Plant Physiol. (1993) 102: 821-828

Identification of the Main Species of Tetrapyrrolic Pigments in Envelope Membranes from Spinach Chloroplasts

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The chlorophyll precursors protochlorophyllide and chlorophyllide were identified in purified envelope membranes from spinach (*Spinacia oleracea*) chloroplasts. This was shown after pigment separation by high performance liquid chromatography (HPLC) using specific fluorescence detection for these compounds. Protochlorophyllide and chlorophyllide concentrations in envelope membranes were in the range of 0.1 to 1.5 nmol/mg protein. Chlorophyll content of the envelope membranes was extremely low (0.3 nmol chlorophyll *a*/mg protein), but the molar ratios of protochlorophyllide and chlorophyllide to chlorophyll were 100 to 1000 times higher in envelope membranes than in thylakoid membranes. Therefore, envelope tetrapyrrolic pigments consist in large part (approximately one-half) of nonphytylated molecules, whereas only 0.1% of the pigments in thylakoids are nonphytylated molecules. Clear-cut separation of protochlorophyllide and chlorophyllide by HPLC allowed us to confirm the presence of a slight protochlorophyllide reductase activity in isolated envelope membranes from fully developed spinach chloroplasts. The enzyme was active only when envelope membranes were illuminated in the presence of NADPH.

We have previously proposed that envelope membranes from spinach (*Spinacia oleracea*) chloroplasts contain small amounts of Chl precursors such as Pchlide and Chlide (Pineau et al., 1986). This hypothesis was supported by fluorescence data, including properties of the fluorescent signals that were obtained from purified whole envelope membranes with and without solvent extraction (emission and excitation spectra, pigment polarity properties). Preliminary results suggest that Pchlide photoconversion occurred in the presence of NADPH in purified envelope membranes (Pineau et al., 1986; Joyard et al., 1990). Indeed, using specific antibodies, NADPH-Pchlide oxidoreductase (EC 1.6.99.1) has been identified in envelope preparations from spinach chloroplasts as a minor polypeptide of 37,000 D (Joyard et al., 1990).

Until now, however, tetrapyrrolic pigments that are present in purified envelope membranes from higher plant chloroplasts had not been physically separated, and thus were not accurately identified, due to their low amount in these scarce membranes compared with their abundance in thylakoids. Therefore, the purpose of the work described in this article is to confirm the presence of tetrapyrrolic pigments in envelope membranes and to identify them by HPLC separation of pigment extracts, using specific fluorimetric detection and comparison with the chromatographic features of standards. The results obtained demonstrate that Pchlide and Chlide are genuine constituents of all preparations of envelope membranes from spinach chloroplasts. Moreover, this method allowed us to confirm the ability of envelope membranes to photoconvert, in vitro, their endogenous Pchlide pool into Chlide in the presence of NADPH.

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MATERIALS AND METHODS

Purification of Envelope Membranes from Spinach Chloroplasts

Deveined spinach (*Spinacia oleracea*) leaf sections (2–3 kg) were first homogenized for 4 s at 4°C in a Waring Blendor containing 330 mM mannitol, 25 mM tetrasodium pyrophosphate, and 0.1% BSA (pH 7.8). Chloroplasts were prepared by differential centrifugation, and intact chloroplasts were then purified by centrifugation in Percoll gradients using the HS4 Sorvall rotor as described by Douce and Joyard (1982). Osmotic lysis of these chloroplasts in hypotonic medium (10 mM Tricine-NaOH, 4 mM $MgCl_2$, pH 7.8) followed by sedimentation in a discontinuous Suc gradient as first described by Douce et al. (1973) leads to the simultaneous recovery of the two chloroplast membrane fractions, envelope and thylakoids, and the soluble fraction, the stroma.

The envelope membranes were then washed in 10 mM Tricine, pH 7.8, (crude envelope fraction) and purified again by sedimentation on a gradient consisting of three layers (0.6 mL), of 0.6 M, 1 M, and 1.2 M Suc (prepared in 10 mM Tricine, pH 7.8). Centrifugation in a SW60 Ti rotor (Beckman) was for 50 min at 52,000 rpm (360,000*g*). The yellow membranes, which were recovered at the interface of the 0.6 M and 1 M layers, were withdrawn and sedimented.

The thylakoid fraction was diluted in 10 mM Tricine (pH 7.8) and further purified by sedimentation in a gradient consisting of three layers (3 mL each), of 1 M, 1.2 M, and 1.5 M Suc (prepared in 10 mM Tricine, pH 7.8). Centrifugation was for 4 h in a SW41 rotor (Beckman) at 36,000 rpm (220,000*g*). The major part of the material sedimented at the interface between 1.2 M and 1.5 M Suc layers, whereas a faint

yellowish band appeared in the upper part of the 1 M Suc layer (contaminating envelope membranes).

HPLC Separation

Tetrapyrrolic pigments were separated at 25°C by reverse-phase HPLC using a Kromasil C18 column (250 × 4.6 mm). The sample (50 μL of ethanol extract from membrane fractions) was loaded by a Rheodyne 7125 injector and eluted by a gradient of solution B (methanol:ethyl acetate, 50:50, v/v) into solution A (methanol:water, 85:15, v/v), which contained, in addition, 2.5 mM tetrabutylammonium phosphate (Pic A, Waters). The following elution scheme was provided by two pumps (Kontron 420): 0 to 1 min, 0% B; 1 to 23 min, 0 to 80% B; 23 to 26 min, 80% B; 26 to 28 min, 80 to 90% B; 28 to 35 min, 90% B. The absorption of eluent was usually recorded at 440 nm (detector Kontron 430), whereas tetrapyrrolic pigments were selectively and simultaneously detected using a fluorimeter (Hitachi 3010; excitation, 435 nm; emission, 640 or 675 nm) equipped with a flow cell (18 μL). Absorption and fluorimetric data were analyzed with the use of the Kontron data system 450.

Pchlide Reductase Activity

The envelope membrane suspension (at a protein concentration indicated in the figure legend) was incubated for various periods of time, at 20°C under illumination (0.25 W/m^2) with white light from which UV light was excluded by a J423 filter (Métallisations et Traitements Optiques, Marsy, France), in 20 mM Tricine, pH 7.8, 20% glycerol in the presence of an NADPH-regenerating system (6.25 mM Glc-6-P, 0.6 mM NADP, 0.2 units/mL Glc-6-P dehydrogenase) as recommended by Kay and Griffiths (1983). Tetrapyrrolic pigments were followed, at various times of incubation, by recording red fluorescence emission from 600 to 750 nm (under 440 nm excitation).

Decrease in Pchlide and increase in Chlide amounts were estimated after separation by HPLC of pigments from ethanolic extracts of the incubation mixture. Ethanolic extracts were prepared as follows: 50-μL aliquots were withdrawn from the assay medium at given times and pigments were extracted by addition of 500 μL of ethanol. After filtration through a Dynagard filter (Microgon, Inc.), 50 μL of this extract was injected for HPLC analyses as described above.

Preparation of Standards and Estimation of the Pigment Amounts

Pchlide was extracted from etiolated cucumber cotyledons in ammoniacal acetone as described by Rebeiz et al. (1975), and its concentration was estimated in diethyl ether using the molar extinction coefficient reported by these authors (3.56 10^4 M^{-1} cm^{-1} at 624 nm). The HPLC detector was calibrated for Pchlide using fluorescence excitation at 435 nm (bandpath, 10 nm) and emission at 640 nm (bandpath, 5 nm). Chlide was prepared by the same method as Pchlide, but after a brief exposure (1–2 min) of the etiolated cotyledons to strong white light. Its concentration was calculated after transfer in diethyl ether using the molar extinction coefficient of Wu et al. (1989) (8.05 10^4M^{-1} cm^{-1} at 659 nm). The HPLC detector was calibrated for Chlide using fluorescence excitation at 435 nm (bandpath, 10 nm) and emission at 675 nm (bandpath, 5 nm).

Chl *a* and *b* were purified from spinach leaves according to the method of Berger et al. (1990), except that ethanol was used instead of methanol for grinding leaves and prepurification of Chl was carried out on the C18 cartridges (Waters). The concentration of the Chl was estimated in ethanol (95%) using the coefficients (84,600 M^{-1} cm^{-1} for Chl *a* at 664 nm and 41,200 M^{-1} cm^{-1} for Chl *b* at 648 nm) of Lichtenthaler (1987). Calibration of the HPLC detector for Chl *a* and Chl *b* was done with purified Chl *a* and Chl *b* in the two fluorimetric conditions used for detection of Pchlide and Chlide.

Main xanthophylls (neoxanthin, violaxanthin, and lutein) were identified by their *A* spectrum and by comparison with the elution patterns of whole pigments freshly extracted from spinach leaves. Under the experimental conditions used, lutein was not separated from zeaxanthin. Neoxanthin and violaxanthin were further identified after collection by the hypsochromic shift of their *A* maxima in acidic solution (Davies, 1976). β-Carotene was identified by its *A* spectrum and by coelution with a standard (Sigma type 4). Relative amounts of carotenoid species were formally estimated by comparison of the peak area at 440 nm.

Protein Determination

Protein concentration was estimated according to Bradford (1976) using BSA as a standard.

RESULTS

Characterization of Tetrapyrrolic Pigments in Chloroplast Envelope Membranes

The fluorescence emission spectrum of a suspension of envelope membranes is shown in Figure 1A. In good agreement with our previous studies, under 440-nm light excitation at room temperature, two emission peaks were observed at

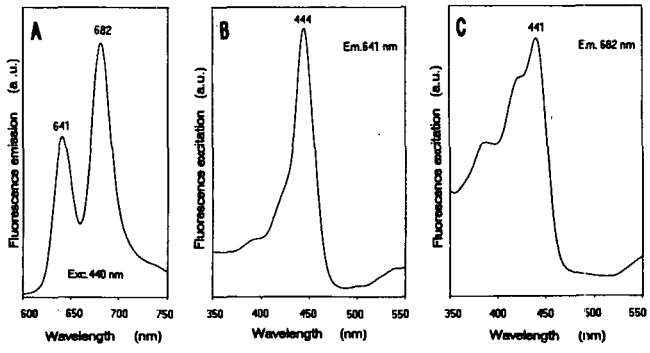

Figure 1. Room temperature fluorescence spectra of envelope membranes (resuspended in 10 mM Tricine-NaOH, pH 7.8) isolated from spinach chloroplasts. A, Emission spectrum, excitation 440 nm; B, excitation spectrum of the 641-nm emission; C, excitation spectrum of the 682-nm emission. All spectra were recorded with emission and excitation bandpaths of 5 nm.

641 and 682 nm. The excitation spectrum of the 641-nm emission (Fig. 1B) was a sharp peak in the blue region (maximum 444 nm), the shape of which was identical to that of the Pchlide isolated from cotyledons of dark-grown cucumber (not shown). The excitation spectrum of the 682-nm emission (Fig. 1C) gave a maximum at 441 nm but no peak at 470 nm, and thus was in accord with the absorption spectra of Chl *a* and/or Chlide *a*.

HPLC separation profiles of tetrapyrroles present in a typical chloroplast membrane preparation, including both polar and apolar pigments, are presented in Figure 2. When the fluorescence emission detector was set at 640 nm, the main signal present in envelope membrane extracts (Fig. 2, B and C) has the same retention time (9.16 min) (half-width 0.22 min) as purified Pchlide from etiolated cucumber cotyledons (Fig. 2A). Moreover, fluorescence emission and excitation spectra of this signal and of the Pchlide standard were similar (Fig. 3). Chl *a* and *b* were present in ethanolic extract of these membranes but were largely reduced with respect to Pchlide when the initial envelope membrane preparation (crude envelope, Fig. 2B) was repurified by a further sedimentation in a Suc gradient (Fig. 2C). In this case, Chls *a* and *b* represent only minor peaks on the chromatogram (the other small peak that was eluted before Pchlide has the same retention time as Chlide, see below). On the other hand, the

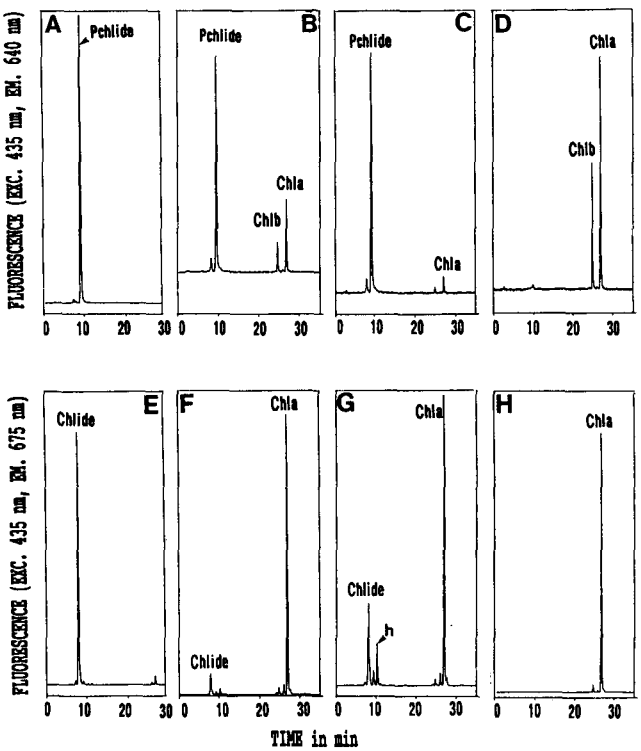

Figure 2. HPLC analysis of Pchlide (A) and Chlide (E) and of pigment extract from crude envelope membranes (B and F), purified envelope membranes (C and G), and purified thylakoid membranes (D and H) from spinach chloroplasts. Fluorescence detection was monitored successively at 640 or 675 nm (excitation, 435 nm) for Pchlide or Chlide detection, respectively. Excitation bandpath, 5 nm; emission bandpath, 5 nm. h, Pheophorbide a.

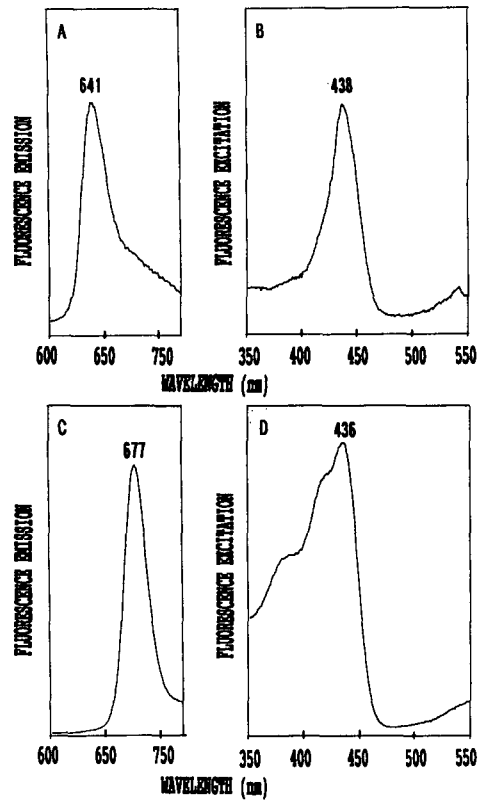

Figure 3. Fluorescence emission (A) and excitation (B) spectra of the peak of Pchlide resolved by HPLC from envelope extract (cf. Fig. 2B). Fluorescence emission (C) and excitation (D) spectra of the peak of Chlide resolved by HPLC from envelope extract (cf. Fig. 2F). Emission and excitation bandpaths, 5 nm.

main signals that were recorded from the thylakoid extracts (always under the same detection conditions) were due to Chls *a* and *b* (Fig. 2D). A small peak at the position of Pchlide was detectable in this sample only at a detector sensitivity that led to saturation of Chl *a* and *b* signals.

When the fluorescence emission detector was set at 675 nm, the first peak (half-width 0.21 min) that was present in envelope membrane extracts (Fig. 2, F and G) had the same retention time (7.93 min) as Chlide prepared from cucumber cotyledons (Fig. 2E). The identification of this component as Chlide was strengthened by the similarities of their fluorescence emission and excitation spectra (Fig. 3). In these experimental conditions, Chl *a* was also detected in every preparation of envelope membranes. However, the Chlide peak was highly increased with respect to the Chl *a* peak in the ethanolic extracts when the initial envelope preparation (Fig. 2F) was repurified by a further sedimentation in a Suc gradient (Fig. 2G). Two other peaks were eluted after Chlide: the first one has the same retention time as Pchlide and the second one was identified as pheophorbide *a* by its spectral characteristics. On the other hand, Chlide was difficult to detect in thylakoid membrane preparations, and its signal appeared only when the sensitivity of the detector led to a large saturation of the Chl *a* peak (Fig. 2H).

The data described above demonstrate that envelope and

Table I. *Distribution of Chl precursors in the main fractions extracted from spinach chloroplasts*

The amounts of Pchlide, Chlide, and Chl *a* in each sample were estimated as reported in "Materials and Methods" after HPLC analysis (as shown in Fig. 2) using fluorescence emission at 640 nm for Pchlide, at 675 nm for Chlide, and at the two wavelengths for the Chls.

Pigment	Thylakoid	Envelope
Chl *a*		
nmol/mg protein	130	0.3
Chl *b*		
nmol/mg protein	50.8	0.05
mol/mol Chl *a*	0.39	0.17
Pchlide		
nmol/mg protein	0–0.2	0.3
mol/mol Chl *a*	0–0.0015	1
Chlide		
nmol/mg protein	0–0.26	0.36
mol/mol Chl *a*	0–0.002	1.2

thylakoid membranes contain completely different levels of polar and apolar tetrapyrrolic pigments. However, the total amounts of Pchlide and Chlide in envelope and thylakoid vary from one experiment to another. On a protein basis, Pchlide and Chlide concentrations were in the range of 0.1 to 1.5 nmol/mg protein in envelope membranes with a mean value around 0.3 nmol/mg protein (Table I). However, proper quantification of these Chl precursors is very difficult in thylakoids because of their low abundance and the high Chl concentration of these samples. We observed a lack of reproducibility in the data, the Pchlide and Chlide signals being usually near (sometimes below) the limit of fluorimetric detection, whereas the signals from Chl were largely saturated. As shown in Table I, the concentration of Pchlide and Chlide was thus lower in thylakoids (range of values) than in envelope membranes on a protein basis.

The content of Chl *a* and Chl *b* is extremely low (respectively 0.3 and 0.05 nmol/mg protein) in the envelope fraction compared with thylakoids, which contain 130 and 51 nmol of Chl *a* and Chl *b*, respectively, per mg protein (Table I). The Pchlide to Chl *a* molar ratio was considerably higher in envelope membranes than in thylakoid membranes: respectively 1 and from 0 to 0.0015 mol of Pchlide per mol of Chl *a* (Table I). Likewise, the molar ratio of Chlide to Chl *a* was several hundred times higher in envelope membranes than in thylakoid membranes: respectively 1.2 and from 0 to 0.002 mol of Chlide per mol of Chl *a* (Table I). From these values, we can also conclude that nonphytylated compounds represent a major part of the envelope tetrapyrroles but a very slight fraction (1/1000) of the thylakoid tetrapyrroles.

Characterization of Envelope NADPH-Pchlide Oxidoreductase Activity

To demonstrate the presence of an active NADPH-Pchlide oxidoreductase in the envelope, the purified membranes were incubated in light (0.25 W/m^2) in the presence or absence of NADPH, and fluorescence emission spectra were recorded at room temperature (as in Fig. 1). Table II shows that the peak at 642 nm (Pchlide) decreased continuously with time when envelope membranes were incubated in light in the presence of NADPH. Depending on the experiments, an increase in the peak at 683 nm (essentially due to Chlide plus Chl *a*)

Table II. *Time course effect of NADPH on the fluorescent signals from envelope membranes isolated from spinach chloroplasts*

A suspension of envelope membranes was incubated in the light, with (NADPH + Light) or without (Light) addition of a NADPH-regenerating system, as indicated in "Materials and Methods." Fluorescence emission was recorded (from 600–750 nm as in Fig. 1; excitation, 440 nm) for each sample at the beginning (Time = 0) or after various times of incubation. Emission and excitation bandpaths were 5 nm. The results were expressed as peak height in arbitrary units. 1 and 2, Two different experiments. The protein concentration was 0.12 mg/mL in the two assays, and fluorescence was read on an aliquot of incubation mixture at the indicated time. 642/683 is the ratio of the two emissions.

Fluorescence	Experiment	Time (min)						
		0	20	25	35	45	65	75
NADPH + Light								
Emission 642 nm	1	6.9	5.8		5.3		4.1	
	2	7.7	6.3		4.7		3.0	
Emission 683 nm	1	17.1	16.8		17.1		17.1	
	2	18.3	19.2		19.2		19.2	
642/683	1	0.40	0.34		0.31		0.24	
	2	0.42	0.33		0.24		0.16	
Light								
Emission 642 nm	1	7.1		6.6		6.6		6.4
	2	8.4		7.9		7.6		7.4
Emission 683 nm	1	16.7		15.4		15		13.9
	2	18.8		17.2		16.2		14.9
642/683	1	0.42		0.43		0.44		0.46
	2	0.45		0.46		0.47		0.50

was usually observed (Table II) and a large decrease in the ratio of the two emission amplitudes (642–683 nm) was thus evident in the presence of light and NADPH (Table II). Without NADPH, both the 642- and the 683-nm emissions decreased at slightly different rates; therefore, we observed a small increase in the ratio of the two emissions. These data thus bear out the previous observations obtained using low-temperature fluorescence (Pineau et al., 1986; Joyard et al., 1990). However, because the fluorescence emission peak at 683 nm was due to Chlide plus Chl *a*, the actual variation of the Chlide level was difficult to monitor.

To provide a more accurate analysis of the changes of the Pchlide and Chlide amounts, the reaction was followed by HPLC analysis. To have internal standards for each injected sample, we simultaneously recorded the A_{440} to quantify the main carotenoid species (neoxanthin, violaxanthin, and lutein plus zeaxanthin), which also were separated using the same elution program (Fig. 4). In good agreement with previous observations (for reviews, see Douce and Joyard, 1979; Joyard et al., 1991), envelope membranes are characterized by a high ratio of violaxanthin to lutein (plus zeaxanthin) (3.3), which is different from that of thylakoids (0.76). The light incubation of envelope membranes with NADPH led to a strong decrease in the amount of Pchlide (Fig. 5, A and D), whereas light incubation without NADPH (Fig. 5B) or dark incubation with NADPH (Fig. 5C) induced only small variations in the amount of Pchlide. Illumination of envelope membranes without NADPH resulted in a decrease in the amounts of both Chlide and Chl *a* (Fig. 6, A and B), probably due to photooxidation. In the presence of NADPH (Fig. 6, C and D), the amount of Chlide increased, whereas the amount of Chl *a* decreased. The carotenoid content of the envelope membranes did not appreciably change, as shown for violaxanthin in Table III. The opposite variation of Pchlide and Chlide contents was clearly confirmed on the basis of the violaxanthin content (Table III).

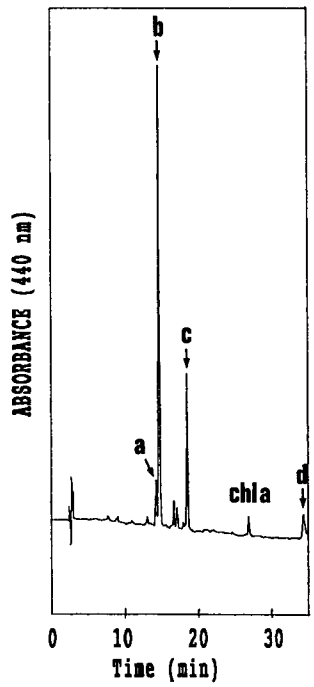

Figure 4. HPLC separation of ethanolic extract of envelope from spinach chloroplasts. Detection was by A_{440}. The main species of carotenoids were separated: a, neoxanthin; b, violaxanthin; c, lutein plus zeaxanthin; d, β-carotene.

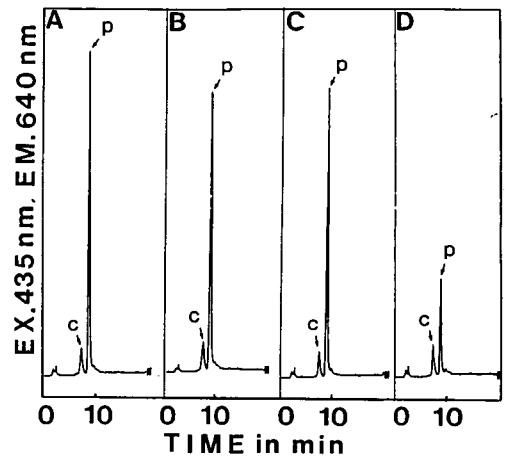

Figure 5. Effect of NADPH on the Pchlide content of the envelope membranes. A suspension of envelope membranes (1 mg/mL protein), pH 7.8, previously in darkness (A), was incubated for 60 min either in the light at 0.25 W/m² (B) or, with addition of an NADPH-regenerating system, in darkness (C) or in the light (D). Fifty microliters of the assay were extracted with ethanol for HPLC analysis. Fluorescence emission at 640 nm (bandpath, 10 nm) with excitation at 435 nm (bandpath, 10 nm) was used for detection of Pchlide (p). c, Chlide (see Fig. 2, B and C). Only the first part of the HPLC elution pattern is shown.

DISCUSSION

The presence of polar precursors of Chl *a* in purified envelope membranes from spinach chloroplasts was unambiguously demonstrated after HPLC separation of ethanol extracts of envelope pigments. Pchlide was shown to be present in all preparations of spinach chloroplast envelope membranes analyzed so far (at least 30). The fluorescence emission at 640 nm (at room temperature) was due to Pchlide without any significant contribution of compounds devoid of Mg. This point was verified by the lack of any other peaks when excitation in the range of 410 to 420 nm was used for fluorescence recording during HPLC separation. The demonstration that the fluorescence emission at 640 nm was due essentially to Pchlide allowed us to compare relative amounts of Pchlide from extracts of envelope membranes directly from their fluorescence emission spectra.

In contrast, and as previously suspected (Pineau et al., 1986), the fluorescence emission at 682 nm was more complex. HPLC separation identified Chlide *a* and Chl *a* and also, in some preparations, faint amounts of pheophorbide *a*, which was eluted slightly after Pchlide (see Fig. 2). Chlide was easily separated from Chl *a* by HPLC and thus its occurrence in envelope membranes is now clearly established. Because Chl *a* and Chlide *a* have very similar fluores-

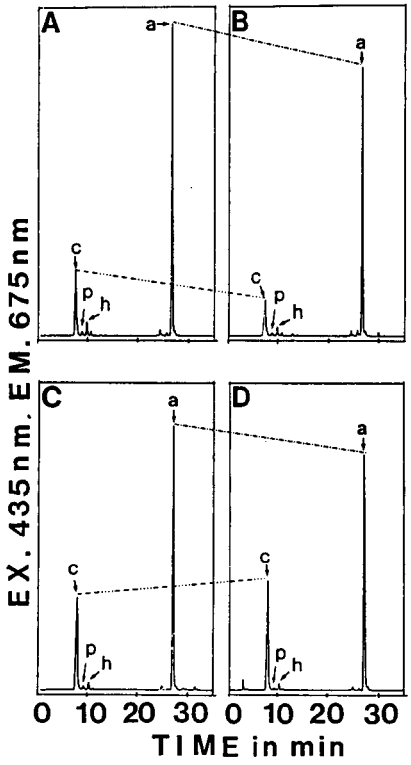

Figure 6. Effect of NADPH on the Chlide content of the envelope membranes. Two suspensions of envelope membranes (1 mg/mL protein) previously in darkness (A and C) were incubated in the light (0.25 W/m^2) with (D) or without (B) the addition of an NADPH-regenerating system. Fifty microliters of the assay were extracted with ethanol for HPLC analysis. Fluorescence emission at 675 nm (bandpath, 10 nm) with excitation at 435 nm (bandpath, 10 nm) was used for detection of Chlide (c). p, Pchlide; h, pheophorbide a; a, Chl a (see Fig. 2, F and H).

cence properties, they could not be distinguished easily by fluorescence analysis of whole membrane extract in our previous work (Pineau et al., 1986; Joyard et al., 1990). Chl *a* was also present in each preparation of envelope membranes, but at a very low concentration (0.3 μg/mg protein) compared with that of thylakoids (140 μg/mg protein). Because the Chl *a* to *b* ratio in the purest envelope membrane preparations was around 6, one can suggest that these preparations were mostly devoid of Chl *b* (see HPLC and fluorimetric data). Therefore, Chl that occurred in the envelope membrane fraction probably did not come from contamination by bulk thylakoid fragments but could correspond to discrete thylakoid pieces (stromal parts of thylakoids?). However, the possibility that they are genuine constituents of envelope membranes cannot be entirely ruled out.

The results described above also demonstrate that Pchlide and Chlide accumulated in envelope membranes, compared with thylakoids, and thus cannot result from thylakoid contamination. Chlide *a*, pheophorbide *a*, and pheophytin *a* represent the natural products of breakdown of Chl *a* (Amir-Shapira et al., 1987), resulting in part from the enzymic action of the chlorophyllase. In the great majority of HPLC analyses, the peak of pheophorbide was very small and the peak of pheophytin was difficult to discern even when we used appropriate excitation light. In addition, chlorophyllase is considered to be an intrinsic thylakoid membrane protein (Terpstra and Lambers, 1983). Thus, it is highly unlikely that, in our experimental conditions, the envelope Chlide could be the result of Chl degradation by such an enzyme.

In contrast, one can question whether the presence of small amounts of Pchlide and Chlide in thylakoid preparations really represents the situation in vivo. First, one should keep in mind that the level of fluorescence due to Chl is very high in thylakoids and does not allow reproducible analyses of Pchlide and Chlide in these membranes. Despite this problem, the presence of very low levels of Chlide in these membranes is not surprising because the last step in Chl biosynthesis, i.e. the esterification of Chlide by phytol or geranylgeraniol, takes place in thylakoids (Block et al., 1980). In addition, Chlide could be produced by chlorophyllase activity, which can also be active in thylakoids (Terpstra and Lambers, 1983). However, these two series of observations do not explain why Pchlide could sometimes also be detected in thylakoids. We should keep in mind that thylakoid preparations usually contain significant levels of envelope membranes as a contaminant (Dorne et al., 1990). Therefore, at least part of the polar tetrapyrroles found in thylakoids could be derived from envelope membranes. Thus, from our data, we can only estimate that thylakoid preparations contain less than one molecule of polar tetrapyrrolic pigments for 1000 Chl molecules, a value that is extremely low.

The envelope endogenous Pchlide pool could be converted into Chlide when purified envelope membranes were incubated in vitro under light in the presence of NADPH. This conversion was analyzed by room temperature spectrofluorimetry of whole envelopes and also by following the evo-

Table III. *Evolution of tetrapyrrolic and carotenoid pigments during light incubation of isolated envelope membranes with NADPH*

A suspension of envelope membranes was incubated in light with addition of a NADPH-regenerating system as described in "Materials and Methods." At the beginning (Time = 0) or after 15 or 55 min of incubation, 50 μL of assay medium were removed. The pigments (solubilized in 500 μL of ethanol) were separated by HPLC (see Figs. 5 and 6). The amplitude of the fluorescence emission was used for determination of the tetrapyrrolic pigments (fluorescence excitation, 435 nm; bandpath, 10 min). Emission at 640 nm (bandpath, 10 nm) was for Pchlide; emission at 675 nm (bandpath, 10 nm) was for Chlide and Chl a. A_{440} was simultaneously recorded for each sample for determination of carotenoids (see Fig. 4), and the relative amount of violaxanthin (area of the 440-nm peak) was used as an internal reference.

Pigment	Time (min)		
	0	15	55
	arbitrary units		
Pchlide	2.62	2.23	1.14
Violaxanthin	3.73	3.83	3.77
Chlide	20.3	21.4	23.6
Chl *a*	56.9	50.6	49.9
Violaxanthin	3.74	3.41	3.60

lution of pigment amounts after HPLC identification. The data presented above confirm the occurrence of a Pchlide reductase activity in envelope membranes from fully developed chloroplasts (Pineau et al., 1986; Joyard et al., 1990). This suggests that the envelope Pchlide and Chlide pools could be associated with events in the biosynthesis of Chl. Moreover, this hypothesis is supported by the observation that purified envelope membranes can be immunodecorated by a serum raised against NADPH-Pchlide oxidoreductase (Joyard et al., 1990). However, the rate of photoconversion observed in our in vitro experiments is very low (0.1–1 nmol Pchlide converted h^{-1} mg^{-1} protein). There could be at least two reasons for such a low rate: (a) we have assayed this enzymic activity only with the endogenous precursor Pchlide, present in low concentration in envelope membranes; and (b) not all Pchlide molecules that are present in etiolated or greening tissues are usually directly phototransformable (Cohen and Rebeiz, 1981; Oliver and Griffiths, 1982).

The NADPH-Pchlide reductase was shown to be negatively regulated by light at the level of RNA (decrease of translatable mRNA) and protein (proteolytic breakdown) (Kay and Griffiths, 1983; Griffiths et al., 1985; Haüser et al., 1987; Darrah et al., 1990; Forreiter et al., 1990), at least in most angiosperms. Nevertheless, the presence of discrete amounts of this enzyme, in vitro activity of which is largely compatible with the requirement of Chl turnover, is now confirmed in green tissues of oat and barley plants (Griffiths et al., 1985), and it is not surprising to detect such an activity in fully green spinach leaves.

The location of the enzymic steps that catalyze the conversion of δ-aminolevulinic acid to Pchlide via protoporphyrin IX was recently reinvestigated (for review, see Castelfranco and Beale, 1983; Rüdiger and Schoch, 1988). All the enzymes that convert δ-aminolevulinic acid into Chl via protoporphyrin IX were shown to be associated with chloroplasts (Bhaya and Castelfranco, 1985; Lee et al., 1991; Walker and Weinstein, 1991). Some of these activities, or at least part of them such as protoporphyrinogen oxidase (Matringe et al., 1992) or Mg-chelatase (Fuesler et al., 1984) were shown to be bound to plastid envelope membranes. Other activity, such as Mg-protoporphyrin IX monomethyl ester cyclase activity, could possibly involve envelope membrane components (Nasrulhaq-Boyce et al., 1987; Walker et al., 1991).

Our data provide additional support for a possible function of envelope membranes of mature chloroplasts in the biosynthetic sequence that leads from protoporphyrin IX to Chlide. One should keep in mind, however, that NADPH-Pchlide oxidoreductase and substrate concentrations are very low in the envelope membranes derived from fully developed spinach chloroplasts. In addition, our results do not preclude the presence in thylakoids of very low amounts of such an enzyme. However, immunological assays were unable to provide evidence for this (Joyard et al., 1990). Obviously, further work is required to confirm this hypothesis.

Moreover, envelope-associated porphyrin compounds could be related to other processes such as biosynthetic regulation between plastids and cytosol. In *Chlamydomonas reinhardtii*, for instance, some Chl precursors regulate the accumulation of several light-induced transcripts of *cab* nuclear genes (in the cytosol). However, the way of the regulatory process remains unknown (Johanningmeier, 1988; Jasper et al., 1991).

Received December 7, 1992; accepted March 29, 1993.

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