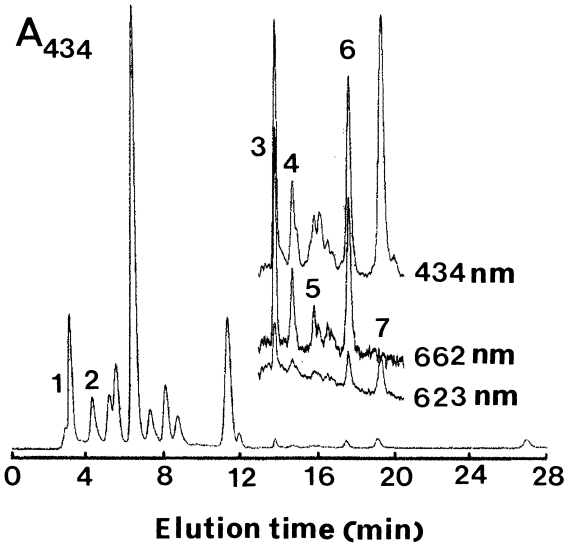

Fig. 1. Actual HPLC chromatograms recorded at 434, 623 and 662 nm (the two last ones only in the inset) of the pigments extracted from 2 days old bean leaves having received a saturating polychromatic flash and replaced in the dark for 4 min. The inset presents an amplified view of the part of the chromatograms where the different Chlide esters were eluted. Only the peaks corresponding to (P)Chl(ide) are denoted by a number: Peak 1, Chlide; peak 2, Pchlide; peak 3, Chlide geranylgeraniol; peak 4, Chlide dihydrogeranylgeraniol; peak 5, Chlide tetrahydrogeranylgeraniol; peak 6, Chlide phytol; and peak 7, Pchlide phytol.

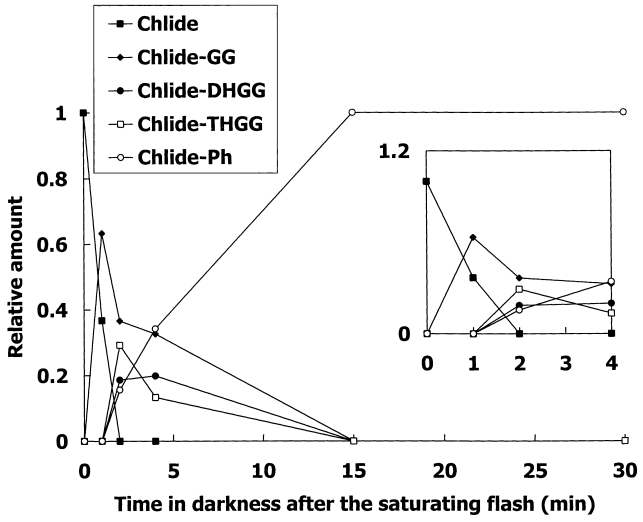

Fig. 2. Time courses of the relative amount of Chlide (■), Chlide-GG (●), Chlide-DHGG (♦), Chlide-THGG (□) and Chlide-Ph (○) during the formation of authentic Chl. Each point is the mean of two independent runs.

Fig. 2 shows the variations of the relative amount of the four Chlide esters during the first 30 min of darkness following the saturating flash which triggered the complete phototransformation of photoactive Pchlide to Chlide. Immediately after the flash only Chlide was observed. Its proportion decreased rapidly and it was no longer observed in the extracts of leaves placed in the dark for more than 2 min after the flash. During this period Chlide geranylgeraniol appeared first and then the other esters were detected. Their relative amount increased transiently and then decreased while Chlide phytol was accumulated. The entire pool of Chlide was transformed in Chlide phytol within the 15 min of darkness following the

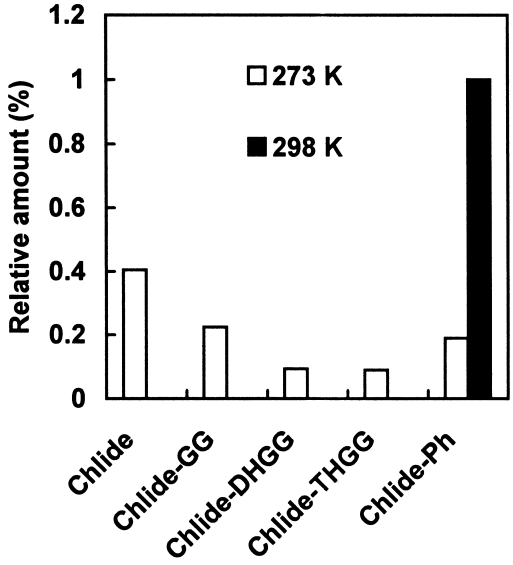

Fig. 3. Comparison of the relative amount of the different chemical forms of Chlide present in 2 days old leaves having received one single saturating flash and incubated during 15 min in the dark before pigment extraction. The experiments were performed either at 298 K or 273 K.

flash. No direct esterification of Chlide with phytol pyrophosphate was detected.

In order to determine whether lowering the temperature down to 273 K will differentially reduce the speed of either reactions (i.e. esterification and hydrogenations), the previous experiment was partially repeated with leaves incubated at this temperature prior the illumination. Fig. 3 compares the relative amounts of the different Chlide esters contained in leaves incubated for 15 min in the dark after the flash. After this period the leaves incubated at 273 K mainly contained non-esterified Chlide and Chlide geranylgeraniol. This result was expected since a low temperature affects more the hydrogenation steps than the esterification one [8].

To explain why the esterification process is faster in leaves with proplastids than in leaves with etioplasts, it is necessary to consider the particularities of the 'Pchlide–Chlide' cycle (reviewed in [5]) and of the plastid ultrastructure of these leaves ([21], reviewed in [22]).

In young leaves, the Shibata shift is not observed [21,23,24] and Chlide is immediately released from the active site of the enzyme [23]. This corresponds to the formation of Chlide emitting fluorescence at 675 nm. This Chlide is immediately available for esterification. Rapid esterification is only possible if both NADPH:Pchlide oxidoreductase and chlorophyll synthase are in proximity. This seems to be the case in the proplastid membranes as well as in etioplast ones. In fact, in etioplasts, both enzymes are associated to the prolamellar body membranes [25,26] but in this case chlorophyll synthase is latent since Chlide remains bound to LPOR. Esterification should await for the dissociation of the large aggregates of Chlide–NADPH:Pchlide oxidoreductase [26]. This occurs during the Shibata shift (reviewed in [27]). Nevertheless, a small fraction, approximately 15% of the initial Chlide pool [28] is rapidly esterified [29,30]. It corresponds to the Chlide emitting at approximately 675 nm [21,23]. A large proportion of this Chlide spectral species is formed in leaves treated with delta-aminolevulinic acid [31,32]. Nielsen [32] found that it is fastly esterified. It can be deduced from the studies using isolated internal membranes from etioplasts [33] that this Chlide is mainly formed in the prothylakoids which are in many aspects similar to the non-appressed internal membranes of proplastids. We can, therefore, assume that this Chlide is also esterified along a four-step process.

Fast esterification of Chlide emitting at 675 nm is necessary in the case of young leaves. In fact, the newly formed Chlide is not protected by carotenoids from photooxidation [34,35]. In addition, the 'Pchlide–Chlide' cycle of leaves with proplastids does not include the appendice which protects the newly formed Chlide against photooxidation in leaves with etioplasts ([36,37], reviewed in [5]). Chl(ide) photooxidation results not only in pigment destruction but also in generation of very reactive oxygen species (see also [38]). Therefore, the formation of Chl–protein complexes containing carotenoids seems to be the only possible way to protect the newly formed Chl(ide) from photooxidation. The identity of these proteins remains to be determined in the case of proplastids. This may involve proteins such as ELIPs or smaller analogues of Lhcb [39,40]. Connected to this, fluorescence spectra recorded during the first photoperiod of greening of bean leaves with proplastids present one single band at 683 nm until the activation of the oxygen evolving system started, i.e. after approximately 12 h of illumination [16,41].

4. Conclusions

We demonstrated that the esterification process in leaves containing proplastids is a four-step process very similar to the one observed in leaves with etioplasts. This indicates that at the beginning of greening, this mechanism is not a particularity of etioplasts. In proplastids the reactions are however much faster than in etioplasts. This is probably due to the fact that the Chlide is rapidly released from the active site of NADPH:Pchlide oxidoreductase. Lowering temperature mainly affects the speed of the hydrogenation steps. This is a clue for the existence of a different enzyme than Chl synthase which would catalyze the hydrogenation steps. No direct esterification of Chlide by phytol pyrophosphate was observed. It remains to determine at which stage of chloroplast development and for which reasons the formation of Chl from Chlide switches from the four-step reaction to direct esterification by phytol pyrophosphate. It is not impossible that different Chl synthases are involved in the reaction.

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References

[1] Paulsen, H., Hose, S. and Eisen, C. (1992) in: Regulation of Chloroplast Biogenesis (Argyroudi-Akoyunoglou, J.D., Ed.), pp. 343–349, Plenum Press, New York.
[2] Maloney, M.A., Hoober, J.K. and Marks, D.B. (1989) Plant Physiol. 91, 100–106.
[3] Henry, A., Powles, R. and Pennock, J.F. (1986) Biochem. Soc. Trans. 14, 958–959.
[4] Griffiths, W.T. (1974) FEBS Lett. 49, 196–200.
[5] Schoefs, B. (1999) Photosynthetica 36, 481–496.
[6] Rüdiger, W., Benz, J. and Guthoff, C. (1980) Eur. J. Biochem. 190, 193–200.
[7] Schoch, S., Lempert, U. and Rüdiger, W. (1977) Z. Pflanzenphysiol. 83, 427–436.
[8] Rüdiger, W. (1993) in: Pigment-Protein Complexes: Synthesis and Assembly (Sundqvist, C. and Ryberg, M., Eds.), pp. 219–240, Academic Press, San Diego, CA.
[9] Addlessee, H.A., Gibson, L.L.C., Jensen, P.E. and Hunter, C.E. (1996) FEBS Lett. 389, 126–130.
[10] Schoch, S. (1978) Z. Naturforsch. 33c, 712–714.
[11] Soll, J., Schultz, G., Rüdiger, W. and Benz, J. (1983) Plant Physiol. 71, 849–854.
[12] Rüdiger, W. (1987) in: Progress in Photosynthesis Research (Biggins, J.J., Ed.), Vol. 4, pp. 461–467, Martinus Nijhoff, Dordrecht.
[13] Rüdiger, W. (1992) in: Regulation of Chloroplast Biogenesis (Argyroudi-Akoyunoglou, J.D., Ed.), pp. 183–191, Plenum Press, New York.
[14] Thomson, W.W. and Whatley, J.M. (1980) Annu. Rev. Plant Physiol. 31, 375–394.
[15] Schoefs, B., Bertrand, M. and Lemoine, Y. (1998) Photosynth. Res. 57, 203–213.
[16] Schoefs, B., Bertrand, M. and Franck, F. (1992) Photosynthetica 27, 497–504.
[17] Bertrand, M. and Schoefs, B. (1997) in: Handbook of Photosynthesis (Pessarakli, M., Ed.), pp. 151–173, Marcel Dekker, New York.
[18] Drazkiewick, M. (1994) Photosynthetica 30, 303–313.
[19] Schoefs, B., Bertrand, M. and Lemoine, Y. (1995) J. Chromatogr. 692A, 239–245.
[20] Schoefs, B., Lemoine, Y. and Bertrand, M. (1996) Am. Biotechnol. Lab. 14, 18–22.
[21] Klein, S. and Schiff, J.A. (1972) Plant Physiol. 49, 619–626.
[22] Hudak, J. (1997) in: Handbook of Photosynthesis (Pessarakli, M., Ed.), pp. 27–49, Marcel Dekker, New York.

[23] Schoefs, B. and Franck, F. (1993) J. Exp. Bot. 44, 1053–1057.
[24] Schoefs, B., Garnir, H.-P. and Bertrand, M. (1994) Photosynth. Res. 41, 405–417.
[25] Lindsten, A., Welch, C.J., Schoch, S., Ryberg, M., Rüdiger, W. and Sundqvist, C. (1990) Physiol. Plant. 80, 277–285.
[26] Lindsten, A., Wiktorsson, B., Ryberg, M. and Sundqvist, C. (1993) Physiol. Plant. 88, 29–36.
[27] Ryberg, M., Artus, N., Böddi, B., Lindsten, A., Wiktorsson, B. and Sundqvist, C. (1992) in: Regulation of Chloroplast Biogenesis (Argyroudi-Akoyunoglou, J.D., Ed.), pp. 217–225, Plenum Press, New York.
[28] Granick, S. and Gassman, M. (1970) Plant Physiol. 45, 201–205.
[29] Bovey, F., Ogawa, T. and Shibata, K. (1974) Plant Cell Physiol. 15, 1133–1137.
[30] Ogawa, T., Bovey, F., Inoue, Y. and Shibata, K. (1974) in: Proceedings of the 3rd International Congress on Photosynthesis (Avron, M., Ed.), pp. 1829–1832, Elsevier Scientific Publishing Company, Amsterdam.
[31] Sundqvist, C. (1969) Physiol. Plant. 22, 147–156.
[32] Nielsen, O.F. (1971) Biochem. Physiol. Pflanz. 167, 195–206.
[33] Böddi, B., Lindsten, A., Ryberg, M. and Sundqvist, C. (1990) Photochem. Photobiol. 52, 83–87.
[34] Butler, W.L. (1961) Arch. Biochem. Biophys. 92, 287–295.
[35] Fradkin, L.I., Shlyk, F.F., Kalinina, M. and Faludi-Daniel, A. (1969) Photosynthetica 3, 326–337.
[36] Franck, F., Schoefs, B., Barthélemy, X., Mysliwa-Kurziev, B., Strzalka, K. and Popovic, C. (1995) Acta Physiol. Plant. 17, 123–132.
[37] Schoefs, B. and Franck, F. (1995) in: Photosynthesis: From Light to Biosphere (Mathis, P., Ed.), Vol. 3, pp. 1013–1017, Kluwer Academic Publishers, Dordrecht.
[38] Hoober, J.K. and Eggink, L.L. (1999) Photosynth. Res. 61, 197–215.
[39] Adamska, I. (1997) Physiol. Plant. 100, 794–805.
[40] Funk, C., Lindström, V. and Vermaas, W. (1999) in: The Chloroplast: From Molecular Biology to Biotechnology (Argyroudi-Akoyunoglou, J.H. and Senger, H., Eds.), pp. 103–106, Kluwer Academic Publishers, Dordrecht.
[41] Schoefs, B. and Franck, F. (1991) C. R. Acad. Sci. Paris 313, 441–445.