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Development of Oat Prothylakoids into Thylakoids during Greening Does Not Change Transmembrane Galactolipid Asymmetry but Preserves the Thylakoid Bilayer[1]

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ABSTRACT

The lipase from *Rhizopus arrhizus* and the lipolytic acyl hydrolase from potato tubers have been used to determine the transmembrane distribution of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) in prothylakoids and thylakoids from oat (*Avena sativa*). Both galactolipids were found to be asymmetrically distributed. The molar outside/inside distribution was 70 ± 8/30 ± 8 for MGDG and 10 ± 4/90 ± 4 for DGDG in the prothylakoid membrane. Mature thylakoids presented a similar distribution, *i.e.* 63 ± 4/37 ± 4 for MGDG and 12 ± 3/88 ± 3 for DGDG. This distribution has been assessed under a variety of different conditions, namely (a) in media favoring thylakoid stacking or unstacking and inducing various membrane surface potentials, (b) in the presence of defatted bovine serum albumin which removed free fatty acids and partially lyso-galactolipids, (c) under various temperature conditions which resulted in different hydrolysis rates and degrees of fluidity of the membrane, and (d) in the presence of different enzyme concentrations which influenced the hydrolysis rate. The above distribution was found to be independent of the type of conditions used. Nonbilayer forming/bilayer forming lipid ratios suggest that both monolayers of the prothylakoid and the inner monolayer of oat thylakoid membranes should display lamellar structures (*e.g.* ratios <2.5). In contrast the outer monolayer of the thylakoid membrane should display non-lamellar configurations (*e.g.* ratio >2.5). Thus, it is concluded that the incorporation of chlorophyll-protein complexes into the nascent thylakoid membrane modifies neither the galactolipid nor the phospholipid transmembrane distribution. However, these complexes appear to be crucial to preserve a bilayer configuration to the greening membrane which, otherwise, would adopt nonlamellar structures. The possible origin of galactolipid transversal asymmetry which appears very early during the biogenesis of oat thylakoid membranes is discussed.

Photosynthetic electron transport gives rise to a vectorial transport of protons across the thylakoid membrane so as to establish an electrochemical potential gradient necessary to drive ATP synthesis at the CF_0-CF_1 complex. This potential is achieved through laterally separated protein complexes, the PSII, and PSI, and the Cyt b_6-f complexes. The transfer of reducing equivalents between these complexes is mediated by the hydrophobic redox agent plastoquinone and by the two water-soluble proteins plastocyanin and ferredoxin which are localized on each side of the membrane. Thus, the asymmetric arrangement of proteins supports the vectorial properties of the membrane (10, 20).

The particular spatial organization of proteins raises the question of whether a similar heterogeneity exists also for acyl lipids which are the second major components of the thylakoid membrane (10, 20). Indeed, it has been shown recently that both galactolipids and phospholipids are asymmetrically distributed between the two monolayers of the thylakoid membrane in several higher plant species (10, 24, 25, 31–33, 37).

A second intriguing question is to assess to what extent the heterogeneous arrangement of acyl lipids is dependent on and/or induced by the asymmetrical distribution of proteins. In order to approach this question we have recently compared the transverse distribution of phospholipids in prothylakoids and mature thylakoids from oat (31). Prothylakoids are the precursors of mature thylakoids but their function (39) and protein composition are quite different, *e.g.* prothylakoids are devoid of Chl-protein complexes and contain a relatively large amount of CF_1-ATPase (17). Surprisingly, we have found that the distribution of phospholipids is similar in both types of membranes, suggesting that Chl-protein complexes are unlikely to be involved in the origin of the asymmetric transmembrane distribution of these lipids (31). However, in order to get a more thorough insight into the reciprocal influence of acyl lipids and proteins on their respective organization, it was desirable to investigate and compare the distribution of galactolipids in prothylakoid and mature thylakoid membranes. Galactolipids are the predominant acyl lipids in these membranes and, in this respect, may have a greater "organizing potential" than phospholipids.

Here, various lipolytic enzymes were used as a tool to assess the transmembrane distribution of galactolipids in both prothylakoids and thylakoids from oat. In addition to earlier reports (24, 30), the validity of the enzymatic approach has been confirmed unambiguously by using spinach thylakoid inside-out vesicles in which the galactolipid distribution was found to be the opposite of that observed in intact thylakoids, *i.e.* the molar outside/inside distribution is 42/58 for MGDG[3] and 82/18 for DGDG in inside-out vesicles compared to 62/38 for MGDG

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[3] Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; LAH, potato lipolytic acyl hydrolase; LRa, lipase from *Rhizopus arrhizus*; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; SQDG, sulphoquinovosyldiacylglycerol.

and 20/80 for DGDG in intact thylakoids (34). In this investigation, we show that, as for phospholipids (31), the transversal distribution of galactolipids was similar in both prothylakoid and thylakoid membranes.

MATERIALS AND METHODS

Isolation of Membrane Fractions. Etioplasts were isolated from dark-grown oat (*Avena sativa* L. cv Borrus) and purified on a Percoll gradient (31). After osmotic shock and centrifugation, the resuspended fraction was sonicated to release the prothylakoids from the prolamellar bodies. The sonicated suspension was then loaded at the bottom of a sucrose gradient and centrifuged as described earlier (31). The purified prothylakoids were pelleted by ultracentrifugation and resuspended in various media as indicated in the figure legends.

Chloroplasts and thylakoids were purified by repeated differential centrifugations as described earlier (31).

Lipid Analysis. Lipids were extracted and separated by high performance TLC in chloroform:methanol:acetic acid:H_2O, 85:15:10:3 (v/v/v/v), as described previously (24). After I_2 staining, MGDG and DGDG were scraped off for galactose determination (24).

Enzymic Analyses. Ferredoxin-$NADP^+$ oxidoreductase was measured as a diaphorase by the rate of ferricyanide reduction. A NADPH-generating system was used to keep the electron donor concentration constant (42). NADPH-protochlorophyllide oxidoreductase activity was assayed according to Griffiths (11): the rate of chlorophyllide synthesis induced by blue light flashes was measured spectrophotometrically (Aminco DW2A, dual mode, 675–710 nm) in the presence of saturating amounts of protochlorophyllide and NADPH. The trypsin-activated Ca^{2+}dependent ATPase of CF_1 was assayed as described by Nelson (21). Phosphate was determined by the method of Lebel *et al.* (14). The procedure of Wood and Bendall (40) was used to determine the Cyt b_6/f-dependent electron flow activity. Cyt c_{552} from *Euglena gracilis* was substituted for plastocyanin to increase the sensitivity of the method and duroquinol was used as an electron donor. Rates were corrected for uncatalyzed reactions.

Incubation Conditions. All incubations in the presence of lipase were carried out in darkness in different media and conditions, as indicated in the legend of each figure. Hydrolysis curves were expressed as semilog plots of the percentage of residual galactolipids (24, 31). In this investigation, two lipolytic enzymes were used: (a) the lipase from *Rhizopus arrhizus* (from Boehringer Mannheim), which splits the acyl chain of MGDG and DGDG at the *sn*-1 position, (b) the lipolytic acyl hydrolase which was isolated from potato tubers by the method of Galliard (7). This enzyme splits lipid ester bonds at both *sn*-1 and *sn*-2 positions.

Other Methods. Pigments were extracted with acetone:water, 80:20 (v/v), and Chl was determined according to Bruinsma (4). Protein was quantified according to Markwell *et al.* (18).

RESULTS

Table I shows that prothylakoids and thylakoids had a rather similar lipid composition. However, the former membrane fraction contained relatively less MGDG and more SQDG than the thylakoid fraction. As a consequence, the molar MGDG/DGDG ratio (1.6) was smaller in prothylakoids. This value is similar to that reported for prothylakoids isolated from etioplasts devoid of saponins (compare results in Ref. 15 and 16). Although the lipid composition of both fractions did not show great differences, the amount and the nature of proteins changed drastically during etioplast greening. First, the lipid/protein weight ratio was 32% higher in prothylakoids than in thylakoids (Table I). Second, the NADPH-protochlorophyllide oxido-reductase disappeared in the prothylakoid while the three main Chl-protein complexes appeared in the thylakoid (results not shown). This observation is in agreement with other reports (1). Prothylakoids displayed, in decreasing order, the following activities: CF_1-ATPase (642 ± 120 nmol Pi/mg protein·min), ferredoxin-$NADP^+$ reductase (123 ± 14 nmol ferricyanide/mg protein·min), duroquinol-Cyt c_{552} reductase (3.3 ± 1 nmol Cyt reduced/mg protein·min) and NADPH-protochlorophyllide oxido-reductase (1.95 nmol protochlorophyllide formed/mg protein·min). In contrast, thylakoids were characterized by light-dependent electron transport activities (*e.g.* 80 μmol $NADP^+$ reduced/mg Chl·h).

Table I. *Acyl Lipid Composition of Prothylakoid and Thylakoid Membranes from Oat*

Lipids were first separated by TLC in acetone:benzene:water (91:30:8; v:v:v) and then quantified by gas chromatography. Heptadecanoic acid was used as internal standard. The amount of proteins was determined according to Markwell *et al.* (18). Mean (± SE) values of *n* (four and nine) experiments.

Lipid Class	Prothylakoid (*n* = 4)	Thylakoid (*n* = 9)
	mol %	
MGDG	47 ± 3	61 ± 3
DGDG	30 ± 2	28 ± 2
SQDG	10 ± 1	3 ± 1
PG	8 ± 2	6 ± 1
PC	6 ± 2	2 ± 1
MGDG/DGDG	1.6 ± 0.1	2.2 ± 0.1
Lipid/protein (μg/μg)	0.560	0.425

In order to evaluate the transmembrane distribution of galactolipids, both prothylakoid and thylakoid fractions were treated with the lipase from *Rhizopus arrhizus*. The rationale of this enzymatic approach has been discussed elsewhere, as well as the prerequisites for the success of this method with thylakoids (24, 30, 34) and prothylakoids (31). Thus, it has been verified that (*a*) both prothylakoids and thylakoids formed closed vesicles; (*b*) each type of structure had, within the same population, an identical orientation; (*c*) the prothylakoid fraction was highly purified; (*d*) total galactolipid digestion occurred in control experiments where both sides of the membranes were attacked by the enzyme, *i.e.* that there were no inaccessible galactolipids.

Figure 1 illustrates hydrolysis kinetics of galactolipids in prothylakoids, expressed as semilog plots. The justification of this representation is discussed elsewhere (24). The hydrolysis kinetics displayed three phases for MGDG and two phases for DGDG. In the case of MGDG, the two first phases are typical kinetic pools, as defined by Rawyler and Siegenthaler (24). The slope of the first kinetic pool depended on the lipid/enzyme ratio. The decrease in the slope of the second kinetic pool may be explained by the formation of free fatty acids and lyso-MGDG during the enzymatic treatment which progressively increases the lateral surface pressure to an extent that will decrease the rate and extent of hydrolysis, as demonstrated earlier (24). The slope of the third kinetic pool is likely to depend on the transbilayer movement rate of MGDG (24). The magnitudes of the two first pools for MGDG and the first one for DGDG correspond to the amount of galactolipids localized in the outer monolayer of the prothylakoid membrane. In contrast, the magnitude of the last reactive pool corresponds to the amount of MGDG and DGDG which are located in the inner leaflet (24). Thus, the extrapolation to zero time of the last phase (Fig. 1) allows the estimation of the transverse distribution of galactolipids. The molar outside/inside ratio was 70 ± 8/30 ± 8 for MGDG and 10 ± 4/90 ± 4 for DGDG in the prothylakoid membrane.

In order to determine whether the insertion of new proteins (namely the Chl-protein complexes) in the prothylakoid membrane induces a redistribution of acyl lipids between the two

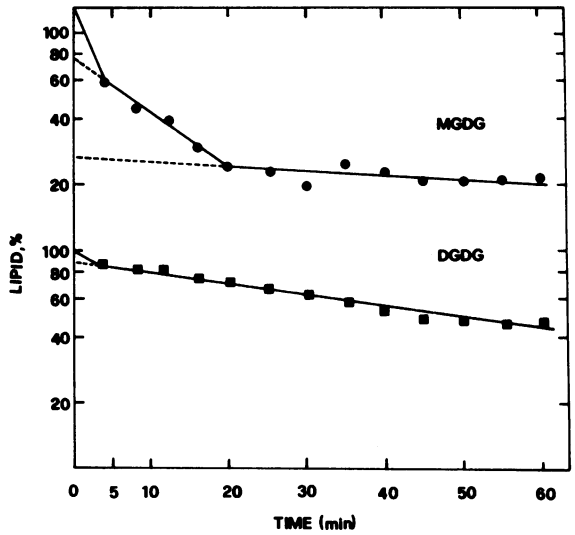

FIG. 1. Time course of galactolipid hydrolysis, expressed as semilog plots, in oat prothylakoids (0.5 mg protein/mL) treated with the lipase from *R. arrhizus* (14 Boehringer units/mg protein) at 10°C. The incubation medium contained 0.3 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM MOPS-NaOH (pH 7.4).

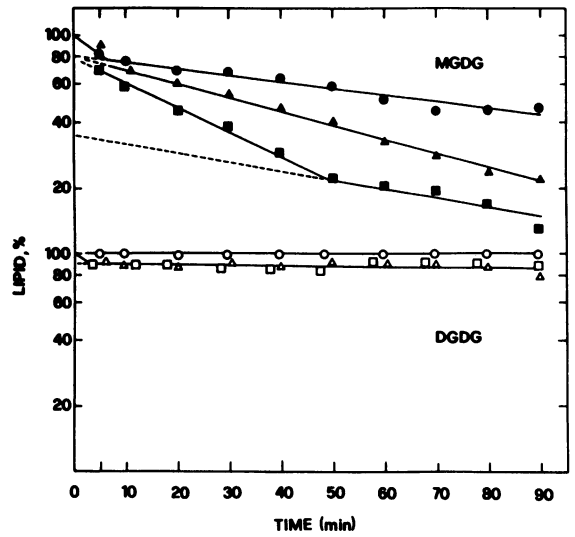

FIG. 2. Time course of galactolipid hydrolysis, expressed as semilog plots, in thylakoids as a function of temperature (●, ○ = 0°C; ▲, △ = 10°C; ■, □ = 20°C). Thylakoids (1 mg Chl/mL) were incubated in 0.3 M sorbitol, 10 mM NaCl, 5 mM MOPS-NaOH (pH 7.4) and 10 Boehringer units/mg Chl of lipase from *R. arrhizus*.

leaflets of the membrane, we have carried out similar experiments with mature oat thylakoids. Figure 2 illustrates the hydrolysis kinetics of both galactolipids at different temperatures. The hydrolysis kinetics at 20°C displayed three phases for MGDG and two phases for DGDG. It is important to emphasize that the lower degradation extent of DGDG compared to that of MGDG is a consequence of the different amount of these two galactolipids in the outer monolayer as demonstrated with thylakoid inside-out vesicles (34), but not the result of the difference in substrate specificity of the enzyme used. The extrapolation to zero time of the last phase of the kinetics obtained at 20°C showed an outside/inside molar ratio of 63 ± 4/37 ± 4 for MGDG and 12 ± 3/88 ± 3 for DGDG (average of 5 experiments). Thus, the transverse distribution of both galactolipids did not change during greening.

Figure 2 shows also that lowering the temperature decreased both the rate and extent of galactolipid hydrolysis. For instance, at 0° and 10°C, the outer MGDG molecules were not completely degraded within the experimental period. This is the result of a decrease of the enzyme activity due to a lowering of the temperature and to an increase of the viscosity of the membrane lipid phase as discussed above and elsewhere (24). Galactolipid transmembrane distribution has been studied under a variety of other conditions: (a) at high salt media (corresponding to a surface membrane potential varying from −45 to −52 mV) under which thylakoids were stacked; (b) at low salt media (from −67 to −152 mV) under which thylakoids were unstacked; (c) in the presence of various amounts of defatted bovine serum albumin which removed free fatty acids and partially lyso-galactolipids; and (d) in the presence of different enzyme concentrations. Although the rates and extents of the kinetic pools were different under the above conditions (a to d), the extrapolation to zero time of the last phases of the hydrolysis kinetics always gave the same galactolipid transmembrane distribution (results not shown).

In order to verify that galactolipid distribution is independent of the type of lipolytic enzyme used, we have carried out similar experiments with the lipolytic acyl hydrolase (LAH) from potato which displays different properties from the lipase from *R. arrhizus* (LRa). In contrast to LRa which catalyzes a specific hydrolysis at the *sn*-1 position, LAH splits lipid ester bonds at both *sn*-1 and *sn*-2 positions. Moreover, it is known that the release by LRa of one free fatty acid and one lyso-compound in the membrane results in an increase of the packing pressure of the lipid phase (24), while the release of two free fatty acids and the loss of the large polar head group of the molecule in the surrounding medium cause a decrease of the initial packing pressure (2). This interesting property of LAH allows the degradation of galactolipids in the outer monolayer without any restriction due to increase in packing pressure. Figure 3 shows that in the presence of LAH, the hydrolysis kinetics of both galactolipids displayed three phases. Due to lowering of membrane lipid pressure, the galactolipids localized in the outer leaflet were degraded in a single step, in contrast to the two steps occurring in LRa-treated thylakoids membranes (Fig. 2). The extrapolation to zero time of the second phase allowed the estimation of the outer/inner galactolipid distribution which was

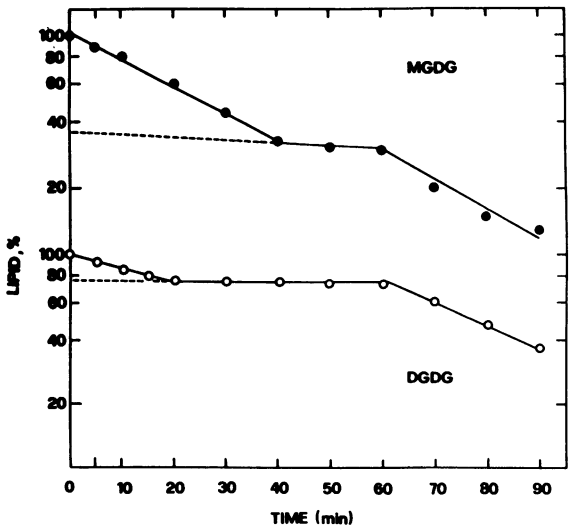

FIG. 3. Time course of galactolipid hydrolysis, expressed as semilog plots, in oat thylakoids (1 mg Chl/mL) treated with the lipolytic acyl hydrolase from potato (100 μg/mL) at 20°C. The incubation medium contained 0.3 M sorbitol, 35 mM NaCl, 50 mM MES-NaOH (pH 6.0) and 2 mg/mL defatted bovine serum albumin.

in this particular case 62/38 for MGDG and 22/78 for DGDG. Furthermore, the extensive hydrolysis of both galactolipids (and phospholipids, see also Ref. 23) occurring during the third phase (Fig. 3) should correspond to a rupture of the thylakoid structure allowing a progressive hydrolysis of galactolipids located in the inner monolayer. Thus, it appears that the galactolipid distribution in mature oat thylakoids does not depend on the type of lipase used.

Since no differences between transverse galactolipid distribution of pro- and mature thylakoids were observed, it was of interest to follow the evolution of this distribution during "natural" greening. Accordingly, thylakoids of increasing age isolated from leaf sections (about 3 cm long) taken at increasing distances from the basal meristem were tested for their galactolipid distribution. Again, it was found to be independent of the stage of oat development (results not shown).

DISCUSSION

Our results show several interesting features. Firstly, the transverse lipid distribution is independent of the conditions used for its estimation (*e.g.* temperature, presence or absence of bovine serum albumin, salt and enzyme concentrations, enzyme nature). These conditions, which are known to support different degrees of stacking, hydrolysis rates, fluidity levels and surface membrane potentials (24), influence the rate of lipid hydrolysis but not the degree of galactolipid asymmetry. Secondly, the molar outside/inside distribution of MGDG and DGDG is identical (within standard deviation) in both prothylakoid and thylakoid membranes from oat, *i.e.* 2/3 of the total MGDG and 1/10 of the total DGDG are localized in the outer monolayer. It is most remarkable and unexpected that during the biogenesis of the thylakoid in the light, which is accompanied by a significant enrichment in the relative protein amount (see lipid/protein ratio changes in Table I) and by modification of the protein nature (12, 19), the distribution of phospholipids (31) and galactolipids (Figs. 1–3) between the two leaflets of the membrane remained the same. This is true even though the molar ratio MGDG/DGDG increased by a factor of about 1.4 when the prothylakoid evolved into a mature thylakoid (Table I).

The distribution of all lipid classes between the two leaflets of prothylakoid and mature thylakoid membranes is summarized in Figure 4. When lipids are expressed in mol % of each lipid class (Fig. 4A), it appears that in both types of membranes the outer leaflet is highly enriched in MGDG and PG, while the inner one contains mainly DGDG and probably SQDG molecules. In contrast, when acyl lipids are expressed in mol % of total lipids (Fig. 4B), MGDG, in the outer monolayer, and DGDG plus MGDG, in the inner monolayer, are prominent in the two types of membranes. However, both monolayers contained almost identical amounts of polar lipids (TL in Fig. 4B). As discussed earlier (25) the thylakoid membrane is a bilayer structure in spite of the fact that its outer monolayer is highly enriched in MGDG, a non-bilayer forming lipid (Fig. 4). In order to evaluate the potential of acyl lipids to form lamellar or hexagonal configuration in each monolayer of prothylakoid and thylakoid membranes, we have calculated two acyl lipid ratios: (a) the molar MGDG/DGDG ratio which gives a first approximation on this potential; (b) the non-bilayer forming/bilayer forming acyl lipid molar ratios which takes into account all other acyl lipids present in these membranes (Table II). It is worth mentioning that Sprague and Staehelin (35) have demonstrated that the transition of bilayer to non-bilayer structures starts at a molar ratio of MGDG/DGDG of about 2.5. Taking a ratio of 2.5 as being critical for the formation of nonlamellar phases means that a mixture of pure galactolipids of similar composition to that of both prothylakoid and thylakoid outer monolayer, would contain mostly nonlamellar structures, whereas that of

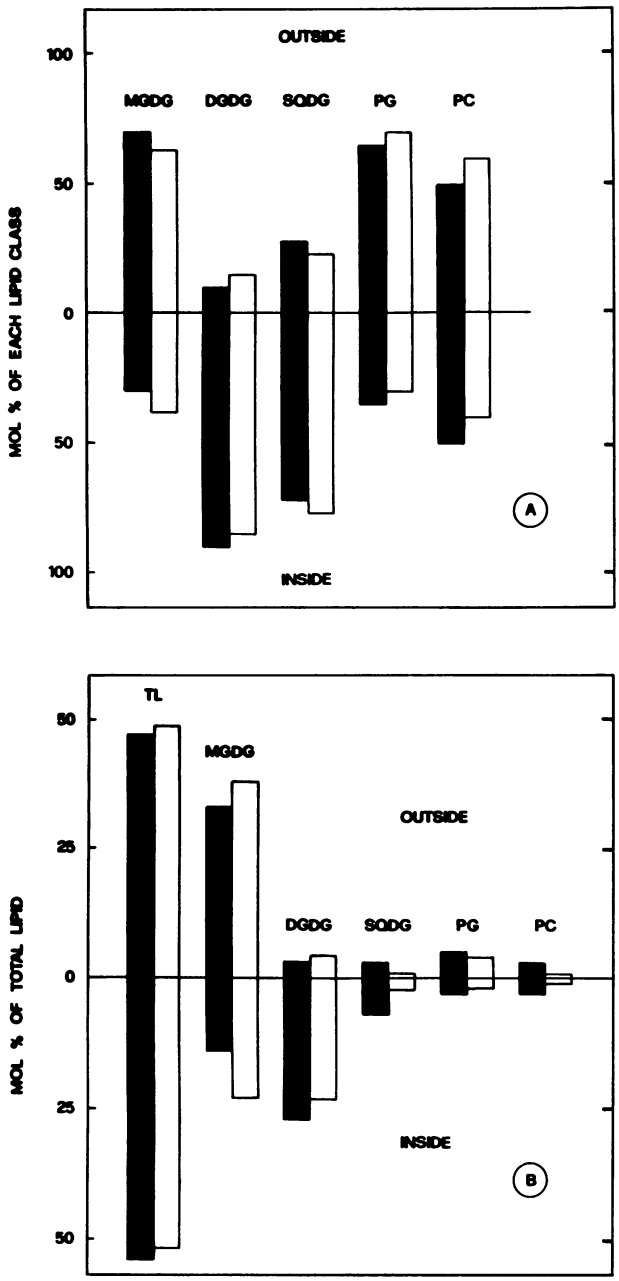

FIG. 4. Distribution of acyl lipids between the outer and inner monolayers of oat prothylakoid (■) and thylakoid (□) membranes, expressed as (A) mol % of each lipid class or (B) mol % of total lipid. Values for phospholipid distribution are from Siegenthaler and Giroud (31); those for galactolipid distribution are from Figures 1 to 3; due to its low reactivity toward lipolytic enzymes, SQDG has been tentatively estimated between the two monolayers as in Rawyler *et al.* (25).

the inner monolayers would promote exclusively lamellar structures (see Table II). When taking all acyl lipids into consideration and calculating the non-bilayer forming/bilayer forming lipid ratios, it is seen that the outer monolayer of the prothylakoid membrane now displayed the ability to form lamellar structures (*e.g.* ratio <2.5). In contrast, the outer monolayer of the thylakoid membrane should display nonlamellar configuration (*e.g.* ratio >2.5). Table II shows also that lamellar structures should be present in the inner monolayers of both prothylakoid and thylakoid membranes (*e.g.* all the ratios were inferior to 2.5).

Although this way of reasoning is appropriate for a mixture of

Table II. *MGDG/DGDG and Non-Bilayer Forming/Bilayer Forming Acyl Lipid Molar Ratios in the Two Monolayers of Prothylakoids and Thylakoids from Oat*

Amounts are calculated assuming that all of the MGDG is potentially non-bilayer forming but that all the other acyl lipids (DGDG, SQDG, PG, and PC) are bilayer forming. For the distribution of acyl lipids, see legend of Figure 4.

Lipid	Prothylakoid		Thylakoid	
	Outer leaflet	Inner leaflet	Outer leaflet	Inner leaflet
MGDG/DGDG [mol:mol]	11.0	0.52	12.7	0.92
Non-bilayer/bilayer forming lipids [mol:mol]	2.38	0.36	4.16	0.77

acyl lipids, one should keep in mind that the potential of acyl lipids to form lamellar or nonlamellar configurations is dependent on several other factors, such as the degree of unsaturation of the lipid fatty acyl chains (8), the water content (3), temperature (3), the pH and the cation type and concentration (9). Proteins also have been demonstrated to modulate the bilayer/non-bilayer preference of lipids (5). For instance, Taraschi *et al.* (38) have shown that 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine (which, like MGDG, is a non-bilayer forming lipid) undergoes, in the presence of excess water, a bilayer to hexagonal (H_{II}) arrangement as the temperature is increased above 0°C. Incorporation of glycophorin, an intrinsic protein, into this system stabilizes the bilayer configuration, prohibiting the formation of H_{II} phase. As discussed above, the biogenesis of oat thylakoids is accompanied by a remarkable increase in the potential of the outer monolayer lipids to form non-bilayer structures (Table II). Thus, incorporation of large amount of Chl-protein complexes (18), which are known to interact with MGDG (29) and PG (6) molecules, into the greening membrane may be necessary to preserve lamellar structures. In this respect, Chl-protein complexes may have an effect similar to that of glycophorin. On the other hand, acyl lipids alone in both prothylakoid monolayers are able to form by themselves stable lamellar structures (Table II). We may therefore postulate that part of MGDG molecules which, by their insertion into the prothylakoid membrane, could destabilize the bilayer structure are diverted to prolamellar bodies, which, indeed, are known to contain a large amount of MGDG and to adopt nonlamellar structures (15–17). Thus, the third interesting feature of this investigation is that the incorporation of Chl-protein complexes into the nascent thylakoid membrane modifies neither the galactolipid (this investigation) nor the phospholipid (31) transmembrane distribution. In contrast, these complexes appear to be crucial to preserve a bilayer configuration to the greening membrane which otherwise, would adopt a nonlamellar structure.

Although our results do not allow us to draw conclusions about the mechanism by which lipid asymmetry takes place in the thylakoid membrane, they show, however, that the asymmetry appears very early during the biogenesis of this membrane. Thus the appearance of lipid asymmetry has to be looked for during the formation of the prothylakoid membrane itself; this asymmetry may take place in the following ways: (a) by a self-induced mechanism due to the lipid properties themselves (26); (b) by a lipid-protein interaction mechanism due to the presence, in the nascent membrane, of preexistent hydrophobic proteins such as the CF_0-CF_1 ATP synthetase, the $NADP^+$-protochlorophyllide oxidoreductase and to some extent ferredoxin, ferredoxin-$NADP^+$ reductase and plastocyanin; (c) by the pre-existence of lipid asymmetry in the etioplast inner envelope membrane which generates the prothylakoid membrane (28 and discussion in Ref. 31). However, the evidence showing that prothylakoids have originated from the inner envelope membrane by invagination is still controversial (36); (d) finally, acyl lipid transfer proteins (13) combined with acyl lipid translocase (41) may be involved in the asymmetric assembly of acyl lipids in the prothylakoid membrane. This may be true for galactolipids but not for phospholipids since a galactolipid-transfer protein (22) but not a phospholipid-transfer protein (27) has been found in the chloroplast stroma.

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