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A Comparison between Prolamellar Bodies and Prothylakoid Membranes of Etioplasts of Dark-Grown Wheat Concerning Lipid and Polypeptide Composition[1]

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ABSTRACT

The aim of the present investigation was to find factors critical for the co-existence of prolamellar bodies and prothylakoids in etioplasts of wheat (*Triticum aestivum* L. cv Starke II). The lipid composition of the prolamellar body and prothylakoid fractions was qualitatively similar. However, the molar ratio of monogalactosyl diacylglycerol to digalactosyl diacylglycerol was higher in the prolamellar body fraction (1.6 ± 0.1), as was the lipid content on a protein basis. Protochlorophyllide was present in both fractions. The dominating protein of the prolamellar body fraction was protochlorophyllide oxidoreductase. This protein was present also in prothylakoid fractions. The other major protein of the prothylakoid fraction was the coupling factor 1, subunit of the chloroplast ATPase. From the lipid and protein data, we conclude that prolamellar bodies are formed when monogalactosyl diacylglycerol is present in larger amounts than can be stabilized into planar bilayer prothylakoid membranes by lamellar lipids or proteins.

The generally accepted model for the organization of lipids and proteins in a membrane is the fluid mosaic membrane model, proposed by Singer and Nicolson (26). According to this model, the lipids form a planar bilayer in which the proteins are more or less embedded. Membrane lipids and proteins can also form branched bilayer structures in the cells. In plants, the most conspicuous ones are the crystalline PLBs[2], found in plastids. Based on ultrastructural studies, the structure of the PLB is considered to be a branched lattice constructed of four- or six-armed units (5, 8, 30). The PLBs are found in developing plastids (33), in etioplasts (32), and in nonirradiated chloroplasts (9, 12). It has been proposed that the PLBs store material to be utilized in the assembly of membranes at later stages of plastid development (20, 34).

Ultrastructural studies of etioplasts have shown that the branched membranes of PLB form a continuous membrane system with the planar bilayer membranes of PT (13, 20). It is therefore reasonable to assume that several membrane components are common between PLBs and PTs. We previously showed that glycolipid composition of PLB and PT fractions was qualitatively similar (22). Ryberg and Sundqvist (21) and Ikeuchi and Murakami (10) showed that PT fractions contained several polypeptides while the PLB fraction was dominated by one polypeptide. The methods used in these studies (10, 21, 22) for separating PLBs and PTs resulted in pure PLB fractions whereas the PT fractions probably contained PLB fragments and/or envelope membranes.

To study which factors could be important for the existence of the continuous system of prothylakoid membranes and branched prolamellar body membranes, a PT fraction representing prothylakoids that *in situ* had been connected with PLBs was needed. A method for isolating such a PT fraction, as well as a PLB fraction, has been previously described (23). In the present investigation, this method was used for isolating a PLB and a PT fraction. These fractions were compared with respect to lipid, pigment, and polypeptide composition with the aim of finding which compositional differences could explain the structural differences in membrane organization between PLBs and PTs.

MATERIALS AND METHODS

Isolation of Membrane Fractions. Etioplasts were isolated from dark-grown wheat (*Triticum aestivum* L. cv Starke II Weibull, Sweden) and purified on a Percoll gradient. After osmotical and mechanical rupture of the plastids, a fraction rich in envelope membranes ('env'), a PLB fraction, and a PT fraction were isolated according to Figure 1. After centrifugation of the ruptured etioplasts, the supernatant was collected ('env fraction'). The pellet was resuspended in 1.46 M sucrose buffer and loaded at the bottom of a continuous sucrose density gradient, made from 0.50 and 1.40 M sucrose in buffer. After centrifugation, the upper band containing PLBs and membrane vesicles (probably PTs connected to the PLBs), was collected. The lower band, containing membrane vesicles only, was discarded. The PLB-, PT-band was pelleted, resuspended in 1.46 M sucrose buffer, and divided into two aliquots that were sonicated with the purpose of separating the PTs from the PLBs. After sonication and sucrose gradient centrifugation, a PT fraction was collected from the aliquot subjected to weak sonication and a PLB fraction from the aliquot subjected to strong sonication. Details on plant cultivation, isolation of etioplasts, 'env', PLB, and PT fractions have been described elsewhere (23). In that description (23), the 'env', PLB, and PT fractions were dominated PS, $son3_{PLB}$, and $son1_{PT}$, respectively.

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[2] Abbreviations; PLB, prolamellar body; PT, prothylakoid; MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; SQDG, sulfoquinovosyl diacylglycerol; PG, phosphatidyl glycerol; PC, phosphatidyl choline; PI, phosphatidyl inositol; PE, phosphatidyl ethanolamine; PA, phosphatidic acid; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid.

Lipid Analyses. Lipids were extracted from the membrane fractions according to Bligh and Dyer (2). The individual lipid classes were isolated by two-dimensional TLC (3) on silica gel plates (Merck 60H, 0.25 mm). The solvent system in the first dimension was chloroform:methanol:water (65:25:4 by volume) and, in the second, chloroform:acetone:methanol:acetic acid:water (100:40:20:20:10 by volume). After the second development, the plates were sprayed with 0.02% (w/v) 2,7-dichlorofluorescein in methanol and the lipid-containing areas were visualized under UV light. Identification of the phospholipids was achieved by comparing their chromatographic properties with those of reference lipids (Phosphoglyceride Kit, Supelco). Silica gel areas containing lipids were transferred into screw-cap tubes and the acyl groups of the lipids were methylated with 14% BF_3 in methanol (17). The methyl esters of the acyl groups were separated by GC. A 0.13- (i.d.) × 180-cm glass column packed with 10% SP-2330 on Chromosorb W/AW 100/120 mesh (Supelco) was used in a temperature program from 140 to 185°C. The flow of carrier gas, N_2, was 20 ml·min^{-1}. A Varian 3200 gas chromatograph with a flame ionization detector was used. Heptadecanoic acid methyl ester was used as internal standard and a Hewlett-Packard 3390 integrator was used for quantification.

Pigment Analyses. Pigments were extracted with acetone:water (85:15, v/v) and PChlide was determined according to Koski and Smith (14). After addition of water to the pigment extract, carotenoids were extracted with petrol fraction (bp 40–60°C). Total carotenoids, dissolved in petrol fraction (bp 40–60°C):acetone (98:2, v/v) were determined spectrophotometrically at 440 nm using the extinction coefficient $\epsilon = 253$ l g^{-1} cm^{-1}.

Protein Analyses. Proteins were solubilized from the membrane fractions with 0.08 M KOH at 4°C overnight and determined by a color reaction using Coomassie Brilliant Blue G-250 (19; source of dye: Serva, FRG). BSA (essentially fatty acid free) was used as standard.

SDS-PAGE. Polypeptides of the membrane fractions were separated by gradient SDS-PAGE (10–20%) according to Laemmli (15) as previously described (23). In the present investigation, the membranes were not defatted with acetone prior to solubilization of the proteins with SDS, and the membranes were solubilized for 30 min at 40°C.

Mol wt of the major polypeptide bands were estimated by comparing R_F values with those of reference proteins: phosphorylase *b* (mol wt 94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,000; LMW Calibration Kit, Pharmacia Fine Chemicals, Sweden). Reflection spectras of the gels were recorded at 600 nm with a Zeiss KM3 Chromatogram Spectrophotometer. Relative weights of the different polypeptides were estimated gravimetrically from the reflection spectras.

Electron Microscopy. Isolated 'env', PT, and PLB fractions were fixed in glutaraldehyde, embedded in agar, and postfixed in OsO_4 as described by Ryberg and Sundqvist (21) and further prepared for electron microscopy as described by Sundqvist and Ryberg (28).

RESULTS AND DISCUSSION

Recovery of Plastids and Membrane Fractions. The purified etioplasts represented 1 to 3% of the plastids of the leaf segments used, based on carotenoid content. The isolated 'env', PT, and PLB fractions (Fig. 1) each represented between 4 and 6% of the amount of carotenoids of the purified etioplasts.

Purity of Membrane Fractions. No PE could be detected in any fraction. Since PE has been shown to be present in all plant membrane fractions analyzed except those of plastids (6), this shows that the 'env', PT, and PLB fractions were not contaminated with membranes of extra-plastic origin. The 'env' fraction consisted of single and double membrane vesicles (Fig. 2a). SDS-PAGE of the polypeptides of the solubiized membrane proteins (Fig. 3) showed that the env fraction yielded more numerous polypeptides than did the PT and PLB fractions. In the 'env' fraction, polypeptides with mol wt of $51 \cdot 10^3$ (large subunit of ribulose-1,5-bisphosphate carboxylase; 32) and $56–58 \cdot 10^3$ (α and β subunits of coupling factor of chloroplast ATPase [27]) always dominated, although the contribution of the $51 \cdot 10^3$ polypeptide varied between different isolations. Other major polypeptides of the 'env' fraction had mol wt of 114, 100, 65, 36, 33, 24, 18, 16, and $14 \cdot 10^3$. The 'env' fraction, thus, qualitatively resembled an envelope fraction of etioplasts concerning polypeptide pattern (23). In addition to the $56–58 \cdot 10^3$ polypeptide, the 'env' fraction also contained some other polypeptides that probably were identical with polypeptides of the PT fraction (Fig. 3). Also, PChlide was present in the 'env' fraction (Table I). Thus, the 'env' fraction contained both envelope and prothylakoid membranes.

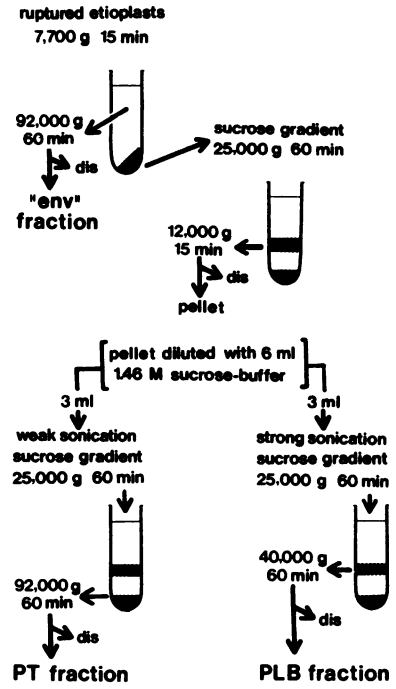

FIG. 1. A schematic representation of the method used for the isolation of membrane fractions from etioplasts. 'env', Envelope-rich fractions; PT, prothylakoid fraction; PLB, prolamellar body fractions; dis, discarded supernatant.

Before separating the inner membranes of the etioplasts into a PT and a PLB fraction, envelope vesicles and peripheral prothylakoids were removed by a sucrose density gradient centrifugation (Fig. 1; Ref. 23). A PT fraction was isolated from the resulting PLB-PT complex using a weak force of sonication. This treatment removed a fraction of the PTs from the PLBs without fragmenting the PLBs to any great extent. A PLB fraction was isolated by subjecting the PLB-PT complex to a strong force of sonication, which removed most of the PTs from the PLBs. Electron micrographs of the fraction showed that the PT fraction consisted mainly of single membrane vesicles but also contained a few small PLB fragments (Fig. 2b), whereas the PLB fraction besides PLBs also contained some membrane vesicles (Fig. 2c). The presence of PT membranes in the PLB fraction could also be seen on the SDS-PAGE of the fractions. In both the PT and PLB fractions, the dominating polypeptides had mol wt of 56–$58 \cdot 10^3$ (α and β subunits of coupling factor of chloroplast ATPase [27]) and $35 \cdot 10^3$ (NADPH:PChlide oxidoreductase [1,

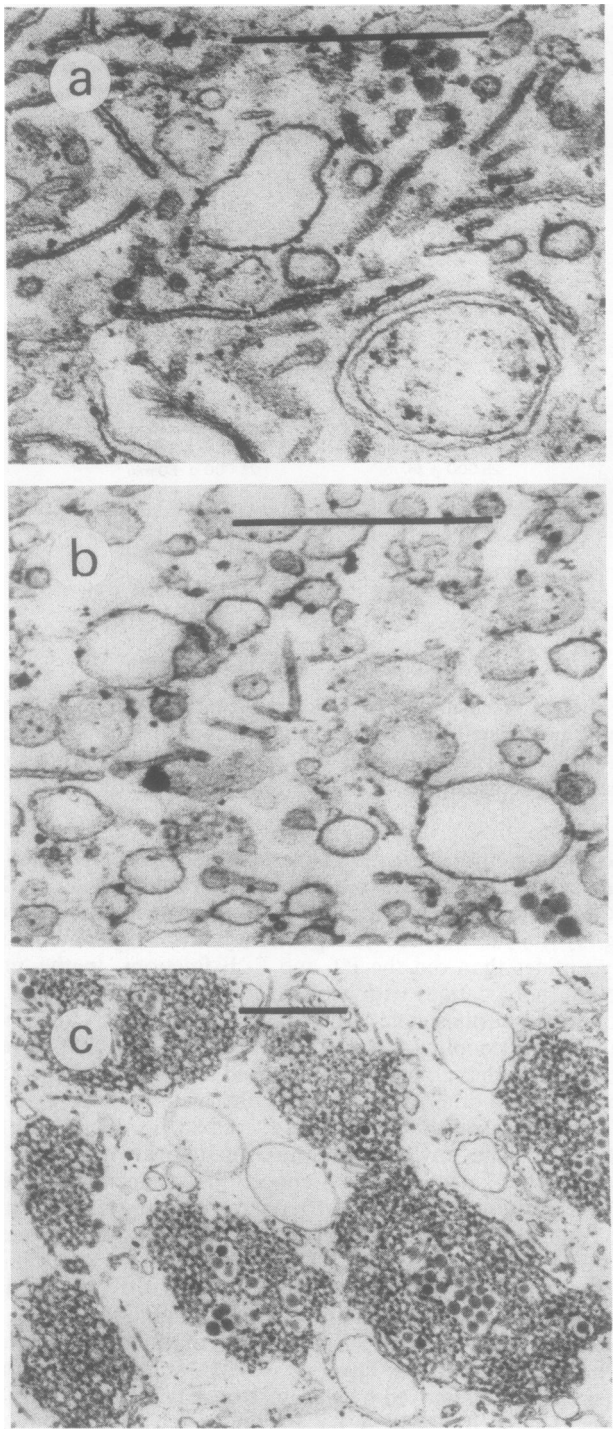

FIG. 2. Electron micrographs of isolated membrane fractions. a, 'env' fraction; b, PT fraction; c, PLB fraction. The bars represent 0.5 μm. Abbreviations as in Figure 1.

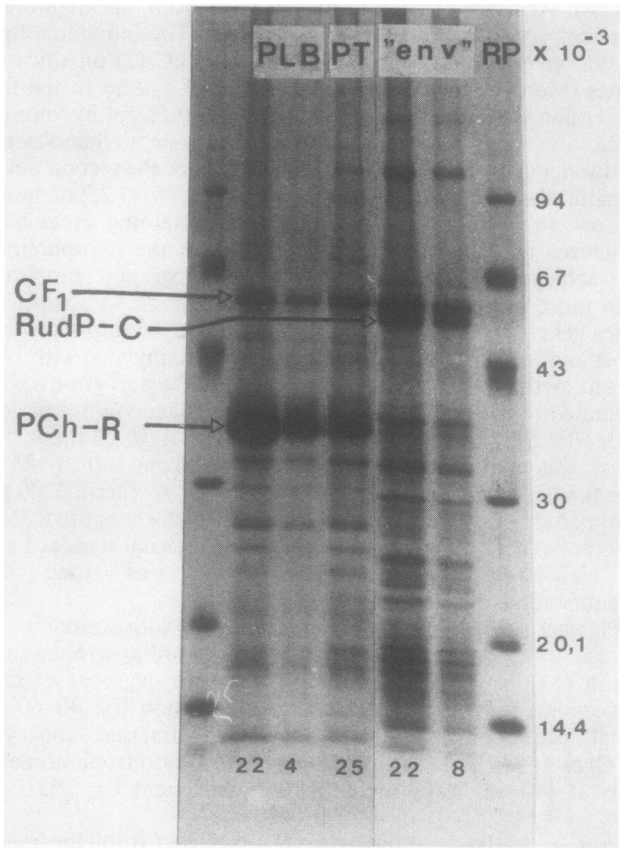

FIG. 3. SDS-PAGE of solubilizable polypeptides of etioplast membrane fractions. Mol wt of the reference polypeptides (RP) are shown, as are the locations and mol wt estimations of the major polypeptides of the membrane fractions. CF_1 α- and β-subunits of the coupling factor of the plastid (chloroplast) ATPase; RudP-C, ribulose-1,5-bisphosphate carboxylase; PCh-R, NADPH:PChlide oxidoreductase; PLB, PT, and 'env' as in Figure 1. The total amount of polypeptides (in μg) in each lane is indicated at the bottom of the lanes.

Table I. *Lipid and Pigment Content of Etioplast Membrane Fractions*
Statistical variation is given as SD.

	Envelope-Rich Fraction	n[a]	PT Fraction	n	PLB Fraction	n
nmol lipid[b] / μg protein	0.60 ± 0.03	4	0.97 ± 0.19	3	1.26 ± 0.16	3
pmol PChlide / μg protein	1.5 ± 0.3	5	7.8 ± 0.2	3	14.3 ± 3.3	4
pmol carotenoids / μg protein	33 ± 4	5	73 ± 4	3	92 ± 22	4
pmol carotenoids / pmol PChlide	24 ± 9	5	9.4 ± 0.8	3	6.5 ± 0.6	4

[a] Number of replicates.
[b] MGDG + DGDG + SQDG + PG.

18]). The weight ratio of the $35 \cdot 10^3$ polypeptide to the $56 \cdot 10^3$ polypeptide was for the PT fraction 1.47 ± 0.21 and for the PLB fraction 4.55 ± 0.20 (n = 3). The presence of the $56–58 \cdot 10^3$ polypeptide in the PLB fraction indicated the contamination of PT membranes since it has been shown that the coupling factor of the ATPase is not attached to the PLB proper (31, 32). Plastoglobuli were frequent in the PLB fraction (Fig. 2c). However, the galactolipids, the major lipid components of PLBs and PTs, are probably not constituents of plastoglobuli (C. Dahlin, personal communication).

The Lateral Heterogeneity of Pigments, Lipids, and Proteins in the PLB-PT System. The pigment analyses show that the PChlide content on a protein and lipid basis was higher in the PLB than in the PT fraction (Table I). The PChlide content of the PLB fraction was on a protein basis about 60% of the value previously presented (22) but similar to a value (10–11 nmol/mg protein) presented by Ikeuchi and Murakami (10) for PLB fractions isolated from squash etioplasts. The PT fraction isolated by Ikeuchi and Murakami (10) was obtained similarly to our 'env' fraction and the low PChlide content of their PT fraction

Table II. *Lipid Composition of Membrane Fractions of Etioplasts*

Lipid	Envelope-Rich Fraction ($n = 4$)	PT Fraction ($n = 3$)	PLB Fraction ($n = 3$)
		mol %	
MGDG	47 ± 2	45 ± 2	52 ± 2
DGDG	40 ± 4	40 ± 3	32 ± 4
SQDG	6 ± 1	8 ± 2	8 ± 1
PG	7 ± 1	7 ± 1	8 ± 1
MGDG / DGDG	1.2 ± 0.2	1.1 ± 0.1	1.6 ± 0.3

(0.9–1.4 nmol/mg protein) is close to the value of our 'env' fraction (1.5 ± 0.3; Table I).

The molar proportions of PChlide to lipids was 1:90 for the PLB fraction and 1:120 for the PT fraction whereas Chl (*a*+*b*) to lipids is approximately 1:3 in chloroplasts (13). Thus, the content of Chl pigments is very low in etioplast membranes as compared to chloroplast membranes.

The content of lipids (MGDG + DGDG + SQDG + PQ) was on a protein basis highest in the PLB fraction (Table I). The difference in lipid per protein content between the PLB and PT fractions was less pronounced than previously reported (23). This is probably because, in the present study, the PT fraction used represented only membranes that *in situ* had been connected with PLB. MGDG was the major lipid class of all fractions (Table II), followed by DGDG. Other than MGDG, DGDG, SQDG, and PG, all fractions contained small amounts of PC and PI. PA could not be detected in any fraction. When PC and PI were included, the molar lipid composition of the 'env' fraction was: MGDG, 44 ± 3; DGDG, 37 ± 4; SQDG, 6 ± 1; PG, 6 ± 1; PC, 5 ± 2; PI, 2 ± 1 mol% ($n = 4$). PC and PI constituted a slightly smaller part of the lipids of the PLB and PT fractions. However, these fractions were usually too small to make possible a reproducible quantification of the contents of these minor lipids.

When comparing the lipid composition of the PLB and PT fractions, the shape of the different lipid molecules should be considered (11). This is influenced by the acyl group composition and by the size, charge, and hydration of the polar group. There were no significant differences between the PLB and PT fractions concerning neither the acyl group composition of the individual lipid components (Table III) nor the proportion of uncharged (MGDG + DGDG) to charged (SQDG + PG) lipids (Table II). The main difference between the fractions was in the ratio of MGDG to DGDG, which was higher in the PLB than in the PT fraction (Table II). The molecular shape differs between these lipids because the polar group of MGDG is smaller than that of DGDG. Thus, MGDG is a conically shaped and DGDG a cylindrically shaped molecule; and, at physiologically relevant temperatures and with an acyl group composition representative of chloroplast and etioplast galactolipids, MGDG will form a reversed hexagonal and DGDG a lamellar phase with water (24, 22; Brentel, Selstam, Lindblom, BBA, in press). In addition to DGDG, SQDG, PG, PC, and PI also form lamellar phases when separately mixed with water (11, 24). Thus, the lipids of the PLB fraction consisted of 52% and 48% of lipids forming $hexagonal_{II}$ and lamellar phase, respectively, while the lipids of the PT fraction consisted of 45% and 55% of $hexagonal_{II}$ and lamellar lipids, respectively (Table II). The higher content of MGDG in the PLB than in the PT fraction was accompanied by a lower content of DGDG only (Table II). The significance of a high proportion of MGDG in the branched bilayer structure of PLB has also been indicated by the observation that mixtures of MGDG and DGDG (1.2:1 and 2:1, molar ratio) in water can form a bicontinuous cubic phase (Brentel, Selstam, Lindblom, BBA, in press). Moreover, when mixtures of chloroplast acyl lipids are dispersed in water, a structure interpreted as inverted micelles in a lipid bilayer is formed (4). Formation of these structures, the cubic phase and the bilayer containing inverted micelles, are favored by cone-shaped molecules like MGDG. Thus, the lipids of the inner membrane systems of chloroplasts and etioplasts may not alone be able to form planar bilayer structures.

In chloroplast thylakoids, large amounts of intrinsic proteins, such as the Chl-protein complexes and the CF_o subunit of the chloroplast ATPase, are constituents of the membranes. The presence of intrinsic proteins may stabilize the lipid components

Table III. *Acyl Group Composition of the Individual Lipids of Membrane Fractions of Etioplasts*

Lipid	Fraction	Fatty Acid						
		16:0	16:1	18:0	18:1	18:2	18:3	*n*
					mol %			
MGDG	env[a]	<1	<1	<1	<1	3 ± 1	95 ± 1	7
	PT	2 ± 1	<1	<1	<1	3 ± 1	94 ± 3	5
	PLB	2 ± 1	<1	<1	<1	4 ± 1	93 ± 2	5
DGDG	env	9 ± 1	<1	<1	<1	2 ± 1	87 ± 2	7
	PT	9 ± 1	<1	1 ± 1	<1	2 ± 0	87 ± 2	5
	PLB	9 ± 0	<1	1 ± 1	<1	2 ± 1	87 ± 1	4
SQDG	env	31 ± 9	<1	2 ± 1	1 ± 1	5 ± 1	60 ± 8	7
	PT	30 ± 5	<1	2 ± 1	2 ± 1	6 ± 2	56 ± 1	4
	PLB	37 ± 4	<1	4 ± 2	1 ± 2	4 ± 1	54 ± 2	3
PG	env	34 ± 1	20 ± 3	2 ± 1	2 ± 1	3 ± 0	40 ± 4	4
	PT	36 ± 8	14 ± 2	6 ± 2	3 ± 3	6 ± 4	36 ± 11	4
	PLB	41 ± 2	13 ± 5	6 ± 3	4 ± 2	4 ± 1	33 ± 2	3
PC	env	29 ± 10	1 ± 1	2 ± 1	6 ± 2	28 ± 4	34 ± 6	6
PI	env	42 ± 4	1 ± 3	7 ± 1	8 ± 2	19 ± 4	24 ± 8	4

[a] Envelope-rich fraction.

into planar bilayer membranes. Few proteins present in chloroplast thylakoids have been found in PTs of etioplasts (7). However, the ATPase is a major protein both of chloroplast thylakoids and of PTs of etioplasts. (Fig. 3; Ref. 31) but is probably not present in the PLB proper (10, 21, 31). The CF_o subunit of the ATPase as well as other intrinsic proteins could be involved in stabilizing a part of the etioplast lipids into planar PT membranes. The protein yielding the $35 \cdot 10^3$ polypeptide NADPH:PChlide oxidoreductase (1, 18) was the dominating protein of PLBs (Fig. 3; Refs. 15, 27), and it was also present in the PT fraction. The structural role of this protein in the PLB-PT system is not clear. NADPH:PChlide oxidoreductase may facilitate or induce the formation of the PLB structure, but the presence of the enzyme does not lead to formation of branched bilayer structures when enough amounts of other proteins are present or when the MGDG to DGDG ratio is too low, which is indicated by the planar bilayer structure of PTs. PLBs without PChlide have been reported (16, 29), but in these cases, the presence of NADPH:PChlide oxidoreductase was not analyzed.

To summarize, we propose that PLBs in equilibrium with PTs are formed when the cone-shaped lipid MGDG is synthesized in larger amounts than the membrane proteins synthesized can stabilize into lamellar membranes.

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