Planta (1998) 206: 673–680

Planta

Isolation and characterization of photoactive complexes of NADPH:protochlorophyllide oxidoreductase from wheat

Mohammed Aziz Ouazzani Chahdi, Benoît Schoefs*, Fabrice Franck

Laboratory of Photobiology, Institute of Plant Biology B22, University of Liège B-4000 Liège/Sart-Tilman, Belgium

Received: 25 February 1998 / Accepted: 8 June 1998

Abstract. A photoactive substrate-enzyme complex of the NADPH:protochlorophyllide oxidoreductase (POR; EC 1. 3. 1. 33) was purified from etiolated *Triticum aestivum* L. by gel chromatography after solubilization of prolamellar bodies by dodecyl-maltoside. Irradiation by a 1-ms flash induced the phototransformation of protocholorophyllide *a* (Pchlide) with −196 °C absorbance and emission maxima at 640 and 643 nm, respectively. The apparent molecular weight of this complex was 112 ± 24 kDa, which indicates aggregation of enzyme subunits. By lowering the detergent concentration in the elution buffer, a 1080 ± 250-kDa particle was obtained which displayed the spectral properties of the predominant form of photoactive Pchlide in vivo (−196 °C absorbance and fluorescence maxima at 650 and 653 nm). In this complex, POR was the dominant polypeptide. Gel chromatography in the same conditions of an irradiated sample of solubilized prolamellar bodies indicated rapid disaggregation of the complex after Pchlide phototransformation. High performance liquid chromatographic analysis of the POR complexes obtained using two detergent concentrations indicates a possible association of zeaxanthin and violaxanthin with the photoactive complex.

Key words: Carotenoid – Etioplast – NADPH:protochlorophyllide oxidoreductase – Protochlorophyllide – *Triticum*

Introduction

The enzyme NADPH:protochlorophyllide oxidoreductase (POR; EC 1. 3. 1. 33) accumulates in prolamellar bodies (PLBs) and prothylakoids (PTs) of the etioplast during growth of angiosperms in darkness (Griffiths 1978; Dehesh and Ryberg 1985). By forming dark-stable, photoactive complexes with NADPH and protochlorophyllide *a* (Pchlide) substrates, this photoenzyme catalyzes the photoreduction of Pchlide to chlorophyllide *a* (Chlide), an important light-requiring step in plant greening (reviewed by Schulz and Senger 1993; Fujita 1996; Schoefs and Bertrand 1996). Three major Pchlide forms with different spectroscopic properties have been found in vivo. Two photoactive forms with −196 °C absorbance and fluorescence maxima at 650 and 655 nm ($Pchlide_{650–655}$) and 638 and 645 nm ($Pchlide_{638–645}$), respectively, have been suggested to correspond to different aggregation states of the enzyme-substrates complex (Ryberg et al. 1992). A single millisecond flash triggers the complete phototransformation of these two forms. A third spectral form, $Pchlide_{628–632}$, [presumably a mixture of Pchlide and protochlorophyll (Pchl)] is nonphotoactive and usually present in minor amounts in etiolated leaves.

The first studies on Pchlide phototransformation in vitro were carried out by Krasnovsky and Kosobutskaya (1952) and by Smith and Benitez (1953) who isolated a soluble photoactive Pchlide-protein complex from etiolated bean leaves, the so-called Pchlide-holochrome. Boardman (1966) characterized the holochrome as a particle of 600–1000 kDa. By using Triton X-100 as detergent, Schopfer and Siegelman (1968) prepared a holochrome of an estimated molecular weight of 600 kDa from etiolated bean leaves. Subsequent use of saponin as detergent led to the isolation of a solubilized photoactive Pchlide-protein complex of only 63 kDa from etiolated barley. The same procedure applied to etiolated bean holochrome gave a preparation of 100 kDa or greater (Henningsen and Kahn 1971). The subunit of bean Pchlide holochrome was first identified as a pigment-binding polypeptide of 45 kDa by SDS-PAGE (Canaani

**Present address*: Biomembrane laboratory, University of South Bohemia, Branisovska 31, 370 05 Ceske Budejovice, Czech Republic

Abbreviations: Chlide = chlorophyllide *a*; DM = *n*-dodecyl-β-D-maltoside; EM = etioplast membrane; Pchlide = protochlorophyllide *a*; $Pchlide_{X-Y}$ = protochlorophyllide *a* with −196 °C absorbance and fluorescence maxima at X and Y nm respectively; PLB = prolamellar body; POR = NADPH:protochlorophyllide oxidoreductase; PT = prothylakoid

Correspondence to: F. Franck; E-mail: F. Franck@ulg.ac.be; Fax: 32(4)3662926

and Sauer 1977). Apel et al. (1980) and Beer and Griffiths (1981) later purified the photoenzyme POR as a 36-kDa polypeptide. Recent studies have demonstrated that two nuclear genes code for two POR photoenzymes (POR-A and POR-B) with high homology and close molecular weight in barley (Holtorf et al. 1995) and in Arabidopsis (Armstrong et al. 1995).

The molecular structure of the native photoactive POR complexes that accumulate in dark-grown leaves is largely unknown. From studies on pigment-pigment interactions by circular dichroism (Böddi et al. 1989), on excitation energy migration (Thorne 1971; Sironval 1972) and from cross-linking experiments (Wiktorsson et al. 1993), the general concept emerged that $Pchlide_{650–655}$ consists of an aggregate of several POR subunits that binds several Pchlide molecules (Ryberg et al. 1992; Sundqvist and Dahlin 1997). The $Pchlide_{638–645}$ complex would represent a less-aggregated form of the complex that can easily be derived from $Pchlide_{650–655}$ through detergent solubilization or, more generally, by chaotropic agent (Butler and Briggs 1961; Franck et al. 1994). In order to gain more insight into the structure of the photoactive complexes, biochemical procedures are needed for their isolation in a pure and active state. Classical holochrome preparations derived from crude homogenates of etiolated leaves do not meet these criteria since they contain polypeptides other than POR (Canaani and Sauer 1977; Dujardin et al. 1987). In recent studies, purified POR (either native POR from etiolated leaves or the enzyme expressed in *Escherichia coli*) catalyzed Pchlide photoreduction under weak continuous light or repeated flashes (Knaust et al. 1993; Holtorf et al. 1995; Birve et al. 1996; Martin et al. 1997). In such preparations, however, the reconstitution of a single-flash-transformable photoactive complex with spectroscopic properties similar to those of native complexes was not reported.

The aim of the present study was to design a procedure to isolate flash-transformable photoactive Pchlide-POR complexes from wheat etioplasts. In a previous report we have shown that the non-ionic detergent dodecyl-maltoside can be used to solubilize inner etioplast membranes with little loss of photoactivity (Franck et al. 1994). Here we report on the spectroscopic properties, photoactivity and polypeptide content of Pchlide-POR complexes obtained using this detergent and further purified by gel filtration. The pigment content throughout the isolation procedure was analyzed by reversed-phase HPLC.

Materials and methods

Plant material. Seeds of wheat (*Triticum aestivum*. L. cv. Minaret, Clovis-Maton, Avelgem-Kerkhove, Belgium) were germinated on vermiculite and tap water for 6–7 d in darkness at 22 °C.

Isolation of etioplast membranes. Approximately 200 g of etiolated leaves of wheat were homogenized in a MX 322 mixer (Braun, Melsungen, Germany) with 900 (300 × 3) ml of extraction buffer containing 0.4 M sorbitol, 25 mM Hepes-NaOH (pH 7.5), 1 mM $MgCl_2$, 1 mM EDTA. The homogenate was filtered through four layers of gauze and one layer of nylon mesh (ϕ = 31.5 μm) and centrifuged at 500 *g* for 5 min to sediment nuclei, unbroken cells and cell debris. Etioplasts were sedimented from the supernatant by acceleration to 2100 *g* for 10 min. The etioplast pellet was resuspended in 2 ml of extraction buffer, to which were added 25 ml of hypotonic buffer containing 25 mM Hepes-NaOH (pH 7.5), 1 mM $MgCl_2$, 1 mM EDTA and 0.25 mM NADPH. Etioplast membranes were sedimented by centrifugation at 7000 *g* for 7 min. The pellet was washed once with 15 ml of hypotonic buffer.

Preparation and solubilization of PLBs. The procedure for preparation of an enriched PLB membrane material was based on the separation of PLBs and PTs by sonication (Wellburn and Hampp 1979). Etioplast membranes were resuspended in 5 ml of extraction buffer supplemented with 20% glycerol (v/v) and 1 mM NADPH, and were sonicated twice for 5 s in a Sonics VC 375 sonicator (Sonics, Danbury, Conn., USA), with a 3-mm-diameter probe at amplitude 5 and frequency of 12.5 kHz. The PLB-enriched material was sedimented by centrifugation at 3000 *g* for 10 min at 5 °C. The pellet was resuspended in 4 ml of extraction buffer suplemented with 0.5 mM *n*-dodecyl-β-D-maltoside (DM) and 1 mM NADPH and kept on ice for 15 min. Insoluble material was removed by centrifugation at 13 000 *g* for 10 min. For gel filtration, 2.8 ml of the supernatant was used.

Gel filtration of solubilized POR complexes. The isolation of the solubilized POR-Pchlide complexes was carried out by gel filtration (Sephacryl S-400 packed in a column 25 cm long, 1.5 cm i.d.). Sephacryl S-400 (Pharmacia) was selected in view of the relatively high-molecular-weight values of Pchlide holochrome reported in the literature (Schopfer and Siegelman 1968). The column was equilibrated with the filtered elution buffer containing 25 mM Hepes-NaOH, (pH 7.5), 5% sorbitol, 1 mM $MgCl_2$, 1 mM EDTA, 0.1 mM NADPH and 0.1 or 0.5 mM DM by allowing 120 ml to pass through before sample application. Elution buffer was delivered at a rate of 24 ml/h by a peristaltic pump and the eluate was collected in 1.5-ml fractions. The column was calibrated using the following proteins: carbonic anhydrase (29 kDa), aldolase (158 kDa), catalase (232 kDa), ferritine (440 kDa), thyroglobulin (669 kDa). The linear relationship between the partition coefficient and the logarithm of molecular weight (correlation coefficient: 0.982) was used for apparent-molecular-weight estimations.

Analysis by SDS-PAGE. Proteins were precipitated by incubation with 80% acetone at −20 °C for at least 1 h followed by centrifugation at 6500 *g* for 15 min at 5 °C. The pellet was washed once with cooled acetone. Samples were incubated with 0.06 M Tris-HCl (pH 6.8) buffer containing 20% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 2% (w/v) SDS and 0.0025% (w/v) bromophenol blue. Before application to the gel, the samples were heated to 100 °C for 2 min and cooled on ice, then the insoluble material was removed by centrifugation at 13 000 *g* at 5 °C for 10 min. The SDS-PAGE was performed according to Laemmli (1970) with minor modifications in 10.5-cm gel casts. Stacking gels with 4% (w/v) acrylamide and 0.1% (w/v) bisacrylamide, and separating gels with 12% (w/v) acrylamide and 0.3% (w/v) bisacrylamide were used. The gels were stained for 20 min in 0.25% Coomassie Brilliant Blue R-250 (w/v), 5% methanol (v/v) and 7.50% acetic acid (v/v), and destained overnight in 5% methanol (v/v), 7.50% acetic acid (v/v) by diffusion facilitated by gentle shaking. As reference proteins, the SDS-PAGE molecular-weight standards, low range (Merck), were used (cytochrome *c*, 12.38 kDa; myoglobin, 16.95 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 42.70 kDa; albumin, 66.25 kDa; ovotransferrin, 78 kDa).

Low-temperature spectroscopy. Fluorescence emission spectra were recorded under 440 nm excitation at −196 °C using a spectrofluorometer (LS50-B; Perkin Elmer, Bucks, UK). The excitation and emission slits were 10 and 5 nm, respectively. The spectra were corrected for the baseline and the wavelength-dependent sensitivity

of the fluorometer. Absorption spectra were recorded at −196 °C using an optical multichannel analyzer (OMA2; EG&G Princeton Applied Research, Princeton, N.J., USA) according to Franck and Strzalka (1992).

Flash illumination. The samples, stored at −196 °C, were warmed up to −20 °C for 10 min and irradiated by a single 1-ms saturating polychromatic flash (photographic flash Multiblitz 50, Multiblitzgerate, Porz-Westhoven, Germany). They were immediately frozen again at −196 °C. This procedure allows only enzyme-bound Pchlide to be phototransformed and fluorescence or absorbance spectra of the same sample to be compared before and after phototransformation.

Pigment analysis. Routine Pchl(ide) and Chlide quantification was made by measurements of room-temperature fluorescence emission spectra of 80% acetone extracts as in Sperling et al. (1998). For reverse-phase HPLC the pigments were extracted with HPLC-grade methanol (UCB, Leuven, Belgium). The extract was clarified by centrifugation (50 000 *g*, 4 °C, 10 min). The supernatent was then dried under a stream of nitrogen. When they were not immediately used for HPLC analysis, the dried pigments were stored in darkness under nitrogen at −20 °C according to Bertrand and Schoefs (1996). The pigment separations were performed with a reversed-phase column (particle size: 4.65 μm; 250 mm long, 4.6 mm i.d.; Zorbax, Rockland Technologies, Newport, Del. USA) according to Schoefs et al. (1995).

Total protein determination. Total protein concentration was measured according to Bradford (1976) with bovine serum albumin as standard.

Results

Solubilization of photoactive POR complexes from etioplast membranes. The −196 °C fluorescence spectrum of crude etioplast membranes (EMs) exhibited two emission maxima at 633 nm and at 656 nm originating from nonphotoactive $Pchlide_{628–633}$ and from photoactive $Pchlide_{650–655}$, respectively (Fig. 1). In the following, the ratio of the emission intensity measured at the two maxima (F656/F633) was used as an indicator of the relative amount of photoactive (flash-transformable)

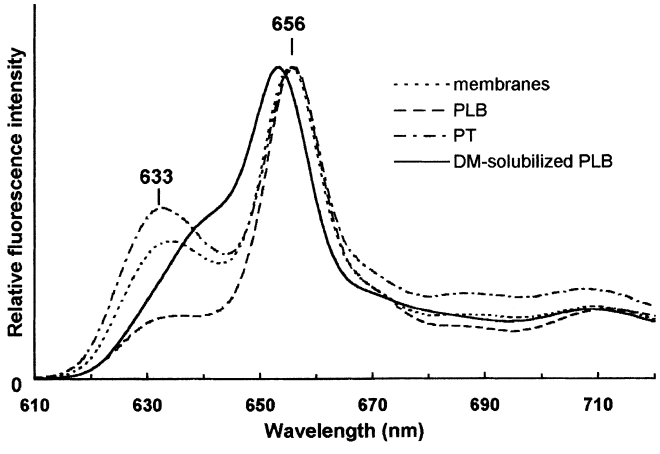

Fig. 1. Emission spectra at −196 °C of crude EMs, of PLB- and PT-enriched fractions after sonication, and of PLBs solubilized with 0.5 mM DM

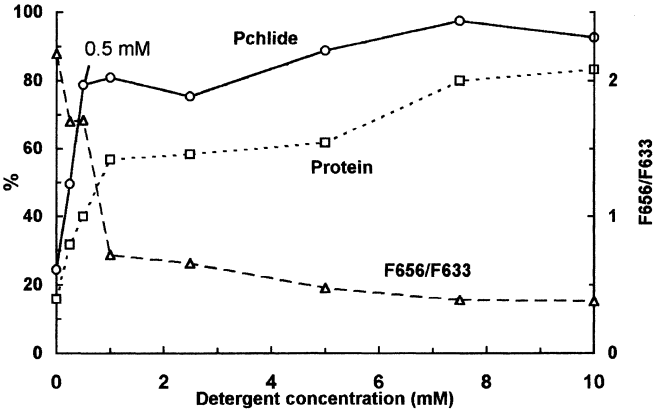

Fig. 2. Effect of DM concentration during a 15-min solubilization step on the F656/F633 ratio and on the protein and Pchlide concentrations found in the soluble fraction (as a percentage of their concentrations in PLBs before solubilization)

Pchlide at successive steps of the isolation of POR complexes. In agreement with previous studies (Böddi et al. 1989), a comparison of the emission spectra of EMs, PTs and PLBs (Fig. 1) showed that the F656/F633 ratio was lower in PTs (1.8), and higher in PLBs (5.0), than in crude membranes (2.2). Therefore, the PLB fraction was used for further solubilization and purification of POR complexes.

Different concentrations of DM were tested to solubilize POR complexes in photoactive form from the PLB-enriched fraction. Figure 2 shows the effect of the DM concentration on the F656/F633 ratio and on the total protein and Pchlide concentration (expressed as percent of the initial protein and Pchlide concentrations in PLBs) after detergent treatment and centrifugation. The F656/F633 ratio decreased progressively at DM concentrations higher than 0.5 mM, leading to the almost complete disappearance of the 656-nm fluorescence band with 7.5 mM DM. The highest F656/F633 ratio (1.7) was obtained with 0.5 mM DM, when protein solubilization was only partial (40%). At the same detergent concentration, around 80–90% Pchlide was found in the supernatant after solubilization. We therefore suspected that a specific solubilization of POR by DM occurs when the detergent is used at relatively low concentration. Nonphotoinactive $Pchlide_{628–633}$ was only present as a shoulder in the −196 °C fluorescence spectrum of solubilized PLBs (Fig. 1). The F656/F633 ratio was stable for at least 3 h at 0 °C (data not shown).

The selective effect of DM on the solubilization of POR was confirmed by SDS-PAGE (Fig. 3). Our PLB preparation showed a prominant 36-kDa band of POR, together with several other EM proteins. Because pure PLBs have been reported to contain mainly POR as integral protein (Dehesh and Ryberg 1985), this result may indicate that the PLB preparation method used here leads to some contamination by other membrane material. The 36-kDa band of POR became predominant after solubilization by DM, which confirms the selective effect of this detergent on POR solubilization.

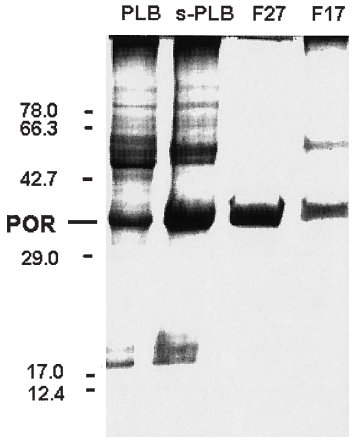

Fig. 3. Analysis by SDS-PAGE of PLBs (*PLB*), solubilized PLBs (*s-PLB*), and fractions 27 (*F27*) and 17 (*F17*) obtained after gel chromatography on Sephacryl S-400 performed with 0.5 or 0.1 mM DM, respectively

Gel filtration of solubilized POR complexes. The elution profiles of total protein and Pchlide (as a percentage of the amounts measured before gel filtration) obtained with 0.5 mM DM in the elution buffer are shown in Fig. 4A. A broad protein distribution was observed, with a maximum in the 27th fraction. The elution profile of Pchlide was more narrow and had a maximum in the same fraction. According to the column calibration, the 27th fraction corresponded to a molecular weight of 112 ± 24 kDa. A similar value was obtained in a similarly run experiment using Sephacryl S-200 (range 5–250 kDa) instead of Sephacryl S-400. Analysis of the polypeptide content of this fraction by SDS/PAGE showed that it contained POR with an apparent molecular weight of 36 kDa and in a practically pure form (Fig. 3). The −196 °C emission spectrum showed a broad band at 643 nm resulting from the overlapping of the 633-nm emission band of nonphotoactive Pchlide and a 643-nm emission band of photoactive, flash-transformable Pchlide (Fig. 5A). The presence of a 643-nm band of photoactive Pchlide was evident from the 'flash minus dark' difference spectrum showing a minimum at that wavelength (Fig. 5A, inset). The emission maximum of Chlide after one flash was at 683 nm. The position of the nonphotoactive Pchlide band appeared clearly at 633 nm in the emission spectrum after a flash. The ratio of quantitatively determined photoactive (flash-transformable) to total Pchlide was 0.55 in this fraction.

In the attempt to isolate photoactive POR complexes complexes in the native state, i.e. having a fluorescence maximum around 655 nm, PLBs solubilized with 0.5 mM DM were chromatographed in the same conditions as above except that the DM concentration in the elution buffer was reduced to 0.1 mM. The total protein and Pchlide elution profiles obtained under these new conditions showed a maximum in the 17th fraction, corresponding to a molecular weight of approx. 1080 ± 250 kDa. This value is in the same range as that previously published for Pchlide holochrome (Boardman 1966). The SDS/PAGE of this fraction

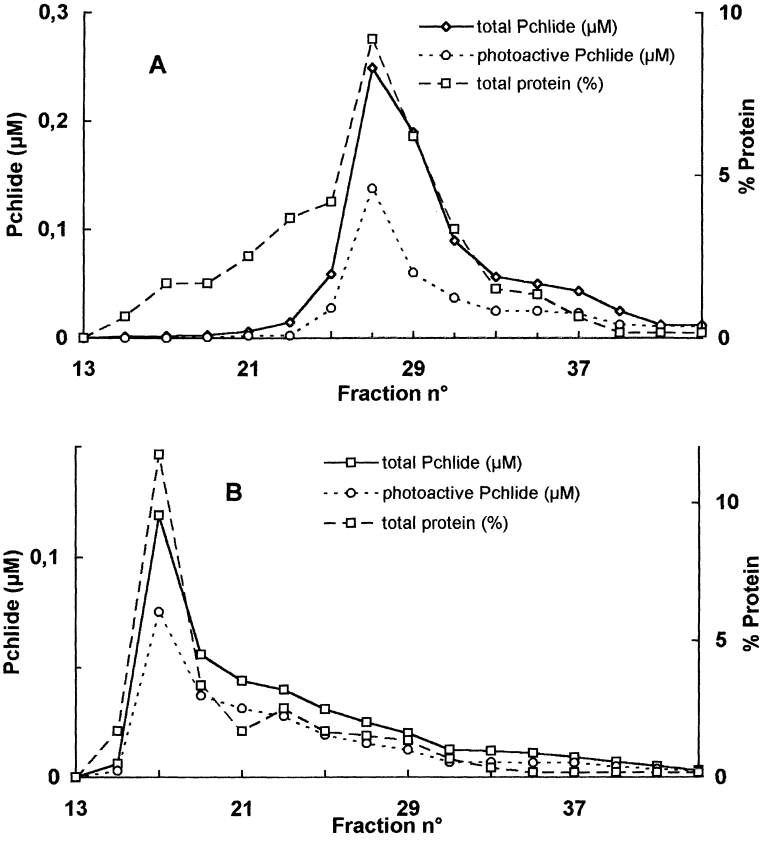

Fig. 4A,B. Sephacryl S-400 gel chromatography of DM-solubilized PLBs: elution profiles of total Pchlide, of photoactive Pchlide and of total protein (as a percentage of total protein concentration in DM-solubilized PLBs). Gel chromatography was performed with 0.5 (**A**) or 0.1 (**B**) mM DM in the elution buffer. The volume of each fraction was 1.5 ml. The void volume corresponds to the first 12 fractions

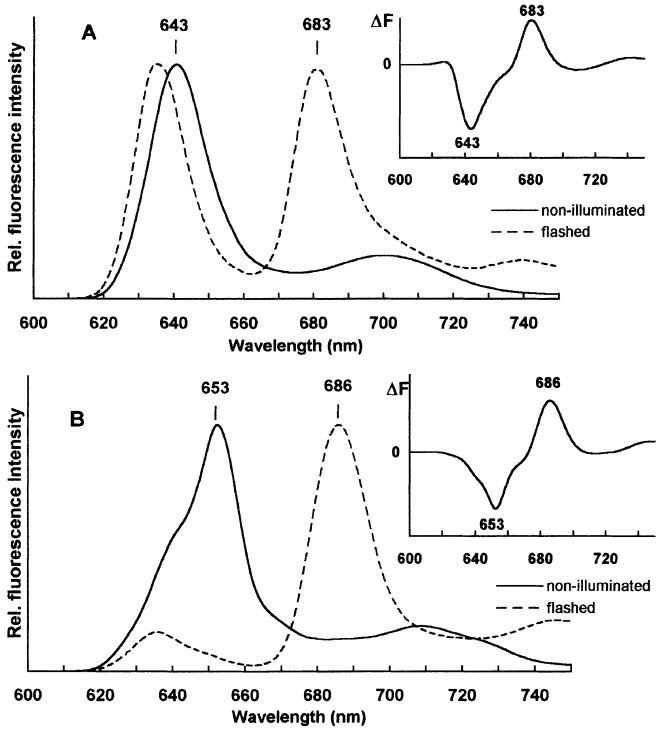

Fig. 5A,B. Emission spectra at −196 °C (normalized at their maximum) of the F27 (**A**) and F17 (**B**) fractions after gel chromatography of DM-solubilized PLBs on Sephacryl S-400 performed with 0.5 or 0.1 mM DM in the elution buffer. Spectra were recorded before (*full line*) or after (*dashed line*) a single 1-ms light flash. Insets, difference 'flashed minus unflashed' spectra obtained after normalization on the non-photoactive Pchlide emission intensity around 630 nm

showed POR as predominent polypeptide together with some contaminants of higher molecular weights (Fig. 3). The −196 °C emission spectrum exhibited a major fluorescence band at 653 nm and a shoulder around 640 nm (Fig. 5B). The F656/F633 ratio was stable at least for 7 h at 0 °C (data not shown). The effect of a 1-ms flash showed that the photoconvertibility was high since almost all the fluorescence at 640 or 653 nm disappeared (Fig. 5B). The emission maximum after the flash was found at 686 nm. A weak emission band at 635 nm from photoinactive Pchlide persisted after the flash. The ratio of quantitatively determined photoactive (flash-transformable) to total Pchlide was 0.70 in this fraction.

The spectroscopic properties of photoactive POR complexes isolated by gel filtration with 0.5 or 0.1 mM DM were further analyzed by −196 °C absorbance spectroscopy (Fig. 6). 'Flash-minus-dark' absorbance spectra showed that the photoactive complex emitting at 643 nm (obtained with 0.5 mM DM) had a main absorbance band at 640 nm and a weak shoulder around 650 nm. The photoactive complex emitting at 653 showed a main band at 650 nm and a weak shoulder around 640 nm. In both preparations, the primary Chlide product after one flash absorbed around 678 nm.

Effect of one light flash on the elution properties of POR. Previously published results suggest that POR complexes undergo rapid disaggregation after Pchlide photoreduction by a short light pulse (Wiktorsson et al. 1992). In order to investigate this process we separated the pigment protein complexes after flash irradiation of solubilized PLBs. With 0.1 mM DM in the elution buffer, the elution profile of total proteins after one flash (Fig. 7) showed an additional peak when compared to the elution profile without flash (compare Figs. 4 and 7). In the irradiated sample, two elution maxima of Chlide and protein were found in the 15th and the 25–27th fraction. Figure 8 shows the SDS-PAGE polypeptide pattern of these two fractions from the same experiment. Although the 15th fraction was enriched in POR protein when the flash was omitted, POR was only present in traces in this fraction in the flashed sample. The NADPH: protochlorophyllide oxidoreductase was found instead as a prominent polypeptide in the 27th fraction.

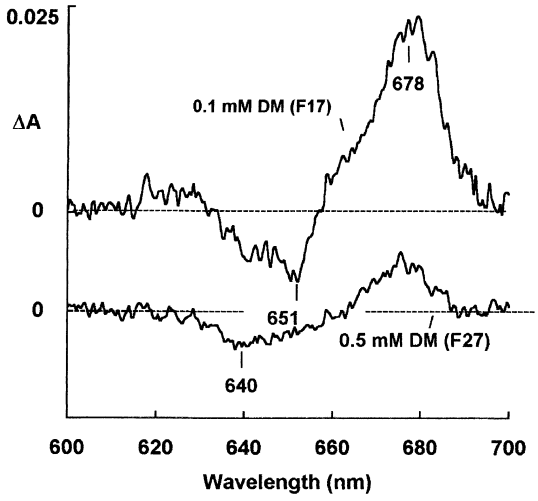

Fig. 6. Flashed minus unflashed' −196 °C absorbance difference spectra of the F27 and F17 fractions after gel chromatography of DM-solubilized PLBs on Sephacryl S-400 performed with 0.5 or 0.1 mM DM in the elution buffer. The optical pathlength was 2 mm

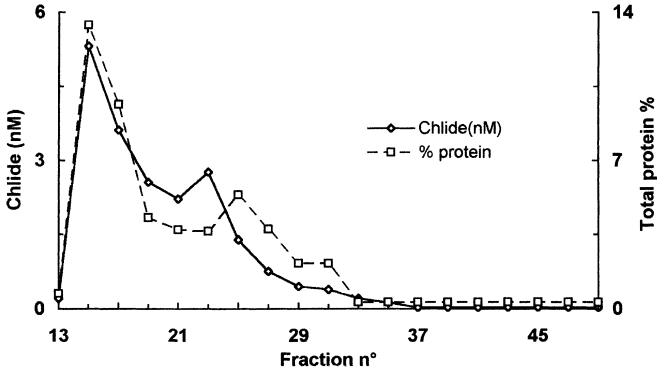

Fig. 7. Sephacryl S-400 gel chromatography of DM-solubilized PLBs irradiated by a 1-ms flash: elution profiles of Chlide and of total protein (as a percentage of total protein concentration in DM-solubilized PLBs). Gel chromatography was performed with 0.1 mM DM in the elution buffer

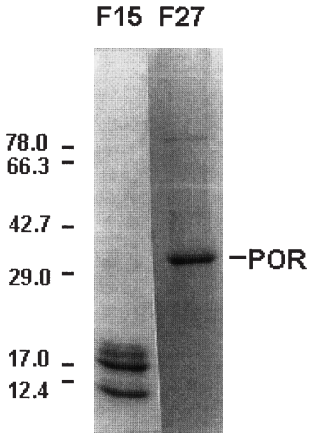

Fig. 8. Analysis by SDS-PAGE of the F15 and F27 fractions after Sephacryl S-400 gel chromatography of flashed, DM-solubilized PLBs with 0.1 mM DM in the elution buffer

Analysis by HPLC of pigment composition during the isolation of photoactive POR complexes. In crude etioplasts the complete set of xanthophylls, β-carotene, Pchlide and the four Pchlide esters were found (Table 1). The main carotenoids were lutein (ca. 50%), violaxanthin (ca. 20%) and antheraxanthin (ca. 15%). Only weak amounts of β-carotene and zeaxanthin were found, and α-carotene was not detected. The main tetrapyrrole pigment was Pchlide (ca. 90%) whereas the main Pchlide ester was Pchlide tetrahydrogeranylgeraniol (ca. 4%). The pigment composition of DM-solubilized PLBs was very similar, except for a decrease in β-carotene. Qualitatively, photoactive POR complexes isolated by gel filtration using 0.1 or 0.5 mM DM in the elution buffer presented the same composition as DM-solubilized PLBs, except that Pchlide esters had been lost. β-carotene was too low for quantitation in the purest POR preparation obtained with 0.5 mM DM in the elution buffer. Relative amounts of antheraxanthin and zeaxanthin increased from solubilized PLBs to the purest POR fraction (F17). The variations in other carotenoids did not seem to reflect specific association with POR complexes.

Table 1. Analysis by HPLC of chlorophyll precursors and carotenoids in crude EMs, solubilized PLBs, and POR-enriched F17 and F27 fractions after gel chromatography with 0.1 or 0.5 mM DM in the elution buffer, respectively. Pigment contents are expressed as a percentage of Pchlide (NQ, not quantified, traces). GG, geranylgeraniol; DHGG, dihydrogeranylgeraniol; THGG, tetrahydrogeranylgeraniol

	Etioplasts	Solub. PLBs	F17	F27
trans-Neoxanthin	21.8	21.2	90.8	16.0
trans-Violaxanthin	106.7	112.3	88.6	214.1
Lutein-5,6-epoxide	10.3	9.9	24.9	17.2
Antheraxanthin	83.4	79.0	117.2	147.0
Lutein	222.6	221.5	529.6	313.1
Zeaxanthin	26.5	20.0	33.6	61.4
β-Carotene	6.1	3.8	6.5	NQ
Pchlide	100	100	100	100
Pchl-GG	3.0	2.5	0	0
Pchl-THGG	4.8	4.0	0	0
Pchl-DHGG	2.4	2.4	0	0

Discussion

The major form of photoactive Pchlide-POR complex in vivo, $Pchlide_{650–655}$, is thought to be an aggregate of several POR subunits housing several Pchlide molecules (Sundqvist and Dahlin 1997). The number of subunits is unknown. From excitation energy migration studies, an energy transfer unit of 18 Pchlide molecules was calculated (Sironval 1972). Although this value has only a statistical meaning, it indicates the grouping of several POR subunits since two to three Pchlide molecules per POR enzyme molecule were found (Apel et al. 1980). The $Pchlide_{650–655}$ holocomplex is expected to be easily disaggregated by detergents. The in vivo $Pchlide_{638–645}$ form is a photoactive complex which was suggested to be less aggregated than $Pchlide_{650–655}$ (Ryberg et al. 1992). Since reconstitution studies have recently indicated that POR activity involves the formation of POR dimers (Martin et al. 1997), one can suggest that $Pchlide_{638–645}$ represents the native substrates-POR dimer in vivo.

In this work $Pchlide_{650–653}$ or $Pchlide_{640–643}$ POR-complexes were obtained from solubilized PLBs by gel chromatography depending on DM concentration in the elution buffer. The high apparent molecular weight (1080 ± 250 kDa) of the $Pchlide_{650–655}$ complex obtained with 0.1 mM DM suggests that it represents small PLB fragments. The polypeptide content of this fraction showed little contamination by polypeptides other than POR, which indicates grouping of the POR holocomplex in the PLB. The $Pchlide_{640–643}$ complex obtained with 0.5 mM DM in elution buffer showed an apparent molecular weight of 112 ± 24 kDa. This value is probably somewhat overestimated due to detergent binding and is compatible with a dimeric nature of $Pchlide_{640–643}$. In this fraction no polypeptide other than POR was detected by SDS-PAGE.

The effect of a pre-flash on the elution properties of POR protein found here confirms the rapid disaggregation of the holocomplex after Pchlide photoreduction (Wiktorsson et al. 1992). Furthermore, the fact that the same POR elution peak in the 110-kDa region was obtainied either from a flashed sample at low detergent concentration, or from an unflashed sample at high detergent concentration suggests that the 110-kDa POR complex is a functional sub-unit that arises either from detergent dissociation of Pchlide-containing complexes or from spontaneous dissociation of less-stable Chlide-containing complexes.

The pigment composition of wheat etioplasts and of DM-solubilized PLBs found in this study is qualitatively similar to that found by others in etiolated leaves of various plants (Lütz 1981; Barry et al. 1991; Schoefs et al. 1995). However, the proportion of esterified Pchlide is different from that found in bean (data not shown) and

cucumber (Shioi and Sasa 1983) but similar to that found by others in wheat EMs (McEwen and Lindsten 1992). The presence of carotenoids in PLBs is documented by the work of Bahl (1977) and confirmed by our study. Until now, no evidence for an association of any carotenoid to POR has been found. An absence of excitation migration from carotenoid to photoactive Pchlide in vivo was reported (Ignatov et al. 1993). Moreover, the flash-induced formation of a long-lived Chlide triplet in crude extracts of etiolated leaves did not support Chlide protection against photooxidation by carotenoids (Franck and Mathis 1980). Our HPLC analysis suggests, however, that antheraxanthin and zeaxanthin (two pigments involved in the xanthophyll cycle) are associated with POR complexes. Further studies are, however, needed to exclude the possibility of unspecific pigment binding.

In conclusion, the methods used here provide a rapid procedure for the isolation of substrate-POR complexes which bear the properties of truly photoactive complexes, as the effect of a single 1-ms light flash demonstrates. The $Pchlide_{640–643}$ complex obtained by this method would be useful for spectroscopic as well as reconstitution studies aimed at understanding the process of $Pchlide_{650–655}$ assembly.

This work was supported by the National Fund of Scientific Research of Belgium (grant FRFC n° 2.4523.95). The authors are grateful to Prof Y. Lemoine (University of Lille, France) for his cooperation in the HPLC analysis.

References

Apel K, Santel J-L, Redlinger TE, Falk H (1980) The protochlorophyllide holochrome of barley (Hordeum vulgare): isolation and characterization of the NADPH-protochlorophyllide oxidoreductase. Eur J Biochem 111: 251–258

Armstrong GA, Runge S, Frick G, Sperling U, Apel K (1995) Identification of NADPH:protochlorophyllide oxidoreductases A and B: a branched pathway for light-dependent chlorophyll biosynthesis in Arabidopsis thaliana. Plant Physiol 108: 1505–1517

Bahl J (1977) Chlorophyll, carotenoid, and lipid content in *Triticum sativum* L. Plastid envelopes, prolamellar bodies, stroma lamellae, and grana. Planta 136: 21–24

Barry P, Young AJ, Britton G (1991) Accumulation of pigments during the greening of etiolated seedlings of *Hordeum vulgare* L. J Exp Bot 42: 229–234

Beer NS, Griffiths WT(1981) Purification of the enzyme NADPH: protochlorophyllide oxydoreductase. Biochem J 195: 83–92

Bertrand M, Schoefs B (1996) Working with photosynthetic pigments: problems and precautions. In: Pessaracli M (ed) Handbook of photosynthesis. Marcel Dekker, New York Basel Hong Kong, pp 151–172

Birve SJ, Selstam E, Johansson BÅ (1996) Secondary structure of NADPH: protochlorophyllide oxidoreductase examined by circular dichroism and prediction methods. Biochem J 317: 549–555

Boardman NK (1966) Protochlorophyll. In: Vernon LP, Seeley GR (eds) The chlorophylls. Academic Press, New York, pp 437–479

Böddi B, Lindsten A, Ryberg M, Sundqvist C (1989) On the aggregational states of protochlorophyllide and its protein complexes in wheat etioplasts. Physiol Plant 76: 135–143

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Butler WI, Briggs WR (1961) The relation between structure and pigments during the first stages of proplastid greening. Biochim Biophys Acta 112: 45–53

Canaani OD, Sauer K (1977) Analysis of subunit structure of protochlorophyllide holochrome by sodium dodecyl sulfate-polyacrilamide gel electrophoresis. Plant Physiol 60: 422–429

Dehesh K, Ryberg M (1985)The NADPH-protochlorophyllide oxidoreductase is the major protein constituent of prolamellar bodies in wheat (*Triticum aestivum* L.). Planta 164: 396–399

Dujardin E, Bertrand M, Radunz A, Schmid GH (1987) Immunological evidence for the presence of proteins of the photosynthetic membrane in etiolated leaves of *Phaseolus vulgaris*. J Plant Physiol 128: 95–107

Franck F, Mathis P (1980) A short-lived intermediate in the photoenzymatic reduction of protochlorophyll(ide) into chlorophyll(ide) at a physiological temperature. Photochem Photobiol 32: 799–803

Franck F, Strzalka K (1992) Detection of the photoactive protochlorophyllide-protein complex in the light during the greening of barley. FEBS Lett 309: 73–77

Franck F, Ouazzani MA, Dujardin E, Popovic R (1994) Optical multichannel analysis of protochlorophyllide phototransformation in detergent-solubilized etioplast membranes of wheat. Z Naturforsch 49c: 125–131

Fujita Y (1996) Protochlorophyllide reduction: a key step in the greening of plants. Plant Cell Physiol 37: 411–421

Griffiths WT (1978) Reconstitution of chlorophyllide formation by isolated etioplast membranes. Biochem J 174: 681–692

Henningsen KW, Kahn A (1971) Photoactive subunits of protochlorophyll(ide) holochrome. Plant Physiol 47: 685–690

Holtorf H, Reinbothe S, Reinbothe C, Bereza B, Apel K (1995) Two routes of chlorophyllide synthesis that are differentially regulated by light in barley (*Hordeum vulgare* L.). Proc Natl Acad Sci USA 92: 3254–3258

Ignatov NV, Belyaeva OB, Litvin FF (1993) Possible role of a flavin component of protochlorophyllide-protein complexes in primary processes of protochlorophyllide photoreduction in etiolated plants. Photosynthetica 29: 235–241

Knaust R, Seyfried B, Schmidt L, Schutz R, Senger H (1993) Phototransformation of monovinyl and divinyl protochlorophyllide oxidoreductase of barley expressed in *Escherichia coli*. J Photochem Photobiol B: Biol. 20: 161–166

Krasnovsky AA, Kosobutskaya LM (1952) Spectral investigations of chlorophyll during its formation in plants and in colloidal solutions of material from etiolated leaves. Dokl Akad Nauk SSSR 85: 177–180

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature 227: 680–685

Lütz C (1981) Distribution of carotenoids and lipids in separated prolamellar bodies and prothylakoids of etioplasts from *Avena sativa* L. J Plant Physiol 104: 43–52

Martin GEM, Timko MP, Wilks HM (1997) Purification and kinetic analysis of pea (*Pisum sativum* L.) NADPH:protochlorophyllide oxidoreductase expressed as fusion with maltose-binding protein in *Escherichia coli*. Biochem J 325: 139–145

McEwen B, Lindsten A (1992) Characterization of protochlorophyllide and protochlorophyllide esters in roots of dark-grown plants. Physiol Plant 84: 343–350

Ryberg M, Artus N, Boddi B, Lindsten A, Wiktorsson B, Sundqvist C (1992) Pigment-protein complexes of chlorophyll precursors. In: Argyroudi -Akoyunoglouh JH (ed) Regulation of chloroplast biogenesis. Plenum Press, New York, pp 217–224

Schoefs B, Bertrand M (1996) Chlorophyll synthesis. In: Pessaracli M (ed) Handbook of photosynthesis. Marcel Dekker, New York Basel Hong Kong, pp 49–69

Schoefs B, Bertrand M, Lemoine Y (1995) Separation of photosynthetic pigments and their precursors by high performance

liquid chromatography using a photodiode array detector. J Chromatography A 692: 239–245
Schopfer P, Siegelman HW (1968) Purification of protochlorophyllide holochrome. Plant Physiol 43: 990–996
Schulz R, Senger H (1993) Protochlorophyllide reductase: a key enzyme in the greening process. In: Sundqvist C, Ryberg M (eds) Pigment-protein complexes in plastids: Synthesis and assembly. Academic Press, San Diego, pp 179–218
Shioi Y, Sasa T (1983) Formation and degradation of protochlorophylls in etiolated and greening cotyledons of cucumber. Plant Cell Physiol 24: 835–840
Sironval C (1972) The reduction of protochlorophyllide into chlorophyllide VI. Calculation of the size of the transfer unit and the initial quantum yield of the reduction in vivo. Photosynthetica 6: 375–380
Smith JHC, Benitez A (1953) The protochlorophyll-chlorophyll transformation: the nature of protochlorophyll in leaves. Carnegie Inst Washington Year book 52: 151–153
Sperling U, Franck F, Van Cleve B, Frick G, Apel K, Armstrong GA (1998) Etioplast differentiation in *Arabidopsis*: both PORA and PORB restore the prolamellar body membrane and photoactive protochlorophyllide-F655 to the *cop1* photomorphogenic mutant. Plant Cell 10: 283–296
Sundqvist C, Dahlin C (1997) With chlorophyll from prolamellar bodies to light-harvesting complexes. Physiol Plant 100: 748–759
Thorne SW (1971) The greening of etiolated bean leaves. I. The initial photoconversion process. Biochim Biophys Acta 226: 113–127
Wellburn AR, Hampp R (1979) Appearance of photochemical functions in prothylakoids during plastid development. Biochim Biophys Acta 547: 380–397
Wiktorsson B, Ryberg M, Gough S, Sundqvist C (1992) Isoelectric focusing of pigment-protein complexes solubilized from non-irradiated prolamellar bodies. Physiol Plant 85: 659–669
Wiktorsson B, Engdahl S, Zhong LB, Böddi B, Ryberg M, Sundqvist C (1993) The effect of cross-linking of the subunits of NADPH-protochlorophyllide oxidoreductase on the aggregational state of protochlorophyllide. Photosynthetica 29: 205–218