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ETUDE COMPARATIVE DE LA DISTRIBUTION ET DU ROLE DES LIPIDES DANS LE PROTHYLACOIDE ET LE THYLACOIDE MATURE D'AVOINE

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STUDY OF THE DISTRIBUTION OF PHOSPHOLIPIDS IN PROTHYLAKOIDS AND THYLAKOIDS FROM OAT AND SPINACH

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INTRODUCTION

The functional and structural asymmetry of protein complexes in TM is well established. A similar heterogeneity has been reported for lipids and has been found to be lateral (1) and transverse (2). In addition, it has been suggested that PL could be associated with different photochemical activities (2). PT, the precursors of TM contain neither chlorophyll nor active photosystems. It was therefore attractive to hypothesize that the PL distribution in PT could be different than in mature TM.

In this investigation, PLA_2 was used as a tool to test the transverse lipid distribution (3) under different experimental conditions (temperature, ionic environment) and with thylakoids at various stages of development.

MATERIAL AND METHODS

Crude Avena etioplasts were purified on a self-generated gradient of Percoll (centrifugation : 100 000 g, 30 min in a Ti-70 angle fixed rotor) made of three layers : 6 ml 27%, 8 ml 45%, 6 ml 65% in a medium derived from (4). Purified etioplasts were then broken by osmotic shock and PT and PLB were separated by sonication and purified by flotation on a linear sucrose gradient as in (5). After collection of the fractions, PT and PLB were sedimented at 300 000 g for 1 h and resuspended in various media (see legends to figures). Intact Avena chloroplasts were isolated by repeated differential centrifugations and intact spinach chloroplasts purified in Percoll gradients as in (6). After an osmotic shock, both types of TM were washed three times and resuspended in various media. The incubation of TM with PLA_2 was carried out in darkness under the conditions described in the legends of the figures. About 1 Sigma unit PLA_2/mg Chl was added to the TM suspension and 0.8 Sigma unit/mg prot to PT. Aliquots of the incubation media were taken at various times for lipid analysis but for the zero time controls, no PLA_2 was present. PLA_2 activity was stopped by an excess of EDTA

Abbreviations: MG and DG, mono- and digalactosyldiacylglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine; PL, phospholipids; PLA_2, pancreatic phospholipase A_2; PT, prothylakoids; PLB, prolamellar bodies; TM, thylakoid membranes.

added in the extraction medium. As no variations of DG and MG were detectable during incubation, DG was used as an internal standard. Lipids were extracted by the method of Bligh and Dyer and separated by HPTLC in chloroform:methanol: acetic acid:H_2O (85:15:10:3). After I_2 staining, PG, PC and DG were scrapped off for phosphate or galactose determination. Hydrolysis curves were expressed as semi-log plots of the % of residual PL (8).

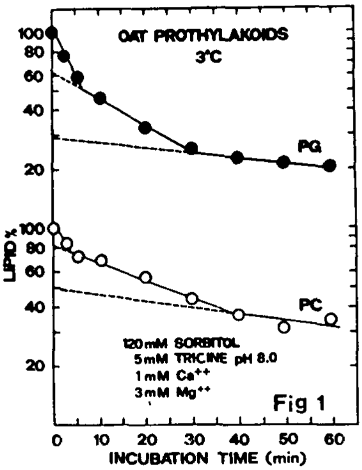
RESULTS AND DISCUSSION

There are good evidences that PT obtained by sonication were right side out vesicles since no CF_1 particles towards the inside are visible (7) and no increase in Fd-$NADP^+$ reductase activity due to entrapped enzyme or inside-out vesicles occurred when PT were disrupted (not shown). No saponins were detectable in acetone extracts. CF_1-ATPase and protochlorophyllide reductase were the two main activities. The different fractions have the following characteristics :

	PT	PLB	Spinach TM	Oat TM
Proteins (% fraction)	75 ± 8	25 ± 8	-	-
PG/PC	1.5 ± 0.6	2.5 ± 1.6	2.5 ± 0.3	3.0 ± 0.5
MG/DG	1.6 ± 0.15	1.9 ± 0.1	1.9 ± 0.1	2.2 ± 0.2

The ratio PG/PC was found to increase during the purification of TM, probably due to PC contaminations. Thus, a great care was taken to minimize unspecific contaminations by using Percoll to obtain intact chloroplasts and by repeated washings of TM.

In PT sonicated in the presence of PLA_2 and 0.1 mM EDTA, all PC and PG were degraded after an addition of Ca^{++}, indicating that PG and PC were fully accessible when PLA_2 had access to both sides of the membrane (not shown). Typical examples of PL hydrolysis kinetics are illustrated in Fig. 1. Fig. 2 summarizes the level of the different PL pools observed at various temperatures. At 0°C, both extent and rate of PC degradation were too weak to detect a break in the hydrolysis rate whereas such a break was detectable for PG hydrolysis. This difference may reflect the preference of PLA_2 for anionic PL. Furthermore, the surface pressure may be too high to allow a full degradation of both PL localized in the outer layer of PT. Raising the temperature increased the rate and amplitude of the hydrolysis for both PL. For instance, at 3°C, only one break for PC but two breaks for PG were detectable in the hydrolysis patterns. At 7°C, two breaks whereas at 10°C only one break presumably corresponding to the total degradation of outer PL were observed for both PG and PC. Thus, the rate and amplitude of the hydrolysis leading to the 2nd pool may be attributed to a packing effect



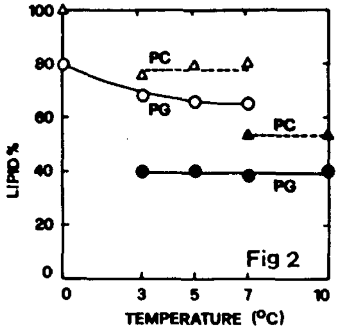
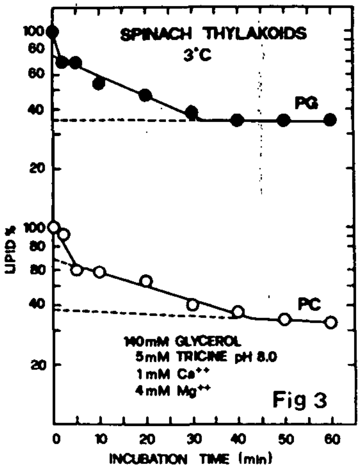


Fig. 1. Hydrolysis of PG and PC in oat PT treated by PLA_2. Lipid hydrolysis kinetics show two breaks revealing the existence of three pools which were hydrolyzed at different rates. The required extent of degradation to reach the less reactive pool was greater for PG (60-70%) than for PC (50%). No abrupt increase in the rate of lipid degradation was visible suggesting that no lysis of PT occurred.

Fig. 2. Extent of PG and PC degradation in PT expressed as the amount of lipid remaining in the membrane as a function of temperature. Values correspond to extrapolation to zero time of the different pools revealed by plotting the log of % lipids remaining in the membrane as a function of incubation time (as in Fig.1). △, ▲ 2nd and 3rd pool of PC; ○, ● 2nd and 3rd pool of PG; medium : 62 mM Tricine pH 8, 1.5 mM $CaCl_2$.

depending on the presence of the hydrolysis products. The last pool may reflect the true amount of PL localized in the inner layer of the membrane. Any further hydrolysis of PL may be due to an outwards transbilayer movement. The time t necessary to destroy all external PL could be modulated by changing the ionic environment. When Mg^{++} was competing with Ca^{++}, an increase of t was observed. Increasing $[Ca^{++}]$ shortened t (not shown). These effects are probably caused by a change in $[Ca^{++}]$ at the membrane interface where Mg^{++} may also compete with Ca^{++}, thus affecting PLA_2 activity. In addition, a direct effect of divalent cations on the compactness of membrane lipids may also affect PLA_2 activity.

In conclusion, our results suggest that : (1) About 2/3 of PG and PC are localized in the outer layer of both oat and spinach TM; (2) Since a higher temperature was required to hydrolyze all PL in the outer layer of oat TM compared to that of spinach, one may infer that the initial surface pressure in oat is greater than in spinach TM; (3) About 2/3 of PG and 1/2 of PC are localized in the outer layer of PT. Thus the distribution of PG is similar in both PT and TM



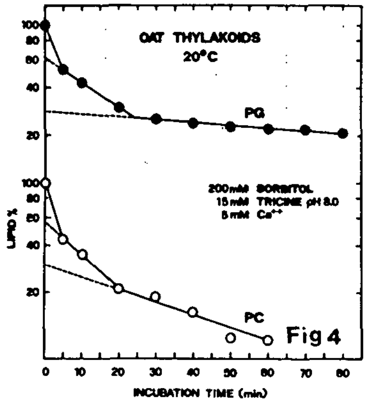


Fig. 3. Hydrolysis of PG and PC in spinach TM treated by PLA_2. As in PT, the lipid hydrolysis kinetics revealed the existence of three pools.

Fig. 4. Hydrolysis of PG and PC in oat TM treated by PLA_2.

suggesting that its localization may not depend on the incorporation of the chlorophyll-protein-complexes in PT during greening. It is proposed that a self-arrangement due to the properties of the lipids themselves rather than to a direct interaction with specific proteins should be responsible for PG distribution.

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Transversal distribution of phospholipids in prothylakoid and thylakoid membranes from oat

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The phospholipases A_2 from porcine pancreas and *Vipera russelli* and the phospholipase D from *Streptomyces chromofuscus* have been used to determine the transmembrane distribution of phosphatidylglycerol (PG) and phosphatidylcholine (PC) in prothylakoid and thylakoid membranes from oat. Both phospholipids were found to be asymmetrically distributed. The molar outside/inside distribution was $70 \pm 5/30 \pm 5$ for PG in both types of membranes. Concerning PC, this ratio was $50 \pm 10/50 \pm 10$ and $65 \pm 10/35 \pm 10$ for prothylakoid and thylakoid membranes, respectively. These results suggest that chlorophyll-protein complexes are unlikely to be involved in the origin and stabilization of the asymmetric transmembrane distribution of phospholipids. The possible origin of this distribution is discussed.

Prothylakoid membrane *Thylakoid membrane* *Phospholipase* *Phospholipid distribution* *Phospholipid asymmetry* *(Oat)*

1. INTRODUCTION

The vectorial properties of the thylakoid membrane lie essentially on the asymmetric (lateral and transversal) distribution of the protein complexes which are involved in electron and proton transport and in photophosphorylation activities [1,2]. These properties underlie the basic concept for the understanding of energy conservation in this particular type of membrane. This raises the question of whether a similar transversal heterogeneity exists for acyl lipids and, if so, what should be its significance in terms of the structure and function of the thylakoid membrane. A better understanding of the role of acyl lipids in thylakoid functions will obviously depend on the knowledge of their molecular organization in the membrane [3].

In thylakoid membranes, PG was found to be asymmetrically distributed. About 70% of this phospholipid is localized in the outer monolayer of thylakoid membranes from spinach [4,5], oat [5] and from barley, pea and lettuce [6]. A similar distribution (60–70% in the outer leaflet) is also found for PC in spinach and oat thylakoids [5]. Galactolipids have also been shown to be asymmetrically distributed within the thylakoid membrane [7–9]. Using the digestion method with the lipase of *Rhizopus arrhizus* and the galactose oxidase method we have found that the outside/inside distribution is around 65/35 for MGDG and 15/85 for DGDG in thylakoid membranes from spinach [9] and from a variety of other higher plant species [10]. Although there is general agreement concerning the above distribution for MGDG, the localization of DGDG is still controversial [8–10].

An intriguing question is whether specific interactions between acyl lipids and certain membrane proteins play a predominant role in inducing and maintaining the asymmetric distribution of

Abbreviations: DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol

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lipids. In such a case, chlorophyll-protein complexes may be good candidates. Indeed, specific associations between particular lipids and these complexes have been reported [11–14]. The knowledge of the distribution of acyl lipids in prothylakoids may provide an answer to the above question. Indeed, prothylakoids are the precursors of mature thylakoids but their protein composition is quite different, e.g. prothylakoids are devoid of chlorophyll-protein complexes and contain a large amount of NADPH protochlorophyllide oxidoreductase [15].

Here, various phospholipases were used as a tool to assess the transmembrane distribution of phospholipids in both prothylakoids and thylakoids from oat. Surprisingly, the distribution of phospholipids is similar in both types of membranes.

2. MATERIALS AND METHODS

Prothylakoids and thylakoids were isolated from 6-day-old leaves from oat (*Avena sativa* v. Borrus). Leaves were cut with scissors then ground in an electrical blendor equipped with razor blades according to [16] in a medium containing 0.5 M sucrose, 50 mM Mops (pH 7.6), 1 mM EDTA, 1 mM $MgCl_2$, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM aminocaproic acid, 5 mM mercaptoethanol, 1 mM thioglycolic acid, 0.1% (w/v) bovine serum albumin (BSA). Crude oat etioplasts were obtained by differential centrifugations [17] and subsequently purified on a self-generated gradient of Percoll (centrifugation: 100000 × *g*, 30 min in a Ti-70 angle fixed rotor) made of three layers: 6 ml of Percoll 27%, 8 ml of 45% and 6 ml of 65% in a medium similar to that in [18]. The lower band of purified etioplasts was collected with a syringe, washed once in the above medium without Percoll (6000 × *g* for 10 min) then broken by osmotic shock in 50 mM Mops-NaOH (pH 7.6), 3 mM $MgCl_2$, 1 mM EDTA-Na, 0.5 mM PMSF and 0.1% (w/v) BSA. Prothylakoids and prolamellar bodies were separated by sonication and purified by flotation on a linear sucrose gradient as in [19]. After collection of the fractions, prothylakoids (and prolamellar bodies) were sedimented at 300000 × *g* for 1 h and resuspended in various media, as indicated in the figure legends. Crude chloroplasts were isolated according to [20], washed twice and osmotically shocked in 10 mM Mops-NaOH (pH 7.6), 2 mM $MgCl_2$ and 2.5 mM KCl to obtain thylakoids which were finally resuspended in various media as indicated in the figure legends.

Incubation of prothylakoids and thylakoids in the presence of various phospholipases were carried out in the dark under the conditions described in the figure legends. Aliquots of the incubation media were taken at various times for lipid analysis but for zero time controls no phospholipase was present. Phospholipase A_2 and D activities were stopped by an excess of EDTA. Since no variations in galactolipid content was detectable during the incubation, DGDG was used as an internal standard. Lipids were extracted according to [21] and separated by HPTLC in chloroform/methanol/acetic acid/H_2O (85:15:10:3). After I_2 staining, PG, PC and DGDG were scraped off for phosphate [22] or galactose [23] determination. Hydrolysis curves were expressed as semi-log plots of the percentage of residual phospholipids [24]. Chlorophyll was determined according to [25] and proteins as in [26].

3. RESULTS

The rationale of the enzymatic approach used for the determination of acyl lipid transmembrane distribution has been discussed elsewhere [9,27]. Among the prerequisites necessary for the success of this approach four are of particular importance in this study. Firstly, the biological structures under investigation should form closed vesicles. Microscopic studies have shown that prothylakoids obtained by a procedure very similar to ours appear as closed vesicles [19,28–30]. Secondly, each type of structure (prothylakoids or thylakoids) must have, within the same population, an identical orientation. At least two observations show that prothylakoids are right-side-out oriented. Electron micrographs of prothylakoids reveal that all vesicles are heavily studded on their outside faces with small particles identical to CF_1 [28,29]. In addition, we have found that in Triton X-100-disrupted prothylakoids no increase in ferredoxin-NADP$^+$ reductase activity occurred which would have been due to entrapped enzyme or to the presence of inside-out vesicles in the preparation (not shown). Thirdly, since phospholipids are minor components of (pro-)thylakoid membranes, it is crucial to work with highly purified membranes. Our prothylakoid preparation was completely devoid of saponins and phosphatidylethanolamine which are the two most common extra-plastidial contaminants of prothylakoids [31,32]. Finally, the enzymatic approach requires that total phospholipid digestion occurs in control experiments where both sides of the membrane are attacked by the enzyme, i.e. that there is no inaccessible phospholipid. Fig.1 shows that when prothylakoids are disrupted by sonication (i.e. when the enzyme has access to both sides of the membrane) more than 90% of both PG and PC was quickly hydrolyzed. In contrast, in intact prothylakoids, the hydrolysis of phospholipids was only partial. The phospholipids which are resistant to the enzyme attack at 5°C are thought to represent those lipids which are localized in the inner monolayer of the membrane [9,27].

Fig.2 illustrates hydrolysis kinetics of PG and PC, expressed as semilog plots. The justification of this representation is discussed elsewhere [9,24]. Two phospholipases A_2, one from pancreas (fig.2A) and the other from snake venom (fig.2B), having different preference for PG and PC were used [4]. All hydrolysis kinetics displayed several pools of different reactivity (expressed by the slope of the curve). The extrapolation to zero time of the last reactive pool allows the estimation of the transversal distribution of phospholipids [9]. The molar outside/inside ratio was 70 ± 5/30 ± 5 for PG and 50 ± 10/50 ± 10 for PC. This distribution was independent of the type of enzymes used and of the rate of the hydrolysis.

The transmembrane distribution of phospholipids in oat prothylakoids has been compared with that of mature oat thylakoids. Fig.3 presents different hydrolysis kinetics of phospholipids in the presence of the pancreatic phospholipase A_2. The rate and extent of the hydrolysis depended upon the temperature and ionic conditions. Within the limits of the experimental period, a temperature of 20°C was necessary to hydrolyze all phospholipids localized in the external monolayer, i.e. to reach the internal pool (cf. fig.3A and B). Moreover, the addition of 4.7 mM $MgCl_2$ in the basic reaction mixture containing 0.3 mM $CaCl_2$ generally resulted in an increase in the time required to

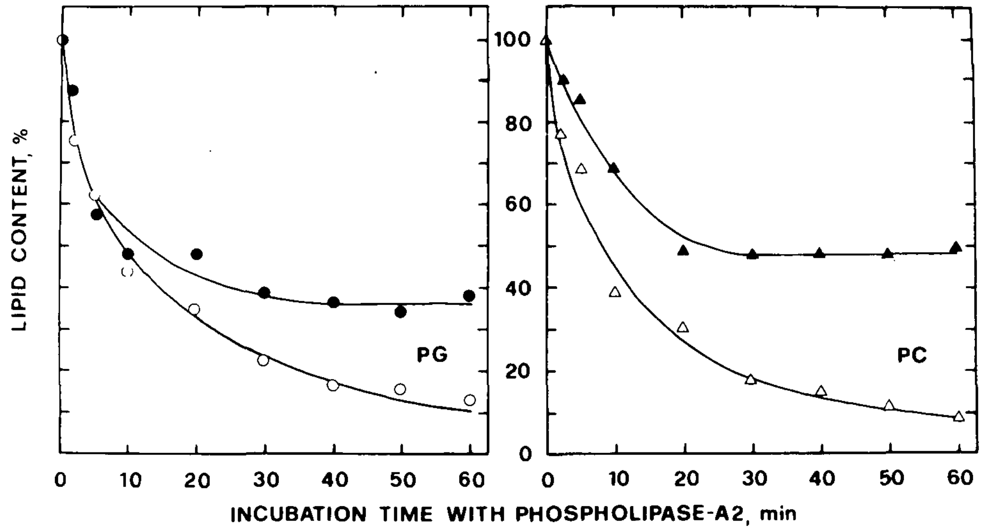

Fig.1. Time course of phospholipid hydrolysis at 5°C of normal (●, ▲) and disrupted (○, △) oat prothylakoids treated with pancreatic phospholipase A_2. Normal prothylakoids were treated as described in section 2. Disrupted prothylakoids were sonicated in the presence of phospholipase A_2 and 0.1 mM EDTA, then the hydrolysis was initiated by adding Ca^{2+}. In both cases, the reaction mixture contained 300 mM sucrose, 50 mM Tricine-NaOH (pH 8), 1 mM $CaCl_2$, 0.8 Sigma units phospholipase A_2/mg protein and prothylakoids (1 mg protein/ml). The 100% values correspond to 88 and 70 nmol/mg protein for PG and PC, respectively.

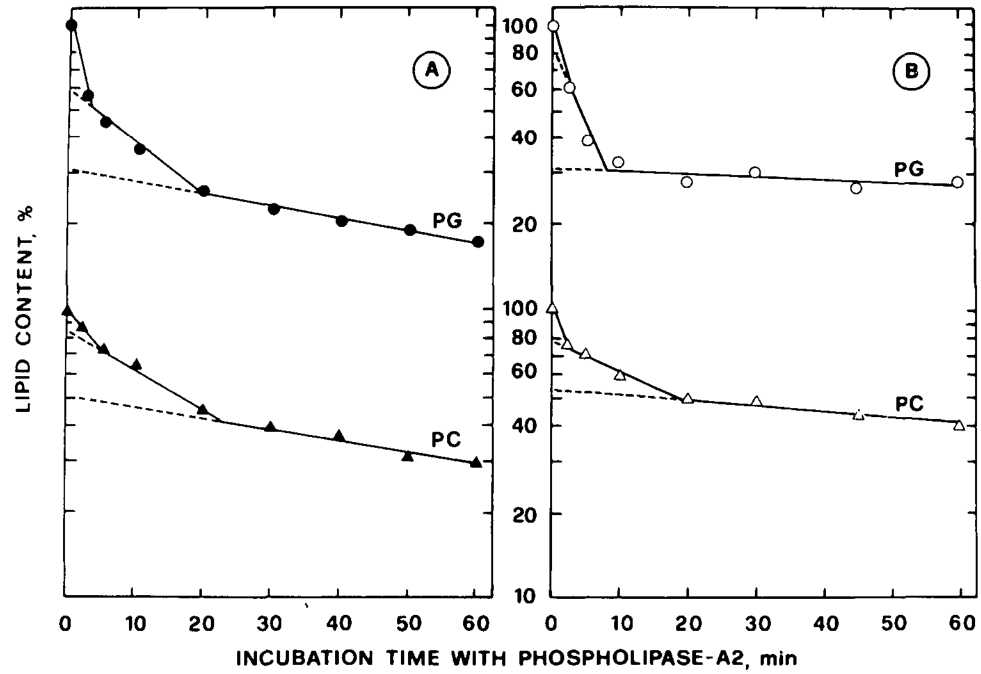

Fig.2. Time course of phospholipid hydrolysis at 4°C of oat prothylakoids treated with either pancreatic (A) or *V. russelli* (B) phospholipase A_2, expressed as semilog plots. In A, the reaction mixture contained 120 mM sorbitil, 5 mM Tricine-NaOH (pH 8), 4 mM $CaCl_2$, 0.8 Sigma units pancreatic phospholipase A_2/mg protein and prothylakoids (1 mg protein/ml). In B, the reaction mixture contained 400 mM sucrose, 50 mM Tricine-NaOH (pH 8), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 mM PMSF, 2 mM mercaptoethanol, 0.02 Sigma units phospholipase A_2 from *V. russelli*/mg protein and prothylakoids (1 mg protein/ml). Symbols and 100% values as in fig.1.

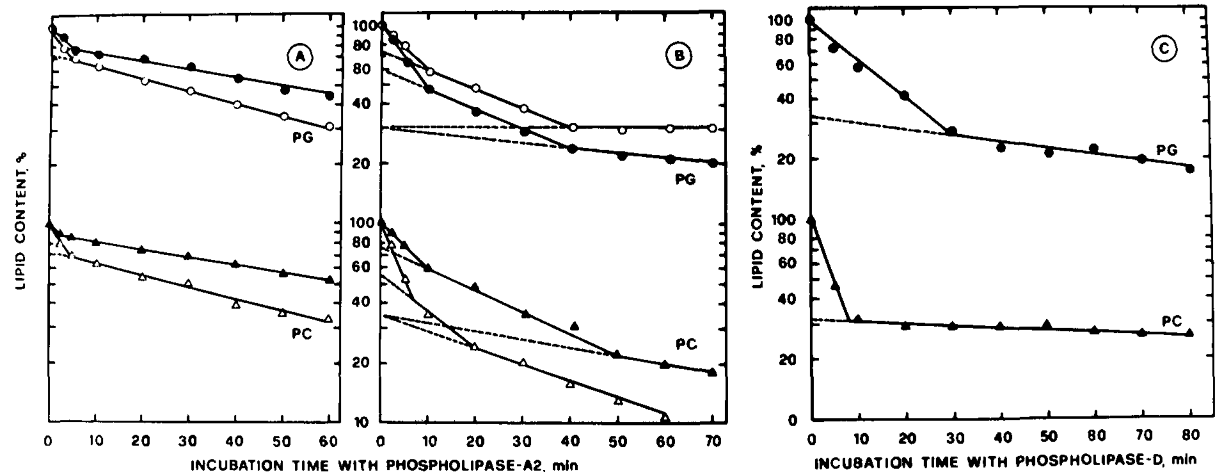

Fig.3. Time course of phospholipid hydrolysis, expressed as semilog plots of oat thylakoids treated with pancreatic phospholipase A_2 (A,B) or with phospholipase D from *S. chromofuscus* (C) under different conditions of temperature and ionic environment. In A and B, the reaction mixture contained 200 mM sorbitol, 15 mM Tricine-NaOH (pH 8), 0.3 mM $CaCl_2$ (open symbols) or 0.3 mM $CaCl_2$ + 4.7 mM $MgCl_2$ (closed symbols), 1 Sigma unit phospholipase A_2/mg chlorophyll and thylakoids (1 mg chlorophyll/ml). Incubations were carried out at 10°C (A) or 20°C (B). In C, the reaction mixture contained 300 mM sucrose, 25 mM Tricine-NaOH (pH 8.4), 35 mM NaCl, 2 mM $CaCl_2$, 20 Calbiochem units/mg chlorophyll of phospholipase D and thylakoids (1 mg chlorophyll/ml). The reaction was carried out at 20°C. Symbols as in fig.1. The 100% values correspond to 176 and 66 nmol/mg chlorophyll for PG and PC, respectively.

hydrolyze both phospholipids localized in the external monolayer. This may be due to competition between Ca^{2+} and Mg^{2+} for the enzyme-binding sites and/or for the interactions with the negative charges at the surface of the membrane. Nevertheless, under any conditions allowing complete phospholipid hydrolysis in the outer monolayer of the thylakoid membrane (fig.3B) the molar outer/inner distribution was 70 ± 5/30 ± 5 for PG and 65 ± 10/35 ± 10 for PC. It is noteworthy that the same distribution was found when another enzyme, i.e. phospholipase D, was used (fig.3C).

4. DISCUSSION

Our results show that the outside/inside distribution of phospholipids is identical for PG (70/30) and similar for PC (50–65/50–35) in prothylakoid and thylakoid membranes from oat. It is known that prothylakoid membranes contain neither light-harvesting chlorophyll *a/b*-protein complex (LHCP) [15] nor PG containing 3(*t*)-hexadecenoic acid [32]. However, the thylakoid membrane is enriched in these two components which have been postulated to be associated with and to preserve the oligomeric structure of LHCP [13]. Therefore, it was expected that the insertion of LHCP together with the appearance of PG containing 3(*t*)-hexadecenoic acid species should modify the prothylakoid transmembrane distribution of both phospholipids. Our results show quite clearly that this is not the case, thereby allowing the following conclusions to be made: (i) Chlorophyll-protein complexes are unlikely to be involved in the origin and stabilization of the asymmetric distribution of PG (and probably of PC); (ii) the incorporation of newly synthesized chlorophyll-protein complexes does not modify the transmembrane arrangement of phospholipids; (iii) lipid asymmetry should be generated at an earlier stage of membrane biogenesis. Microscopic observations [33] and biochemical evidence [34] suggest that prothylakoids have originated from the inner envelope membrane. Thus, if the envelope is the site of lipid asymmetry biogenesis, one may expect, assuming that prothylakoids are formed by invagination of the envelope inner membrane, that the distribution of PG (likely of the other acyl lipids) should be opposite in these two types of membranes. This attractive hypothesis deserves further investigation. An alternative explanation may be that a self-arrangement due to the properties of the lipids themselves is responsible for PG and PC distribution [35].

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SPATIAL ORGANIZATION AND FUNCTIONAL ROLES OF ACYL LIPIDS IN THYLAKOID MEMBRANES

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The structural and functional asymmetry of protein complexes in the plane of and across the thylakoid membrane of higher plants[1] raises the question of whether a similar heterogeneity exists for acyl lipid distribution and if so, what should be its significance in terms of the overall structure and function of thylakoids. In spite of some recent progress concerning the spatial organization of acyl lipids, their role in photosynthetic functions is far from being elucidated.

Lateral asymmetry of acyl lipids in the thylakoid membrane

Several approaches have been adopted to assess the lateral heterogeneity of acyl lipids in the thylakoid membrane (for reports published until Sept. 1984, see ref. in Siegenthaler and Rawyler[2]) : (1) Fractionation of thylakoids into subchloroplast particles enriched in PSI or PSII activities; (2) Separation of appressed and non-appressed regions of thylakoids; (3) and the purification of one of the protein complexes found in the membrane[3,4,5]. The composition in acyl lipids (and proteins) of each of these fractions is determined, compared with the original composition of the thylakoid membrane and, eventually, related to its particular function. It is worth mentioning that almost all procedures for obtaining subchloroplast fractions rely on detergent solubilization of membrane and may therefore present drawbacks but also some advantages. On one hand, it may result in a differential displacement of certain acyl lipids, thereby influencing the final composition of lipids which are associated with the fractions or complexes. But, on the other hand, it may help to reflect the presence of tightly bound and specific associations of particular acyl lipids with membrane proteins.

As an example, a recent study by Murphy and Woodrow[6] illustrates this fractionation strategy. Compared to whole thylakoids, appressed regions

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Abbreviations : BSA, bovine serum albumin; DGDG, digalactosyldiacylglycerol; EGTA, Na_2-ethylene glycol-bis (B-amino-ethyl ether)N,N,N',N' tetraacetate; LHCP, light harvesting chlorophyll a/b protein complex; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; PL, phospholipid; PLA_2, phospholipase A_2; PSI, PSII, photosystem I,II; SQDG, sulphoquinovosyldiacylglycerol.

are characterized by a light-induced extrusion of protons, a depletion of acyl lipids, an equal amount of protein and an enrichment of chlorophyll, a lower (about half) lipid/protein ratio, a depletion of PSI complex and an enrichment of LHCP. In addition to this gross asymmetry, there are also considerable heterogeneities in acyl lipid class distribution between the two membrane fractions. On the basis of mol %, the most striking difference is an increase in PG of appressed regions whilst on the basis of protein there is a decrease in all lipid classes, except in PG. Appressed regions have a much higher MGDG/DGDG (2.4 vs 1.3), contain SQDG molecular species which are depleted in $C_{18:3}$ and enriched in $C_{16:0}$, and PG species which are slightly enriched in 3(t)-$C_{16:1}$.

Recently, we made the proposal that each lipid class consists of several topologically distinct pools which are separated laterally and transversally in the membrane[2]. If this hypothesis is true, one should find an increased heterogeneity of acyl lipids in membrane particles of decreasing size and complexity. Four examples show that this proposal is likely to be correct : (1) The oligomeric form of LHCP is greatly enriched in MGDG and (3t)-$C_{16:1}$ PG species[7]; (2) A photochemically active reaction center complex II contain only two lipid classes (MGDG and SQDG) which are characterized by a low degree of unsaturation and by a complete absence of $C_{16:3}$ in MGDG species; (3) A purified CF_o-CF_1 preparation is highly enriched in SQDG and completely depleted in MGDG and phospholipids[4]; (4) A cytochrome b_6/f complex preparation contains only phospholipids[5]. A general feature of all these particles or complexes is that they are isolated as lipo-protein complexes.

In conclusion, the thylakoid membrane is characterized by considerable lateral heterogeneities in the distribution of all major membrane components, including acyl lipids.

Transversal asymmetry of acyl lipids in the thylakoid membrane

Several methods have been used to assess transversal localization of acyl lipids in the thylakoid membrane [for reports published until Sept. 1984, see ref. in (2)] : (1) Chemical labelling[8,9,10]; (2) Lipid exchange and (3) Immunological procedures; (4) Selective lipid extraction; (5) Enzymatic modification[8,10,12]. In our opinion, this last method has been the most rewarding since it allows one not only to assess the transmembrane distribution of acyl lipids but, simultaneously, to draw relationships between the hydrolysis (or depletion) of certain lipids and the impairment of particular photochemical functions in the thylakoid membrane. The precautions, criteria, special requirements, drawbacks and advantages of this approach have been discussed in detail recently[11,13]. Using this technique, the transversal distribution of galactolipids[10,11] and phospholipids[8,12,14,15] has been estimated, in several higher plant species.

As an example, the distribution in mol % of acyl lipids between the outer and inner monolayers of the spinach thylakoid membrane[10-12,14,15] is illustrated in Fig. 1A. It can be seen that the outer leaflet is mainly enriched in MGDG and PG whilst DGDG is confined essentially in the inner leaflet. When the distribution of each acyl lipid is expressed as % of total lipids, the results show the following striking features (Fig. 1B) : (1) Each class of lipids is asymmetrically distributed across the thylakoid membrane; (2) MGDG constitutes about 70% of the total lipids in the outer monolayer and only 38% in the inner leaflet; (3) Both

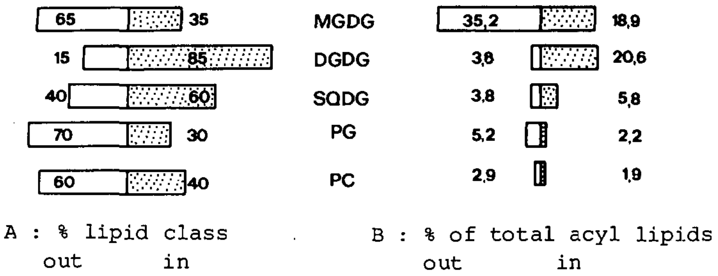

Fig. 1. Distribution of acyl lipids in the two monolayers of the spinach thylakoid membrane

galactolipids (MGDG + DGDG) are equally distributed in both leaflets; (4) The ratio MGDG/DGDG is high (9.8) in the outer leaflet and very low (0.9) in the inner leaflet; (5) The ratio of bilayer/non-bilayer forming lipids is 0.44 and 1.61 in the outer and inner leaflets of the thylakoid membrane. Based on the results reported by Sprague and Staehelin[16] one can conclude that in the spinach thylakoid membrane, the acyl lipid distribution favors a lamellar configuration in the inner leaflet whilst in the outer one both hexagonal and lamellar configurations may coexist. Since the thylakoid membrane is a bilayer structure under physiological conditions, it is obvious that other components (e.g. proteins, ions, etc.) must contribute to the stabilization of lamellar configurations in the outer leaflet, as discussed recently[10].

Role of acyl lipids in photochemical activities

All the approaches proposed in the literature to scrutinize the relationships between acyl lipids and the functions of the thylakoid membrane are aimed at modifying the content, the structure or the composition of acyl lipids and measuring simultaneously one or several photochemical activities. The approaches are (up to Sept. 1984, see ref. in[2]) : (1) Aging of thylakoids in vitro and senescence[17]; (2) Extraction of membrane lipids by organic solvents; (3) Modulation of fatty acid composition by catalytic hydrogenation of acyl chains[18], seasonal variations and treatment by substituted pyridazinones[19], etc.; (4) Reconstitution studies[20,21]; (5) Lipid composition of thylakoids and subchloroplast fractions[3,4,5,22]; (6) Modification of the fluidity of the membrane; (7) Immunological studies; (8) Biogenesis studies; (9) Lipolytic treatments[23,24]. Although the importance of acyl lipids has been recognized to support the photosynthetic competence of leaves, chloroplasts and thylakoids (namely in maintaining the appropriate fluidity of the thylakoid membrane), most of these studies have failed to show a direct correlation between the presence of a certain lipid and a specific photochemical activity. However, very recently, we have succeeded to show that discrete and topologically distinct populations of phospholipids and MGDG are likely to be involved directly in electron transport activity.

Thylakoids were incubated in the presence of either the pancreatic phospholipase A_2 or the lipase from Rhizopus arrhizus under conditions (± bovine serum albumin, BSA, ± EGTA, appropriate pH, T and ionic

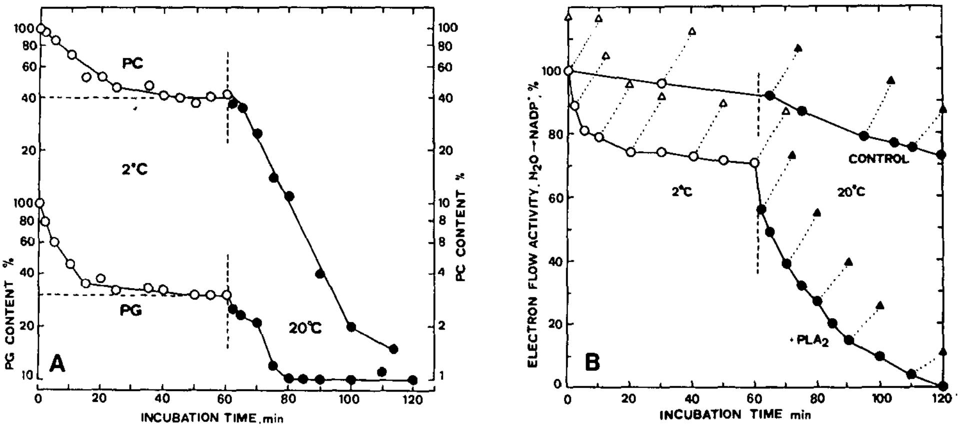

Fig. 2. Kinetics of the hydrolysis of PG and PC in phospholipase A_2-treated thylakoid membranes (A) and of the uncoupled non-cyclic electron flow activity $H_2O/NADP^+$ (B). △,▲ : activity following BSA addition.

strength) allowing depletion of successive pools of phospholipids or MGDG both in the outer then in the inner monolayer of the thylakoid membrane. As an example, Fig. 2 shows that the hydrolysis of phospholipids which are localized in the outer monolayer of the spinach thylakoid membrane (60% of PC and 70% of PG) causes about 20-25% inhibition of the uncoupled non-cyclic electron flow activity. This inhibition is mainly due to hydrolysis products (free fatty acids and lyso-phospholipids) as shown by the restoration effect of BSA. The activity begins to be inhibited greatly only when the hydrolysis of these phospholipids located in the inner monolayer occurs. In the presence of BSA, the restoration of the activity is only partial indicating that phospholipid depletion is the cause of the irreversible BSA-insensitive inhibition. Fig. 3 shows the extent of uncoupled non-cyclic electron flow activity inhibition ($H_2O/NADP^+$) expressed as a function of phospholipids destroyed under conditions where, first, the outer pool of phospholipids and, then the inner pool is hydrolyzed. In the presence of BSA which removes completely free fatty acids and lyso-phospholipids, 4 populations of PG and PC molecules can be depicted : (1) Depletion of the 1st population (about 60% for PG and 40% for PC) which is exclusively located in the outer monolayer affects only slightly the activity ($<10\%$); (2) Depletion of the 2nd population (from 60 to 80% for PG and from 40 to 80% for PC) which is located in both monolayers, causes an inhibition of the activity up to about 30%; (3) Depletion of the 3rd population (from 80 to 90% for both PG and PC) inhibits the greatest part of the activity (30 to 100%); (4) The last population (5-15% remaining in the membrane) which is located in the inner monolayer does not support this type of activity.

In order to ascertain that the "barrier" properties of thylakoids are preserved during PLA_2 treatment, we have studied their osmotic response in sorbitol solutions (Fig. 4). It can be seen that under all experimental conditions, the mean packed volumes of thylakoids vary linearly with the reciprocal of sorbitol concentration. Phospholipase A_2-treated thylakoids have a larger volume after 60 min at 2°C (i.e., when only those phospholipids localized in the outer monolayer are hydrolyzed) and also after 120 min at 20°C (i.e., when all phospholipids are destroyed). These changes can be attributed to the hydrolysis products only since an

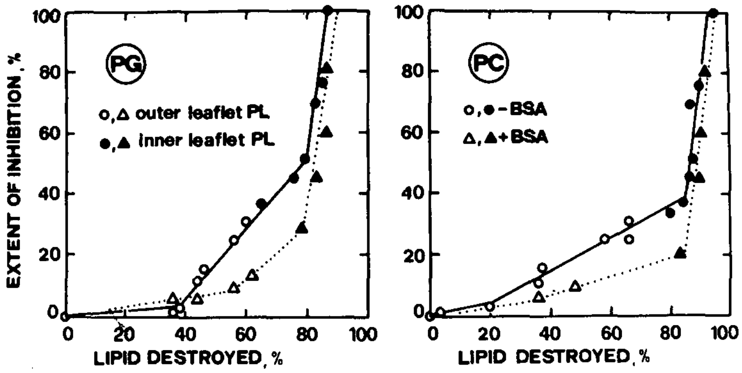

Fig. 3. Relationship between the extent of non-cyclic electron flow activity inhibition and successive phospholipid depletion in the outer and inner monolayers of the spinach thylakoid membrane.

addition of BSA to phospholipase A_2-treated thylakoids decreases the packed volumes close to the values obtained in the BSA-controls. Thus, the osmotic properties of the membrane (toward sorbitol) are essentially preserved in spite of extensive phospholipid breakdown. Therefore, the rapid hydrolysis of both PG and PC observed at 20°C under the conditions of Fig. 2 is not due to thylakoid disruption but to increased outward transbilayer movement of phospholipids.

CONCLUSIONS

(1) Acyl lipids are heterogenously distributed in the plane of the thylakoid membrane. Evidences come mainly from experiments carried out with subchloroplast fractions. A general feature is that all particles or complexes isolated from thylakoids are of lipoprotein nature. Depending on the degree of complexity of the particles, some of them are completely devoided or enriched in certain lipid classes which are generally highly saturated compared to those of the thylakoid membrane.

(2) Several approaches, but mainly the enzymatic one, have revealed that acyl lipids are asymmetrically distributed across the thylakoid membrane : 65 mol % MGDG, 15% DGDG, 70% PG, 60% PC and a maximum of 40% SQDG are localized in the outer monolayer. Glycolipids (MGDG + DGDG + SQDG) as well as phospholipids (PG + PC) are equally distributed in both monolayers but MGDG, a non-bilayer forming lipid, is by far the most predominant class in the outer leaflet. Its role remains to be elucidated. The ratio bilayer/non-bilayer forming lipids (in mol % of total acyl lipids) is 0.44 in the outer leaflet which favors hexagonal configurations. The ratio value which is 1.61 in the inner leaflet imposes lamellar configurations.

(3) The relationships between acyl lipids and photochemical activities are extremely complex. Several parameters are involved in sustaining maximal photosynthesis, the fluidity of the thylakoid membrane being only one of them. Based on "our" enzymatic approach and focusing our attention mainly on phospholipids, we can draw the following conclusions : (a) Phospholipids consist of at least 4 distinct populations

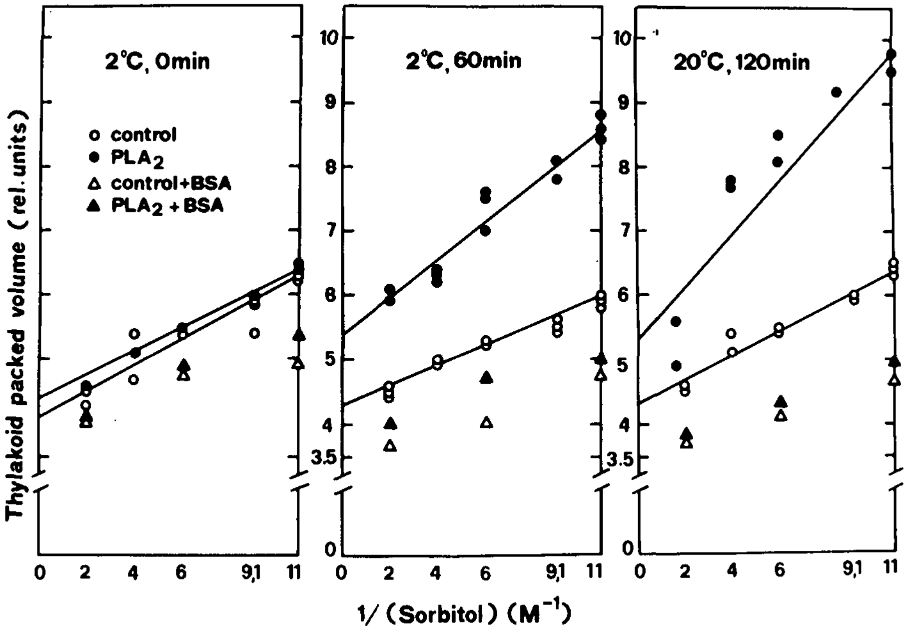

Fig. 4. Mean packed volumes of control and phospholipase A_2-treated thylakoids as a function of the reciprocal of sorbitol concentration. Temperature and time conditions are those of Fig. 2.

which are separated transversally (and laterally ?) in the membrane; (b) We can therefore visualize that each of these populations plays a specific role in the membrane; (c) This is illustrated by our finding that, in the case of uncoupled non-cyclic electron flow, the activity is directly dependent on the 3rd PG and PC populations which are entirely localized in the inner monolayer and which represent only 10% of the total phospholipid molecules. This may explain why in the past, it has been so difficult to establish a correlation between phospholipid depletion and a specific photochemical function; (d) It is noteworthy that in contrast to the above observation, the same activity depends on the MGDG molecule populations which are located in the outer monolayer[24]; (e) These observations do not exclude that other functions may depend on other phospholipid or galactolipid populations; (f) These results give further support to our proposal that acyl lipids are likely to be organized into distinct and discrete pools, each of them having a structural or/and a functional role in the thylakoid membrane[2].

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EFFECTS OF MONOGALACTOSYLDIACYLGLYCEROL (MGDG) DEPLETION ON PHOTOSYNTHETIC ACTIVITIES IN OAT THYLAKOID MEMBRANES

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1. INTRODUCTION

One approach to studying the role of galactolipids in thylakoid membranes has been to incubate membranes with lipolytic enzymes and then to determine the relationship between the changes occuring in their lipid content and composition and simultaneously in their photochemical activities[1]. However, in most studies[2-4], galactolipids have been deacylated by means of unspecific lipases which do not allow an unequivocal assessement of function to each lipid. Moreover, it is often difficult to distinguish between the effects due to the hydrolysis of the parent-lipids themselves and to those resulting from hydrolysis products. In this investigation, special conditions were used allowing lipase from Rhizopus arrhizus to selectively hydrolyze MGDG in the outer monolayer of the thylakoid membrane.

2. MATERIALS AND METHODS

Oat (Avena sativa L. cr. Burrus) seedlings were grown in daylight regime during 6 days. Chloroplasts and thylakoids therefrom were prepared as described previously[5]. Thylakoid preparations were resuspended in 5 mM MOPS-NaOH pH 7.6, 10 mM NaCl, 5 mM $MgCl_2$, 100 mM sorbitol and 30 mg BSA/ml. After a 0 time sampling, the hydrolysis kinetic was initiated by addition of 10 Boehringer units of the lipase from Rhizopus arrhizus. Lipids were extracted and analysed as previously described[6]. Electron flow rates and cytochrome f photooxidation were determined according to[7,8]. Low temperature fluorescence spectra, recorded on a Bausch & Lomb fluorimeter, were uncorrected.

3. RESULTS AND DISCUSSION

Fig. 1 shows that at 0°C, only MGDG is significantly digested. Simultaneously to MGDG hydrolysis, lyso-MGDG and free fatty acids are formed. After 40 min, 62% of the total MGDG is hydrolyzed, which correspond to the amount of MGDG localized in the external monolayer of the thylakoid membrane, as reported recently[6]. Thus, the outside/inside MGDG molar distribution is 62/38, as can be estimated by extrapolation of the last pool to zero time[5]. When oat thylakoids are treated with the lipase in the absence of BSA (Fig. 2), both $H_2O/NADP^+$ and $H_2O/MV-O_2$ activities are quickly

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Abbreviations : BSA, defatted bovine serum albumin; DAD, diaminodurene; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenol-indophenol; DQH_2, durohydroquinone; LRa, lipase from Rhizopus arrhizus; MGDG, monogalactosyldiacylglycerol; MOPS, 3-(N-morpholino)propanesulfonic acid; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine.

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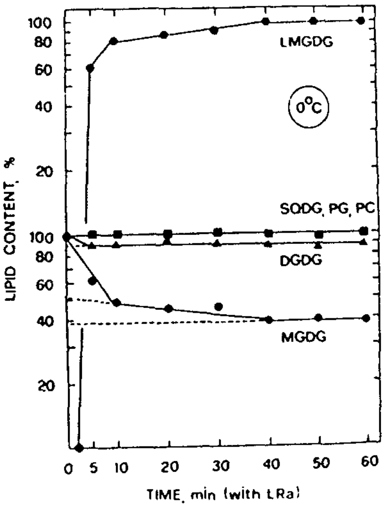


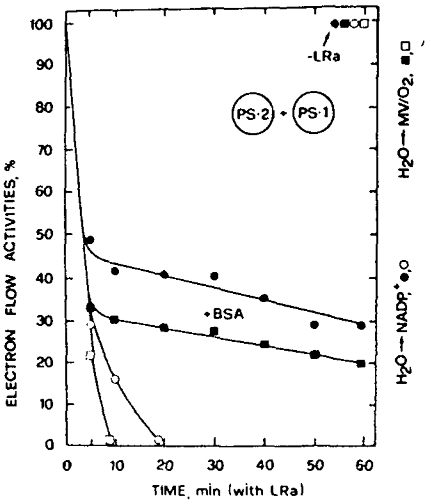


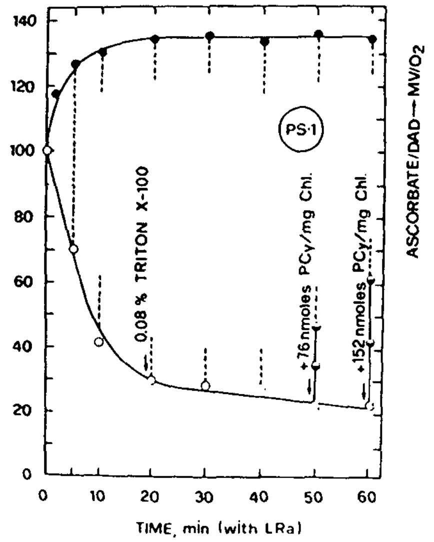
Fig. 1. (left side) : Kinetics of MGDG hydrolysis in oat thylakoids, in the presence of the lipase from Rhizopus arrhizus (LRa) and BSA. The 100% value correspond to 1.11 µmol/µmol Chl for MGDG.

Fig. 2. (right side) : Effect of LRa treatment on non-cyclic electron flow activities, in absence or presence of BSA. The 100% activities correspond to 80 µmol NADPH/mg Chl/h ($H_2O/NADP^+$) and to 130 µmol O_2 consumed/mg Chl/h ($H_2O/MV-O_2$).

inhibited. BSA, which removes all free fatty acids and half of the lyso-MGDG released during the breakdown of MGDG molecules (which are confined in the outer leaflet), protects only partially these photochemical activities. The difference of inhibition between LRa-treated thylakoids in the presence and absence of BSA can be attributed to the release of free fatty acids and lyso-MGDG in the membrane. The residual amount of lyso-MGDG, which is not removed by BSA, is likely to be under the limit thought to be harmful for electron flow activities[9]. Thus, BSA was systematically added in the LRa-containing reaction mixtures.

In other experiments (not shown), it was found that LRa treatment affects similarly several types of PSII electron flows which are sustained by different artificial (1,5-diphenylcarbazide, H_2O_2) or natural (H_2O) donors. Therefore, it is unlikely that the site of inhibition of PSII is at the level of the water splitting system. When silicomolybdic acid is used (in the presence of DCMU) to intercept electrons directly on Q_A, the extent and rate of PSII inhibition are unchanged, indicating that MGDG depletion probably alters the electron transfer between Z and Q_A.

Several types of PSI electron flow activities are inhibited when the outer pool of MGDG is hydrolyzed. The decrease of the $MV-O_2$ reduction rate



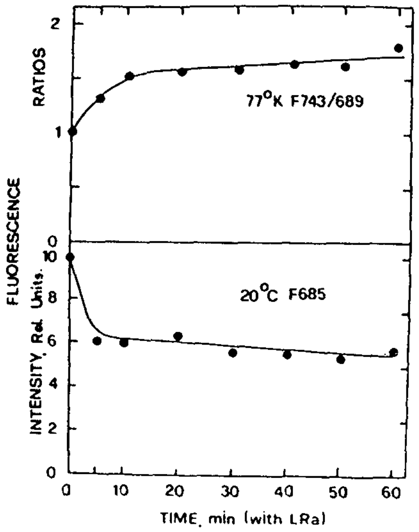


Fig. 3. (left side) : Effect of Triton X-100 and plastocyanin (PCy) on electron flow activity in LRa-treated thylakoids. Electron flow activity (%) of LRa-treated thylakoids (O), restored by Triton X-100 (●) or plastocyanin (●).

Fig. 4. (right side) : Effect of a treatment of LRa on the 20°C fluorescence F_{685} and on the 77°K fluorescence ratio F_{743}/F_{689}.

is dependent neither on the type of electron donors (ascorbate-DCPIP, - DAD, -TMPD, DQH_2) used, nor on the sites of electron donation [plastoquinone (with DQH_2) or the cytochrome f/plastocyanin region], nor on the type of electron flow which can be linked or not to H^+ transport. However, after LRa treatment, all PSI-dependent electron flows can be restored fully by adding Triton X-100 and only partially by large amounts of plastocyanin (Fig. 3) suggesting that the inhibition site is not at the level of PSI itself but elsewhere, e.g. at the level of the cyt b_6/f-plastocyanin region. The restoration effect of Triton X-100 on PSI activity can be explained by a direct accessibility of the artificial donor to P700 (reaction center of PSI). The ineffective effect of plastocyanin on PSI restoration is probably due to its unability to reach its native locus in the membrane or because the inhibition site is upstream. The inhibition of cyt f photooxidation and dark reduction after LRa treatment is in agreement with this last hypothesis.

It was found also that MGDG depletion results in a decrease of the 20°C 685 nm fluorescence intensity and an increase of the 77°K F_{743}/F_{689} nm ratio (Fig. 4). Therefore, it is likely that, besides the alteration of the basic electron flow, energy distribution between the two photosystems is also impaired and this at the expense of PSII. The decrease of the relative quantum yield of PSII, at limiting light intensities when the outer pool of MGDG is degraded (not shown) agrees with this hypothesis.

Thus, it is proposed that the hydrolysis of MGDG in the outer monolayer of the oat thylakoid membrane (1) causes an inhibition of the electron flow at two different sites : one at the level of the cyt b_6/f region and the other one at the level of PSII complex; (2) favors the transfer of energy to PSI at the expense of PSII. In addition, all these effects are likely to be due to the hydrolysis of the parent-MGDG itself but not to the hydrolysis products. It is interesting to mention that in contrast to the above observations, depletion of phospholipids in the outer monolayer of the spinach thylakoid membrane alters the non-cyclic electron flow activity to a much lesser extent (about 20%) than MGDG depletion[10]. These observations give further support to our recent proposal that in the thylakoid membrane acyl lipids are spatially arranged in distinct domains, each of them having a specific role in the photosynthetic function[1,10].

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EVIDENCES FOR DIFFERENT ACYL LIPID DOMAINS IN SPINACH AND OAT THYLAKOID MEMBRANES SUPPORTING VARIOUS PHOTOSYNTHETIC FUNCTIONS

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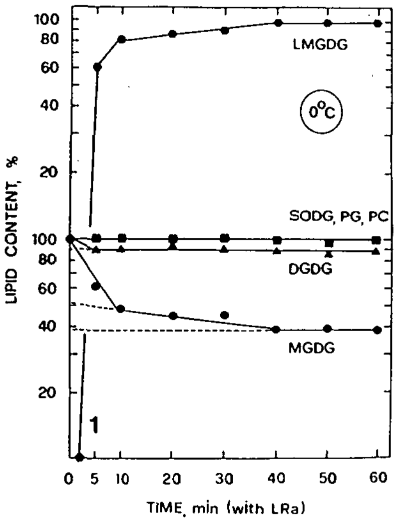
INTRODUCTION

One approach to studying the role of acyl lipids in thylakoid membranes has been to incubate intact thylakoids with lipolytic enzymes and then to determine the relationships between the changes occuring in their lipid content and composition and, simultaneously, in their photochemical activities[1-3]. Whilst phospholipases hydrolyze specifically the two phospholipids (PG and PC) encountered in the thylakoid membrane, lipolytic acyl hydrolases deacylate not only glycolipids (MGDG,DGDG,SQDG) but also phospholipids. The non-specificity of the enzymes used does not allow an unequivocal assessement of function to each of these lipids[4]. Moreover, in most studies, hydrolysis of acyl lipids have been carried out under conditions allowing the attack of all acyl lipids, i.e. of lipids which are localized indistinctly in both monolayers of the membrane. However, it is now well established that all acyl lipids of the thylakoid membrane present a transversal asymmetric distribution[1,3-8], suggesting that different acyl lipid domains may sustain different functions[1,3]. Thus, it would be of great interest to establish relationships between the hydrolysis of specific acyl lipids in the outer membrane monolayer only and the resulting changes occurring in various electron flow activities.

To this aim, we have designed conditions under which (1) only PG + PC or MGDG are hydrolyzed in the outer leaflet of the thylakoid membrane; (2) no transbilayer movement of acyl lipids occurs and (3) the deleterious hydrolysis products (e.g. free fatty acids, lysophospholipids or lyso-MGDG) are removed from the membrane by BSA. Simultaneously, we have measured several photochemical parameters (various electron transport activities and fluorescence characteristics, etc.). Results show that the selective hydrolysis of certain acyl lipids in the outer monolayer of the thylakoid membrane causes preferential effects on these activities.

Abbreviations : BSA, defatted bovine serum albumin; DAD, diaminodurene; DCPIP, 2,6-dichlorophenolindophenol; DGDG, digalactosyldiglyceride; MGDG, monogalactosyldiacylglyceride; PC, phosphatidylcholine; PG, phosphatidylglycerol; PLA_2, phospholipase A_2; SQDG, sulfoquinovosyldiacylglyceride; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine.



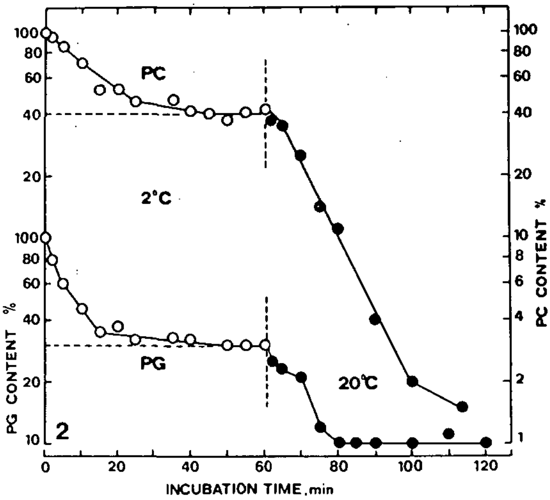


Fig. 1. (left side) Kinetics of MGDG hydrolysis (at 0°C) in oat thylakoid membranes, in the presence of the lipase from Rhizopus arrhizus (LRa) and BSA. The 100% value correspond to 1.11 µmol/µmol Chl for MGDG.

Fig. 2. (right side) Kinetics of PG and PC hydrolysis (at 2° and 20°C) in spinach thylakoid membranes in the presence of porcine pancreas PLA_2. The 100% value correspond to 0.2 (PG) and 0.13 (PC) µmol/µmol Chl.

RESULTS

Fig. 1 shows clearly that at 0°C, only MGDG is significantly digested by the lipase from Rhizopus arrhizus in the presence of BSA. After 40 min, 62% of the total MGDG are hydrolyzed. This amount corresponds to MGDG molecules localized in the outer monolayer of the oat thylakoid membrane. The addition of BSA removes all free fatty acids and about half of lyso-MGDG (LMGDG). Several changes in photochemical activities occur during MGDG depletion in the outer monolayer (not shown) : (1) If BSA is omitted, the two non-cyclic electron flow activities [$H_2O/NADP^+$ and H_2O/MV (methyl-viologen)-O_2] are inhibited more rapidly and at a greater extent than in the presence of BSA; this indicates that the BSA-insensitive inhibition of the activity is due to MGDG depletion; (2) PSII electron flow activities are also inhibited whatever the type of electron donors (H_2O, 1,5-diphenyl-carbazide, H_2O_2) or acceptors (2,5-dimethyl-p-benzoquinone, DCPIP, p-phenylene diamine, silicomolybdate) used. This shows that MGDG depletion in the outer monolayer does not affect the water splitting system; (3) PSI electron flow activities are inhibited rapidly whatever the type of electron donors (durohydroquinone, ascorbate-DCPIP, -DAD or -TMPD) used. The decreased activity can be fully restored by Triton X-100 (likely by allowing direct access of the donor to P_{700}) but not by an excess of plastocyanin; (4) Both rates and amplitudes of photooxidation and dark reduction of cytochrome f are decreased suggesting an inhibition site at the level of cyt b_6/f region; (5) The ratio of 77°K fluorescence F743/F689 increases, suggesting that energy is transferred to PSI at the expense of PSII, as indicated also by the lower quantum yield of the treated samples.

Fig. 2 shows that at 2°C, the hydrolysis of both phospholipids occurs in phospholipase A_2-treated spinach thylakoids. After 60 min 70% of PG and 60% of PC are digested. This amount correspondsto phospholipid molecules localized in the outer monolayer. This important phospholipid depletion causes only about 20-25% inhibition of the non-cyclic and PSII electron flow activities which can be restored partially in the presence of BSA. The activity begins to be inhibited greatly only when the hydrolysis of phospholipids located in the inner monolayer occurs (due to rapid transbilayer movement at 20°C, see Fig. 2). In the presence of BSA, the restoration of the activity is only partial suggesting that phospholipid depletion in the inner monolayer of the membrane is the cause of the BSA-insensitive inhibition.

In conclusion, our results show that optimal electron flow activity is dependent on the integrity of not only a population of MGDG molecules which is localized in the outer monolayer but also of a population of phospholipid molecules which is located in the inner monolayer. This finding is in agreement with our previous proposal[1,3].

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Regular paper

The transmembrane distribution of galactolipids in chloroplast thylakoids is universal in a wide variety of temperate climate plants

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Key words: lipid asymmetry (comparative), mono(di)galactosyldiacylglycerol, (non)-bilayer forming lipid, thylakoid membranes

Abstract. The transmembrane distribution of monogalactosyldiacylglycerol and digalactosyldiacylglycerol was determined in chloroplast thylakoids from a range of temperate climate plants. These plants included dicotyledons, monocotyledons, $C_{16:3}$ and $C_{18:3}$ plants and herbicide-resistant species. In all the thylakoids examined monogalactosyldiacylglycerol was enriched in the outer leaflet (53–65%) while digalactosyldiacylglycerol was highly enriched in the inner leaflet (78–90%). The non-bilayer forming monogalactosyldiacylglycerol represented 55–81% of the total acyl lipids of the outer monolayer. The relative acyl lipid composition of both leaflets of the thylakoid membrane indicates that the lamellar structure is strongly favored in the inner monolayer, whereas the outer one presents a metastable character which allows the probable coexistence of both lamellar and non-lamellar phases. The consequence of this asymmetry for the stability and function of the thylakoid membrane is discussed.

Introduction

The thylakoid membranes of all oxygenic photosynthetic organisms are rich in glycosylglycerides [18]. Two galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) predominate and, because of the preponderance of photosynthetic membranes in nature, MGDG is the world's most prevalent membrane lipid [14, 19].

It seems logical to suggest that the highly conserved lipid composition, which is observed in photosynthetic membranes from cyanobacteria, all types of algae and higher plants, confers specific advantages for these organisms [19]. Indeed, there has been much recent interest on the role of lipids in thylakoid structure and function [23, 44]. Such experiments have been to examine the correlation of lipid composition with photosynthetic function in developing chloroplasts [4, 18], the role of specific lipids in thylakoid structure [8], the effect of lipid hydrolysis on photosynthesis

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[24, 36, 37], the lipid composition of isolated thylakoid chlorophyll-proteins [35] and experiments on reconstitution [17, 39].

A better understanding of the role of acyl lipids in thylakoid function will obviously depend on a detailed knowledge of their molecular organisation in the membrane. Results so far of both the lateral and sided distribution of individual thylakoid lipids have provided data which is not always in good agreement. When lateral distribution is determined through the isolation of appressed and non-appressed membranes from Pea by mechanical methods [6], little difference (apart from a very slight enrichment of MGDG in appressed membranes) is found. With regard to the sided distribution of individual lipids, we only have appreciable data for a single class – the major phospholipid, phosphatidylglycerol. In chloroplast thylakoids from a variety of plants, phosphatidylglycerol has been shown to be enriched (70–75%) in the outer (stromal) leaflet [13, 36, 50]. In contrast, there have been few studies on the major lipid class, the galactolipids, and these have given different results – perhaps due to the various thylakoid preparations and experimental conditions which have been used. In a recent study of the transverse localization of MGDG and DGDG in spinach thylakoids, we examined the experimental conditions for sidedness determination very carefully. The results showed that MGDG was enriched in the outer leaflet while DGDG was highly enriched in the inner leaflet [38]. However, spinach is a dicotyledon which has a particular lipid metabolism which corresponds to a $C_{16:3}$-plant and is indicative of a rather primitive (prokaryotic) emphasis in its acyl lipid metabolism [21]. In order to see whether the sided distribution of MGDG and DGDG, which had been found for spinach thylakoids, applied to those from different plant types we undertook the present comparative study. The results show a remarkable similarity in the sided distribution of galactolipids in thylakoids from a wide variety of plants. This has important implications for experiments designed to test the function of galactosylglycerides in photosynthetic membranes.

Materials and methods

Lipase (EC 3.1.1.3) from *Rhizopus arrhizus* was purchased from Boehringer and used without further purification. Galactose oxidase (*Dactylium dendroides*) and glucose oxidase (*Aspergillus niger*) were from Sigma. Sodium boro[^{3}H]hybride (8.5 GBq/mmol) was purchased from Amersham International (Amersham, Bucks, UK).

Lettuce (*Lactuca sativa*) was obtained from the local supermarket. Pea (*Pisum sativum* cv. Feltham First) and barley (*Hordeum vulgare* cv. Maris Otter) were germinated and grown as described previously [50]. Spinach (*Spinacia oleracea*) and oat (*Avena sativa*) were germinated and grown in controlled conditions [43]. Black nightshade (*Solanum nigrum*, atrazine-resistant and sensitive species) was grown in the greenhouse of the Botanical Garden of Neuchâtel University.

Lipase incubations and lipid analyses

Chloroplasts and thylakoids therefrom were prepared from *Spinacia oleracea, Solanum nigrum,* and *Avena sativa* leaves [38] and from *Lactuca sativa, Hordeum vulgare,* and *Pisum sativum* leaves as previously described [50]. None of the thylakoid fractions contained any endogenous acyl hydrolase activity. The thylakoid preparations were resuspended in 300 mM sorbitol, 0.8 mM MOPS/NaOH (pH 7.6), incubated with lipase and aliquots withdrawn as in [38]. Lipids were extracted and known aliquots of the extracts were spotted on Type 60 silica gel-coated glass plates, separated, the position of the bands revealed and the lipids quantitated as previously described [38]. For analyses by fatty acid quantitation, the bands were revealed by spraying with 0.05% methanolic 8-anilino-4-naphthalene-sulphonic acid and viewing under UV light. The acyl groups were then estimated as fatty acid methyl esters by GLC [50]. Potato leaf acyl hydrolase was prepared using palmitoylated gauze [22] by the method of Matsuda and Hirayama [28]. Incubations with the purified acyl hydrolase were carried out under the same conditions as for the *Rhizopus lipase* and the lipids were extracted and analysed as previously [50].

Galactose oxidase labelling

Galactolipids were labelled in thylakoid preparations using a galactose oxidase preparation from *Dactylium dendroides* and sodium boro[^{3}H] hybride under the conditions described by Steck and Dawson [47]. Control thylakoids to which the galactose oxidase had access from both sides were prepared in the presence of 0.1% Triton X-100 [47] or by bath sonication of the thylakoids in the presence of the labelling reagents [50]. Results were corrected for non-enzymatic blanks and labelling was carried out at 5 °C to reduce non-specific labelling. The reaction was stopped and lipids extracted by the Garbus et al.'s method [12] and the washed lower phase subjected to thin layer chromatography [50]. The separated galactolipids were hydrolyzed with 6% methanolic KOH and their acyl and headgroup moieties analysed separately for radioactivity [20]. Radio-counting was carried out with quench correction as described previously [50].

Chlorophyll and protein determination

Chlorophylls *a* and *b* were estimated by the method of Arnon [1] and proteins by the Bradford [5] or Lowry techniques [27].

Results and discussion

Table 1 shows the values of two important biochemical parameters for the various plant species studied. In all plant species studied, the total chlorophyll/protein ratios were not significantly different within the usual range observed as did also the chlorophyll *a*/*b* ratios (not shown). The lipid/protein ratio showed a greater variability between plant species, *L. sativa* giving the highest and *P. sativum* the lowest values.

Table 1. Chlorophyll- and acyl lipid to protein ratios, galactolipids composition and asymmetry in thylakoids isolated from various plan species. Figures are the averages of n determinations. Values for different preparations were within 5% of each other. Control incubations showed that all MGDG and most of DGDG (> 90 mol %) were available for lipase digestion.

	Spinacia oleracea	*Solanum nigrum*		*Lactuca sativa*	*Pisum sativum*	*Hordeum vulgare*	*Avena sativa*
		R	S				
n =	(5)	(5)	(5)	(2)	(2)	(2)	(3)
Total Chl/protein (μmol/mg)	0.15	0.15	0.16	0.16	0.11	0.12	0.17
Total acyl lipid/protein (μmol/mg)	0.42	0.43	0.37	0.46	0.27	0.32	0.32
MGDG (mol % of total acyl lipids)	55	53	55	43	42	52	61
DGDG (mol % of total acyl lipids)	27	23	24	35	29	26	28
Outer : inner MGDG (mol %)	65 : 35	53 : 47	53 : 47	57 : 43	59 : 41	56 : 44	62 : 38
Outer : inner DGDG (mol %)	15 : 85	16 : 84	12 : 88	18 : 82	18 : 82	22 : 78	20 : 90

R = herbicide-resistant biotype; S = herbicide-sensitive biotype. All species are $C_{18:3}$ plants except *S. oleracea* and *S. nigrum*, which are $C_{16:3}$ plants.

Table 2. Labelling of galactolipids in *L. sativa* thylakoids by the galactose oxidase method. In experiment 1, Triton X-100 was used to break the thylakoids [45]; in experiment 2, sonication was used for the same purpose (see Materials and methods). Figures are the average of triplicates

	Lipid	Labelling (d.p.m./mg chlorophyll)				Outer leaflet (% total labelling)	
		Head group		Acyl chains		Head	Total
Expt. 1			+ *Triton*		+ *Triton*	group	molecule
	MGDG	10660	17835	1745	2890	60	60
	DGDG	2031	9103	1302	2226	22	29
Expt. 2			*Sonicated*		*Sonicated*		
	MGDG	9881	16922	1523	2517	58	59
	DGDG	1745	7992	1007	1436	22	29

Table 3. Galactolipid molar ratios in the inner and outer leaflets of thylakoids from various plant species

	Spinacia oleracea	*Solanum nigrum*		*Lactuca sativa*	*Pisum sativum*	*Hordeum vulgare*	*Avena sativa*
		R	S				
Galactolipid molar ratio	0.95	0.73	0.68	0.66	0.73	0.81	0.87

Expt. 2							
	MGDG	9881	16922	1523	2517	58	59
	DGDG	1745	7992	1007	1436	22	29

Table 3. Galactolipid molar ratios in the inner and outer leaflets of thylakoids from various plant species

	Spinacia oleracea	*Solanum nigrum* R	S	*Lactuca sativa*	*Pisum sativum*	*Hordeum vulgare*	*Avena sativa*
Galactolipid molar ratio (outer/inner leaflet)	0.95	0.73	0.68	0.66	0.73	0.81	0.87
MGDG/DGDG							
– outer leaflet	9.00	7.00	9.67	4.17	5.00	4.83	12.67
– inner leaflet	0.83	1.32	1.24	0.62	0.71	1.15	0.96

Table 4. The amount of non-bilayer forming lipids in the two leaflets of thylakoids from different plant species. Amounts are calculated assuming that all of the MGDG is potentially non-bilayer forming but that all the other acyl lipids (DGDG, sulphoquinovosyldiacylglycerol, phosphatidylglycerol, etc.) are bilayer-forming. For this calculation, we assumed that phosphatidylglycerol was distributed with 70% and sulphoquinovosyldiacylglycerol with 40% in the outer leaflet [see Text]

	Bilayer forming/non-bilayer forming lipids (mol % of total acyl lipids)						
	Spinacia oleracea	*S. nigrum* R	S	*Lactuca sativa*	*Pisum sativum*	*Hordeum vulgare*	*Avena sativa*
Outer leaflet	$\frac{13.5}{36}$ (0.38)	$\frac{16.7}{28}$ (0.60)	$\frac{14.1}{29}$ (0.49)	$\frac{17.7}{25}$ (0.71)	$\frac{20.4}{25}$ (0.82)	$\frac{17.7}{29}$ (0.61)	$\frac{8.8}{38}$ (0.23)
Inner leaflet	$\frac{31.5}{19}$ (1.66)	$\frac{30.3}{25}$ (1.21)	$\frac{30.9}{26}$ (1.19)	$\frac{39.3}{18}$ (2.18)	$\frac{37.6}{17}$ (2.21)	$\frac{30.3}{23}$ (1.32)	$\frac{29.2}{23}$ (1.27)

Ratios are indicated in parentheses.

It is worth mentioning that in *Solanum nigrum*, this ratio was lower in the herbicide-sensitive biotype than in the resistant one, suggesting that thylakoid membranes from the resistant biotype are more fluid than the sensitive ones. However, these parameters do not appear to be correlated with the various types of plants studied, i.e., mono-dicotyledons, $C_{16:3}$ or $C_{18:3}$ plants.

MGDG was the major lipid class in all thylakoid membranes and the molar ratio MGDG/DGDG was generally > 2 [see Table 1]. The total amount of galactolipids varied between 71 mol % for *P. sativum* to 89 mol % for *A. sativa.*

The transmembrane distribution of galactolipids was studied using an enzymatic approach. The rationale for this technique and the interpretation of the results have been discussed recently [38]. For all plants studied, the outer layer was enriched in MGDG (53–65%) whilst DGDG was mainly confined in the inner leaflet [monolayer] (78–90 mol %). Analysis of the hydrolysis products by gas liquid chromatography did not reveal any notable transmembrane asymmetry for the acyl groups of either MGDG or DGDG (unpublished data). Our results on the transmembrane distribution of MGDG and DGDG would appear to give some support to an early model of the thylakoid membrane proposed on the basis of freeze-fracture studies by Mühlethaler [31]. These distributions for the galactolipids in the two leaflets, while not disproving, do not provide support for the suggestion that MDDG and DGDG may be particularly important in the inner and outer leaflets, respectively, at the highly curved ends of the thylakoids [32].

The transmembrane distribution has been checked in the case of *L. sativa* with the galactose oxidase method (Table 2). The results show that, when one considers the specific labelling of headgroups of both galactolipids, this method gives similar values to those found with the enzymatic approach. However, the occurrence of significant labelling of the acyl chains of both galactolipids (cf. non-specific labelling in refs 47, 11) means that caution must be used in the interpretation of the data since the hydrophobic regions of the acyl lipids are clearly available for labelling. However, it is noteworthy that while the contribution of non-specific labelling in the acyl chains did not modify the distribution values of MGDG, it significantly altered those for DGDG. This may explain, at least in part, why our distribution values for MGDG, but not for DGDG, are similar to those found by Sundby and Larsson [48]. The enrichment of MGDG in the outer monolayer also agrees with recent antibody studies [15].

We also attempted to examine the distribution of sulphoquinovosyldiacylglycerol by an analogous labelling procedure using glucose oxidase but without success. Analysis of the membrane topography of this lipid by *Rhizopus lipase* is problematic because of the extensive galactolipid hydrolysis before any sulphoquinovosyldiacylglycerol breakdown occurs [49]. This is due to the specificity of the *Rhizopus lipase* [10]. Therefore,

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we examined the sided distribution of sulphoquinovosyldiacylglycerol with a potato leaf acyl hydrolase which has much higher activity towards this lipid [28]. The results showed that only 12% (*L. sativa*) and 10% (*P. sativum*) of the total sulphoquinovosyldiacylglycerol were digested in the outer leaflet. However, total digestion was only 70% indicating that large amounts of sulphoquinovosyldiacylglycerol were inaccessible. Accordingly, it is difficult to make any definite conclusions about the transmembrane distribution of sulphoquinovosyldiacylglycerol from enzyme digestion studies.

In all plants studied, the molar ratio of total galactolipids in the outer/inner leaflet was < 1 showing an enrichment of galactolipids in the inner monolayer (Table 3). However, in *S. oleracea* the amount of both galactolipids was almost equal in both leaflets. The ratios which are significantly < 1 (as in the case of *L. sativa*) suggest that, given the small proportion of other acyl lipids present, the total acyl lipids will occupy different areas in the two leaflets. This difference is likely to be filled by a larger area occupied by proteins in the outer leaflet, in agreement with recent reports [34].

Although different in the various plants studied, the molar ratio MGDG/DGDG was always > 4.0 in the outer leaflet whereas it was < 1.4 in the inner leaflet. This interspecies variation depends on the composition and on the relative distribution of galactolipids in thylakoid membranes of each plant species. Since MGDG is a non-bilayer forming lipid, one may wonder what is the significance of such high ratios in the outer leaflet. Using a reverse phase evaporation method for the reconstitution of uncharged thylakoid membrane lipids, Sprague and Staehelin [45] demonstrated that the transition of bilayer to non-bilayer structures started at a molar ratio of MGDG/DGDG of 2.5. Sen et al. [40] also using freeze-fracture techniques found extensive amounts of non-lamellar structures at this ratio although other studies using different methods suggested that the exact mixture of phases seen at a MGDG/DGDG ratio of 2:1 was highly dependent on the water content [41, 42]. Taking a ratio of 2.5 as being critical for the formation of non-lamellar phases means that a mixture of pure galactolipids of similar composition to that of the outer leaflet, would contain mostly non-lamellar structures, whereas that of the inner monolayer would contain exclusively lamellar structures. Thus a lamellar configuration for the inner leaflet would be caused by the galactolipid composition alone without considering the contribution by the bilayer-forming acyl lipids such as sulphoquinovosyldiacylglycerol and phosphatidylglycerol. This is certainly not the case for the outer leaflet. However, it is known that the thylakoid membrane is a bilayer structure under physiological conditions [2].

Therefore, it is obvious that other components must contribute to the stabilization of the lamellar configuration of the outer leaflet. The prime candidates to aid in the formation of the lamellar configuration are proteins [7]. In addition, the presence of other lipids may be important. We know from the analysis of thylakoids from five of the plant species studied that about 70% of the phosphatidylglycerol is localized in the outer leaflet [13, 36, 50]. The sulphoquinovosyldiacylglycerol studies showed 30% of the total lipid as being inaccessible to acyl hydrolase digestion (see above). If this 30% is added to the amount of this lipid accessible in the outer leaflet (10%), a figure of about 40% is obtained for *L. sativa* and *P. sativum.* This would represent the maximum contribution of sulphoquinovosyldiacylglycerol in the outer leaflet. Using these figures, we have calculated the amount of non-bilayer forming lipids in the two leaflets (Table 4). Compared with the threshold value of bilayer/non-bilayer lipids (DGDG/MGDG = 0.4) given by Sprague and Staehelin [45] at which hexagonal phase begins to form, and assuming that part of DGDG can be replaced by other bilayer-forming lipids, one can conclude that in all the plant species studied the distribution of lipids largely favours a lamellar configuration in the inner leaflet. Although the most probable configuration of the outer leaflet of thylakoid membranes is also a lamellar structure, one might expect that particularly in *S. oleracea* and *A. sativa* (Table 4) both hexagonal and lamellar configurations could coexist. The lamellar phase is likely to be promoted by molecules such as phosphatidylglycerol [45, 9] but this property has been shown to be dependent on the concentration of divalent cations and the fatty acid composition of the phosphatidylglycerol [9]. In this connection it is worth mentioning that a stromal concentration of 10 mM Mg^{++} [29] may favour the phase separation of particular lipids and consequently promote the appearance of hexagonal phase structures [16].

The presence in the membranes of large amounts of lipid which can form non-lamellar structures in vitro has unknown significance. However, the proportion of MGDG in the outer leaflet is so high that one can expect significant amounts of non-lamellear structures to be easily formed by subtle changes in the membrane's environment such as temperature [13, 51], presence of particular cations [50, 51] or individual membrane proteins [7, 30, 33]. In addition it may have physiological relevance for membrane fusion [7] during chloroplast biogenesis, for morphological adaptation during sun/shade growth [3] or in rapid adaptation during state 1/state 2 transitions [46]. An interesting common feature of energy-transducing membranes may be that non-bilayer lipids are preferentially associated with that part of the bilayer in which the coupling factor structures are embedded. Thus MGDG is found in the outer monolayer of thylakoids (this report), phosphatidylethanolamine on the cytoplasmic face of *Rp. sphaeroides* chromatophores [25] and both phosphatidylethanolamine and diphosphatidylglycerol in the inner monolayer of the internal mitochondrial membrane [26].

The very similar distribution of individual acyl lipids within the thylakoids from a large variety of plants must confer particular advantages for the efficient functioning of such membranes. Certainly, the presence of high amounts of MGDG in the outer leaflet of plant thylakoids is a fact

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which should be carefully considered in experiments concerning the mobility and function of individual membrane components.

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Physiologie cellulaire végétale/*Plant Cell Physiology*

Présence et quelques propriétés d'une activité ATPasique liée aux membranes de l'enveloppe de l'étioplaste d'Avoine et stimulée par le Mg^{2+}, le Ca^{2+} et la calmoduline

Christian GIROUD et Paul-André SIEGENTHALER

Résumé — Dans cette publication, il est montré que les étioplastes d'Avoine possèdent une activité ATPasique liée à l'enveloppe. Cette activité (env. 30 nmoles $P_i.mg$ protéine$^{-1}.mn^{-1}$) est stimulée environ trois fois par de faibles concentrations de $CaCl_2$ (0,2 mM) et 3,5 fois par des concentrations plus élevées de $MgCl_2$ (5-10 mM). Une addition de calmoduline provoque une stimulation de 55% de l'activité ATPasique comparée à celle observée en présence de $CaCl_2$ (0,2 mM) seul. Le K_m apparent pour le substrat Mg-ATP^{2-} est de 0,55 mM. L'enzyme est particulièrement sensible au $LaCl_3$, au vanadate et à l'oligomycine. Ces résultats indiquent que les propriétés enzymatiques de l'ATPase liée à l'enveloppe de l'étioplaste d'Avoine sont presque identiques à celle de l'enveloppe du chloroplaste d'Épinard.

Occurence and some properties of a Mg^{2+}-, Ca^{2+}- and calmodulin-stimulated ATPase activity bound to oat etioplast envelope membranes

Abstract — Oat etioplasts have been found to display an envelope-bound ATPase activity. The ATPase activity (about 30 nmol $P_i.mg$ protein$^{-1}.min.^{-1}$) is stimulated about 3 times at low concentrations of $CaCl_2$ (0.2 mM) and 3.5 times at higher concentrations of $MgCl_2$ (5-10 mM). In the presence of 0.2 mM $CaCl_2$, the activity is further enhanced by calmodulin (55%). The apparent K_m value for Mg-ATP^{2-} is 0.55 mM. The enzyme is particularly sensitive to $LaCl_3$, vanadate and oligomycin. These results show that the enzymatic properties of the bound-ATPase are almost identical for envelopes of both oat etioplasts and spinach chloroplasts.

Version Française abrégée — Lorsque les feuilles croissent à l'obscurité, les proplastes se différencient en étioplastes. L'enveloppe de ces organites ressemble, par sa composition en lipides [1] et en protéines [2], à celle du chloroplaste mature. En outre, toutes deux contiennent des activités qui sont impliquées dans la synthèse des galactolipides ([3], [4], [5]). On peut donc prévoir que les enveloppes d'étioplastes et de chloroplastes présentent d'autres caractéristiques communes, notamment une activité ATPasique dont les propriétés pourraient être pareilles à celles décrites chez les enveloppes de chloroplastes d'Épinard et de Pois ([6]-[10]). Dans ce travail, nous avons tout d'abord recherché la présence ou non d'une activité ATPasique liée à l'enveloppe de l'étioplaste d'Avoine. Dans une seconde phase, nous avons étudié quelques propriétés de cet enzyme, en particulier : (*a*) les effets du rapport ATP/Mg^{2+} sur l'activité de l'ATPase; (*b*) sa dépendance vis-à-vis du Ca^{2+}, du Mg^{2+} et de la calmoduline; (*c*) enfin sa sensibilité à différentes catégories d'inhibiteurs des ATPases.

L'essentiel des méthodes utilisées dans cette recherche est décrit dans les références [6], [11]-[16].

La figure 1 montre quelle est l'activité de l'enzyme en fonction de la concentration d'ATP. Une courbe en cloche est obtenue avec un maximum lorsque la concentration d'ATP (5 mM) est identique à celle de $MgCl_2$ (5 mM). Ces résultats suggèrent que le vrai substrat est le complexe $ATPMg^{2-}$ et non pas l'ATP^{4-}. L'ATPase suit une cinétique de type Michaelis-Menten : son K_m, qui peut être estimé par la méthode des doubles inverses exprimant 1/v en fonction de 1/[ATP] (voir *fig.* 1) est de 0,55 mM. La figure 2 montre qu'à la concentration optimale de $CaCl_2$ (0,2 mM), le $MgCl_2$ reste sans effet sur l'activité. Inversement, aux concentrations (5-10 mM) où le Mg^{++} stimule l'ATPase à son maximum, le $CaCl_2$ est inefficace. Le fait que

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Note présentée par Alexis MOYSE.

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l'enzyme soit sensible à de faibles concentrations de Ca^{2+} suggère que l'activité ATPasique pourrait être modulée par la calmoduline à l'image d'autres enzymes aussi sensibles à de faibles concentrations de $CaCl_2$ (p. ex. la NAD^+-kinase de Concombre [17]). Le tableau révèle, en effet, que l'ATPase est stimulée par la calmoduline en présence de $CaCl_2$ seul (55 %) ou de $CaCl_2$ et de $MgCl_2$ (30 %). Pour mieux caractériser les propriétés de l'ATPase, plusieurs types d'inhibiteurs des ATPases ont été utilisés. L'enzyme est particulièrement sensible au $LaCl_3$ et, dans une moindre mesure, à l'oligomycine et au vanadate, une classe d'inhibiteurs connus pour bloquer les ATPases présentant des intermédiaires phosphorylés ([18], [19]). Par contre, les inhibiteurs de la pompe à H^+ (CF_0-CF_1-ATPase) comme la phlorizine et le dicyclohexylcarbodiimide ([20], [21]) n'ont pas d'effet significatif sur l'ATPase liée à l'enveloppe.

Ces résultats montrent que les ATPases des enveloppes de chloroplaste [6] et d'étioplaste (ce travail) ont des propriétés presque identiques : (*a*) le complexe $MgATP^{2-}$ est le vrai substrat; (*b*) la valeur du K_m est 0,55 mM; (*c*) la sensibilité aux cations Ca^{2+} et Mg^{2+}, à la calmoduline et aux inhibiteurs affectant les enzymes présentant des intermédiaires phosphorylés est pareille. Le fait que l'ATPase soit présente déjà dans l'enveloppe de l'étioplaste et qu'elle possède des propriétés presque identiques à celles de l'enveloppe du chloroplaste mature suggère que son rôle physiologique est indépendant du verdissement. Elle pourrait être impliquée dans le transport des protéines dans le plaste comme l'ont suggéré Nguyen et Siegenthaler [7]. En effet, les étioplastes doivent importer toute une série de polypeptides synthétisés dans le cytoplasme pour achever leur développement (p. ex. la protochlorophyllide réductase).

Récemment cependant, Flügge et Hinz [22] ont montré que l'importation des protéines à l'intérieur du chloroplaste d'Épinard dépendait de la présence de $MgATP^{2-}$ comme source d'énergie du côté cytosolique de cet organite. Ainsi, le rôle physiologique d'une activité ATPasique de l'étioplaste qui est très probablement liée, comme pour le chloroplaste mature [10], à la membrane interne de l'enveloppe, reste encore à être élucidé.

I. INTRODUCTION. — When higher plants are grown in darkness the proplastids of the leaves develop into etioplasts. The properties of the envelope which surrounds this organelle have not been well characterized, probably due to the fact that it is difficult to obtain an envelope fraction which is pure and quantitatively sufficient for biochemical and physiological analyses. However, it has been reported that isolated envelope fractions from etioplasts and chloroplasts are similar, both in their lipid composition [1] and polypeptide pattern [2]. In addition, it has been found recently that the enzymes synthesizing mono- and di-galactosyldiacylglycerol from UDP-galactose, are localized not only in envelope membranes of mature chloroplasts ([3], [4]) but are also present in etioplast envelopes [5]. Thus one may expect to find other similar properties or enzymes in the envelopes of these two organelles. In this investigation, we have studied whether etioplast envelopes contain an (Mg^{2+}, Ca^{2+})-ATPase and if so, whether its main properties are similar to those of the chloroplast envelope ATPase from higher plants ([6]-[10]).

II. MATERIALS AND METHODS. — Oat plants (*Avena sativa* L. v. Borrus) were grown in darkness for 6 days. Etioplasts were purified as described previously by Siegenthaler and Giroud [11]. Leaves were cut with scissors, then ground in an electrical blendor equipped with razor blades in a medium containing 0.5 M sucrose, 50 mM 3-(N-morpholino)propane sulfonic acid (MOPS) [pH 7.6], 1 mM EDTA, 1 mM $MgCl_2$, 0.5 mM phenylmethanesulfonylfluoride (PMSF), 5 mM aminocaproic acid, 5 mM mercaptoethanol, 1 mM thioglycolic acid, 0.1% bovine serum albumin (w/v). Crude oat etioplasts were obtained by differential centrifugations and subsequently purified on a self-generated gradient of percoll (centrifugation 100,000 × g, 30 min. in a Ti70, angle-fixed rotor), made of three layers: 6 ml percoll 27%, 8 ml of 45% and 6 ml of 65% in a medium similar to that used by Takabe *et al.* [12]. The lower band of purified etioplasts was collected with

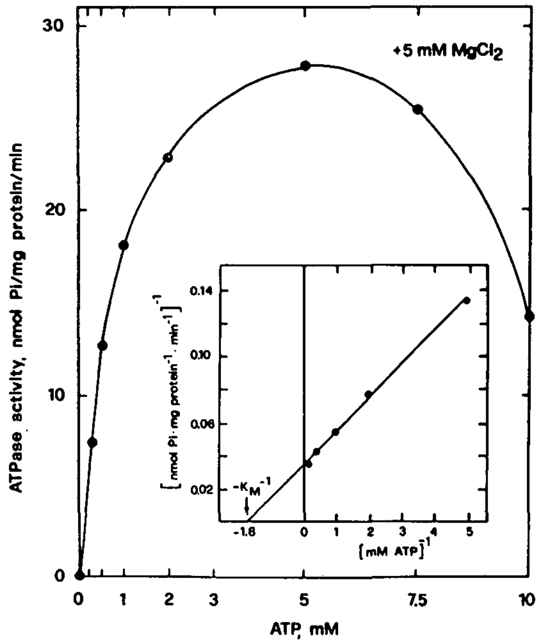

Fig. 1. — ATP concentration dependence of Mg^{2+}-stimulated ATPase activity bound to oat etioplast envelopes. The reaction mixture contained 5 mM $MgCl_2$ and ATP as indicated.

Fig. 1. — Dépendance de l'activité ATPasique liée à l'enveloppe de l'étioplaste d'Avoine vis-à-vis de la concentration en ATP en présence de 5 mM de $MgCl_2$.

a syringe, washed once in the above medium without percoll (6,000 g/10 min.) then broken by osmotic shock in 50 mM MOPS-NaOH (pH 7.6), 3 mM $MgCl_2$, 1 mM EDTA-Na, 0.5 mM PMSF and 0.1% (w/v) bovine serum albumin. After the sedimentation of the prolamellar body and prothylakoid fraction (7,600 × g/10 min.), the enriched envelope fraction (supernatant) was pelleted by ultracentrifugation at 100,000 × g for 1 hr. and resuspended in 0.3 M sucrose, 50 mM Tris-HCl (pH 7.8).

ATPase activity was determined as described by Nguyen and Siegenthaler [6] in a reaction mixture (250 µl) containing 50 mM Tris-HCl (pH 7.8), 300 mM sucrose and 20 µg envelope proteins. Each measurement was carried out in duplicate with adequate controls. After preincubation at 37°C for 10 min., the reaction was started by the addition of ATP and followed for 30 min. at 37°C. Assays were stopped with 50 µl of 60% trichloroacetic acid. Precipitated proteins were removed by centrifugation (1 min. in a Beckman microfuge B) and P_i was measured in the supernatant as described by Lebel *et al.* [13]. The coloration of the phosphomolybdate complex was stabilized as described by Penttinen [14]. Concentration of ATP, $MgCl_2$ and $CaCl_2$ are indicated in the legends of the figures.

Calmodulin was purified from bovine brain according to Gopalakrishna and Anderson [15] and protein concentration was determined according to Bradford [16].

III. Results. — Figure 1 shows that the envelope-bound ATPase activity increased as a function of ATP concentration. The maximum of activity (30 nmol P_i released/mg protein.min.) was reached at 5 mM ATP for an identical concentration of $MgCl_2$. However, the activity decreased progressively when ATP was raised further. These results suggest that $ATPMg^{2-}$ rather than ATP^{4-} alone is the real substrate for the enzyme. The ATPase activity as a function of ATP concentration followed Michaelis-Menten kinetics with an apparent K_m-value for ATP of 0.55 mM as determined by the double-reciprocal plot of 1/V vs 1/sec. (inset of Figure 1).

The ATPase activity was stimulated by divalent cations with maximal rates at 0.2 mM $CaCl_2$ and 5 to 10 mM $MgCl_2$ (*Fig.* 2). It is noteworthy that at 0.2 mM, $CaCl_2$ stimulated the activity whilst $MgCl_2$ had no effect; in contrast, at 5 mM, $CaCl_2$ was

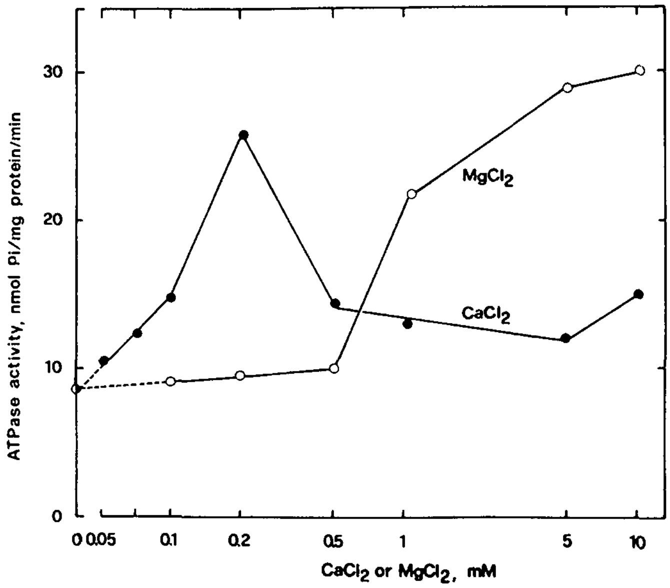

Fig. 2. — Stimulation of etioplast envelope-bound ATPase activity by $MgCl_2$ and $CaCl_2$. The reaction mixture contained 4 mM ATP and salts at the concentrations indicated.

Fig. 2. — Stimulation de l'activité ATPasique liée à l'enveloppe d'étioplaste par le $MgCl_2$ *et le* $CaCl_2$*. Le mélange réactionnel contient 4 mM d'ATP et les sels aux concentrations indiquées.*

inefficient whereas $MgCl_2$ stimulated maximely the activity. Since $CaCl_2$ stimulated the ATPase activity at low concentration, this may suggest that this enzyme is regulated by the Ca^{2+}-calmodulin complex as other enzymes, e. g. NAD^+-kinase from *Cucurbita pepo* L. [17]. Indeed, the Table shows that calmodulin displayed a stimulating effect in the presence of $CaCl_2$ (55%) and to a lesser extent in the presence of both $CaCl_2$ and $MgCl_2$ (30%).

In order to better characterize the ATPase properties, several classical inhibitors of ATPases have been used (*see* Table). The enzyme was especially sensitive to $LaCl_3$ which is known for its effect on the phosphorylated intermediates [18]. At the concentration used, oligomycin and vanadate which have the same effect as $LaCl_3$ [19] inhibited also the activity but to a lesser extent. On the other hand, the ATPase was insensitive to phlorizin and dicyclohexylcarbodiimide which are known for their effects on the proton pump ([20], [21]).

IV. DISCUSSION. — Although it is known that etioplast envelope preparations exhibit a non-latent Mg^{2+}-dependent ATPase [2], it is the first time that some of its main properties are described. The most remarkable feature is that the properties of the envelope-bound ATPase of oat etioplasts are almost identical to those of the envelope-bound ATPase of mature chloroplasts from spinach [6]. Indeed, both enzymes display in common the following characteristics: (*a*) $MgATP^{2-}$, but not ATP^{4-}, is the true substrate for the two bound ATPases; in addition the affinity of both enzymes for their substrate is identical ($K_m = 0.55$ mM); (*b*) for both enzymes, Ca^{2+} stimulates ATP hydrolysis at low concentration (0.2 mM) but has no effect at higher concentrations (> 0.5 mM); (*c*) both enzymes are insensitive to Mg^{2+} at concentrations inferior to 0.5 mM but are strongly stimulated at higher concentrations, optimal conditions depending on the ATP/Mg molar ratio; (*d*) both ATPase activities are further stimulated by

TABLE

Effects of calmodulin and inhibitors on the ATPase activity bound to envelopes from oat etioplasts. All reaction mixtures contained 5 mM ATP. In experiments (1) and (2) 40 µg/ml calmodulin (CaM) were added where indicated. In experiment (3), the reaction mixture contained 5 mM $MgCl_2$ and inhibitors at the indicated concentrations.

Effets de la calmoduline et d'inhibiteurs sur l'activité ATPasique liée aux enveloppes de l'étioplaste d'Avoine. Tous les mélanges réactionnels contiennent 5 mM d'ATP. Dans les expériences (1) *et* (2), *on ajoute, comme indiqué,* 40 µg *de calmoduline/ml. Dans l'expérience* (3), *le mélange réactionnel contient 5 mM de $MgCl_2$ et des inhibiteurs aux concentrations indiquées.*

	Additions	Specific activity (nmol Pi/mg protein . min.)	(%)
(1)	$CaCl_2$ alone (0.2 mM)	27.0	100
	$CaCl_2$ (0.2 mM) + CaM	41.9	155
(2)	$CaCl_2$ (0.2 mM) + $MgCl_2$ (5 mM)	30.0	100
	$CaCl_2$ (0.2 mM) + $MgCl_2$ (5 mM) + CaM	39.0	130
(3)	Control (5 mM $MgCl_2$, 5 mM ATP)	28.0	100
	$LaCl_3$ (2 mM)	0	0
	Oligomycine (40 µg/ml)	16.5	59
	Vanadate (0.2 mM)	12.0	43
	Phlorizine (2 mM)	30.8	110
	Dicyclohexylcarbodiimide (1 mM)	24.6	88

calmodulin. Maximal stimulation occurs in the presence of 0.2 mM $CaCl_2$ for the etioplast envelope-bound ATPase (this study) and in the presence of 0.2 mM $CaCl_2$ + 5 mM $MgCl_2$ for the chloroplast envelope bound ATPase [6]; (*e*) both enzymes are insensitive to inhibitors known for their effect on CF_0-CF_1 complex (phlorizin and dicyclohexylcarbodiimide) but are especially sensitive to inhibitors affecting phosphorylated intermediates ($LaCl_3$, vanadate and oligomycin). However, the specific activity of the envelope bound ATPase from oat etioplasts is smaller (about 30 nmol P_i. mg protein^{-1}. mn^{-1}) than that from spinach chloroplast (about 200 nmol P_i. mg protein^{-1}. mn^{-1}). This difference may be explained by the use of a crude envelope fraction in the case of etioplasts.

The properties of the ATPase which are almost identical for both etioplast and chloroplast envelopes indicate that this enzyme plays a physiological role which is independent of the greening process. Since the etioplast has to import several proteins to achieve its full development (e. g. the $NADP^+$ protochlorophyllide reductase), the envelope-bound ATPase of this organelle may be involved in this process, as suggested by Nguyen and Siegenthaler [7] for mature chloroplasts. Recently, however, Flügge and Hinz [22] have presented several evidences showing that $MgATP^{2-}$ energized protein import into spinach chloroplasts at the cytosolic side of this organelle. Thus, the physiological role of an etioplast ATPase activity which is presumably bound, as for mature chloroplast [10], to the inner envelope membrane, remains to be elucidated.

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PURIFICATION AND CHARACTERIZATION OF PROTHYLAKOIDS AND PROLAMELLAR BODIES FROM OAT ETIOPLASTS

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When oat is grown in darkness, the proplastids of the leaves develop into etioplasts. These organelles contain internal crystalline structures, the prolamellar bodies and flat membranes, the prothylakoids. These structures are devoid of chlorophyll and active photosystems. So far, the purity and characteristics of the two fractions are still controversial. Intact etioplasts were purified by Percoll gradient centrifugation. Prothylakoids were separated from prolamellar bodies by sonication and sucrose gradient centrifugation. The two fractions were analyzed for protein, pigment and lipid content and enzyme activities. Both contained the same lipid classes. No saponins were detected. CF1-ATPase, protochlorophyllide reductase and duroquinol-cytochrome c552 oxidoreductase activities were found mostly in the prothylakoid fraction whereas ferredoxin-NADP$^+$ reductase activity was equally distributed between the two fractions. These results will be discussed in terms of the biogenesis of chloroplasts.

Experientia, 1984, p.607.

TRANSVERSE LOCALIZATION OF GALACTOLIPIDS IN OAT THYLAKOID MEMBRANES DURING GREENING

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The lipase from Rhizopus arrhizus has been used to determine the transmembrane distribution of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) in prothylakoids and mature thylakoids from oat. The hydrolysis kinetics of MGDG and DGDG were characterized by several pools of different reactivity. The number and the rate of these pools were dependent on the temperature, ionic conditions and the presence of bovine serum albumin. The amplitude of each of these pools was different for MGDG and DGDG but similar in both prothylakoids and thylakoids. These results suggest that as for phosphatidylglycerol (1), the transverse distribution of galactolipids is not modified during greening, i.e. by the incorporation of chlorophyll-protein complexes.

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Experientia, 1985, p.782.

CHLOROPHYLL-PROTEIN COMPLEXES IN AVENA SATIVA : CHARACTERIZATION AND EFFECTS OF A LIPOLYTIC TREATMENT

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Chlorophyll-protein complexes from oat thylakoids were separated by mild SDS-polyacrylamide gel electrophoresis. The main complexes (CP1a, CP1, LHCP1, LHCP2, CPa and LHCP3 : nomenclature according to Anderson (1978) FEBS Lett., 92, 227-233) were characterized by their chlorophyll a/b ratios, absorbance and 77 K fluorescence spectra.

Thylakoids were then treated by a lipase from *Rhizopus arrhizus* which, under our experimental conditions, hydrolyzed only monogalactosyldiacylglycerol (MGDG), the major lipid component of thylakoid membranes. Lipase treatment inhibited the Mg^{2+}-induced fluorescence and altered the distribution of chlorophyll-protein complexes. The results suggest that the destruction of MGDG induces a rearrangement of chlorophyll-protein complexes in the thylakoid membrane.

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TRANSMEMBRANE DISTRIBUTION OF GALACTOLIPIDS IN INSIDE-OUT STRUCTURES DERIVED FROM SPINACH GRANA THYLAKOIDS

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Inside-out structures enriched in PS-II were obtained by Triton X-100 treatment (BBY particles) and by the two phase partition method. BBY were fully active as judged by oxygen evolution capacity and depleted in PS-I electron flow activity. Inside-out vesicles displayed reversible proton extrusion and a high 77°K 685/740 nm fluorescence ratio. Treatment of both types of membranes with lipase from Rhizopus arrhizus showed that galactolipids had an asymmetric distribution. However, the partial conversion of monogalactosyldiacylglycerol (MGDG) into its acylated derivative allowed only a semiquantitative estimation. The outer monolayer contained 35% of total MGDG and 80% of DGDG. This distribution was roughly the reverse of that found in whole right-side-out thylakoids. These results confirm our previous proposal that galactolipids are asymmetrically distributed within thylakoid membranes.

Experientia, 1986, p.657.

EFFECTS OF MONOGALACTOLIPID (MGDG) DEPLETION ON PHOTOSYNTHETIC ACTIVITIES IN OAT THYLAKOIDS

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Special conditions were used allowing lipase from Rhizopus arrhizus to selectively hydrolyze MGDG. It was found that both PS-I and PS-II electron flow rates were inhibited and that cyt f photooxydation decreased both in rate and amplitude. However, the PS-I activity could be restored by adding back Triton X-100, but not plastocyanin. Thus, it is proposed that the inhibition site of the lipase is at the level of the cyt b/f complex. Indeed, without TX-100, artificial electron donors reduced mostly cyt f, whereas an addition of detergent allowed a shunt around cyt f. The inhibition of PS-II was much more complex. Electron flow inhibition was observed mainly at high light intensities, and both fluorescence at 20°C and 77°K F685/F740 ratio decreased. Thus, it is proposed that MGDG removal inhibited the electron flow at two different sites : one at the level of the cyt b_6/f complex, and the other at the level of PS-II, by favoring spillover from PS-II to PS-I and inhibiting the basal electron flow.

Experientia, 1986, p.658.

ON THE LATERAL HETEROGENEITY OF ACYL LIPIDS IN THE THYLAKOID MEMBRANE : LIPID COMPOSITION OF OAT CHLOROPHYLL-PROTEIN COMPLEXES

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One of the strategies to studying the lateral distribution of acyl lipids in the thylakoid is to fractionate the membrane by using detergents, then to analyse the lipid composition of each subchloroplast particles.

Chlorophyll-protein complexes obtained by solubilization of the membrane with detergents were separated by SDS/Na-deoxycholate (DOC) polyacrylamide gel electrophoresis. The combination of two detergents for membrane solubilization (Triton X-100 + SDS) and electrophoretic separation (SDS + DOC) was used in order to attenuate the discriminative effect of only one detergent on membrane lipid extraction. The system used for solubilization and electrophoresis generated a negligible amount of free pigments and preserved the oligomeric forms of the complexes. Analysis by thin layer and gas chromatography showed that all lipid classes were present in roughly similar relative proportion in each complex. However, compared to the other complexes, LHCP^1 was slightly enriched in monogalactosyldiacylglycerol and contained higher amount of trans-hexadecenoic acid containing phosphatidylglycerol. Lipids associated with CP$_{1a}$ and CP$_1$ had a lower degree of unsaturation, especially in their sulpholipid molecules.

In conclusion, the present study shows that under conditions which attenuate selective lipid extraction and preserve oligomeric forms of chlorophyll-protein complexes, the acyl lipid composition of these complexes is very similar. The large differences which have been observed in several previous reports are thought to reflect the specificity of detergents rather than true lipid/protein interactions.

Congrès de la FESP, Hambourg, 1986.

RESUME

Le premier objectif de cette thèse était de tenter d'expliquer la structure paracristalline des corps prolamellaires en comparant leur composition à celle des prothylacoïdes et des thylacoïdes. Nous avons montré que les corps prolamellaires différaient des autres membranes non pas par une composition lipidique particulière mais par leur richesse en lipides (rapport lipides/protéines élevé), par la quasi absence de protéines intrinsèques et par la monotonie de leur composition protéique (la protochlorophyllide réductase formant l'essentiel de leurs protéines). La combinaison de ces trois facteurs, et aussi le fait que le monogalactolipide constitue plus de 50% des lipides du corps prolamellaire expliqueraient pourquoi ce dernier adopte une configuration paracristalline.

Un second objectif était de montrer que les prothylacoïdes pouvaient servir de modèle pour élucider les mécanismes qui engendrent puis maintiennent une distribution transversale asymétrique des phospholipides dans le thylacoïde mature. Une interaction spécifique de certains lipides avec des protéines particulières de la membrane, elles-mêmes arrangées d'une manière asymétrique à travers la bicouche du thylacoïde pouvait être la cause majeure responsable de la distribution hétérogène des lipides. Cette hypothèse pouvait être testée en étudiant les modifications de distribution des lipides qui pouvaient accompagner le verdissement du prothylacoïde. Ce dernier voit en effet sa composition protéique se modifier considérablement au cours de sa transformation en thylacoïde mature: la caractérisation des prothylacoïdes par leurs activités enzymatiques a montré que ceux-ci en contenaient quatre de différentes dont trois communes avec les thylacoïdes (la ferrédoxine-$NADP^+$-réductase, la CF1-ATPase et la duroquinol/cytochrome c552 réductase soutenue par le complexe des cytochromes b_6f) et une propre aux membranes étiolées (la protochlorophyllide-réductase). Etant dépourvus de chlorophylle, les prothylacoïdes ne possèdent aucun complexe chlorophylle-protéine et sont par conséquent exempts d'activité de photophosphorylation.

Une étude comparative des distributions transversales des lipides du prothylacoïde et du thylacoïde mature a cependant montré que l'arrangement des lipides polaires restait presque inchangé au cours du verdissement du plaste: 70% du MGDG, 10% du DGDG, 65% du PG et 50% de la PC sont localisés dans le feuillet externe du prothylacoïde; 62% du MGDG, 10% du DGDG, 70% du PG et 60% de la PC sont concentrés dans la monocouche externe du thylacoïde. Dans les deux types de membranes, chacune des deux monocouches contient la même quantité de lipides polaires. Ces résultats indiquent que la distribution transmembranaire des lipides qui caractérise le thylacoïde mature doit apparaître très tôt au cours du développement du plaste. Ils montrent aussi que cette distribution n'est pas modifiée par l'intégration dans la membrane des complexes chlorophylle-protéine.

Pour mettre en évidence une éventuelle hétérogénéité dans la distribution latérale des lipides, les thylacoïdes matures d'Avoine ont été préalablement solubilisés par un mélange de détergents puis séparés par électrophorèse préparative. Après caractérisation par différentes méthodes spectrophotométriques (spectres d'absorption et de fluorescence), leur contenu lipidique a été analysé par chromatographie sur couche mince et en phase gazeuse. La solubilisation des thylacoïdes dans un mélange détersif composé de SDS et de Triton X-100, puis la séparation par électrophorèse des complexes solubilisés ont permis en outre de réduire au minimum la libération des chlorophylles et de préserver la structure oligomérique des complexes. La combinaison de 2 détergents a été utilisée dans le but espéré de réduire la capacité détersive spécifique de chacun d'eux. Les résultats obtenus montrent que les caractéristiques des complexes chlorophylle-protéine d'Avoine sont proches de celles des complexes correspondants des autres plantes supérieures. L'analyse lipidique de chacun d'eux suggère que les thylacoïdes doivent présenter un faible degré d'hétérogénéité latérale. Toutes les classes de lipides sont retrouvées dans chacun des complexes et en compositions relatives similaires. Cependant, le LHCP1 possède une valeur du rapport MGDG/DGDG légèrement supérieure à celle des autres complexes et les molécules de PG qu'il contient sont riches en acide Δ3-trans-hexadécénoïque. Le degré d'insaturation des lipides des complexes dérivés du PS-I est faible, en particulier celui du SQDG.

Pour étudier la dépendance en lipides de certains systèmes enzymatiques des membranes de plastes d'Avoine, nous avons utilisé la stratégie qui consiste à délipider ces membranes à l'aide de différentes lipases puis à corréler la modification de la composition lipidique avec un changement dans l'activité enzymatique des différents systèmes étudiés. Des conditions expérimentales particulières permettant de limiter l'hydrolyse au MGDG de la monocouche externe du thylacoïde ont été recherchées. Les produits d'hydrolyse ont été retirés de la membrane par la BSA, présente tout au long de l'incubation. La dégradation du MGDG a pour effet d'inhiber le transport d'électrons non-cyclique ainsi que les flux partiels liés au PS-II et au PS-I. Le Triton X-100 restaure l'activité liée au PS-I alors que l'addition de plastocyanine reste pratiquement sans effet. Le site d'inhibition des activités impliquant ce photosystème semble être localisé au niveau du cytochrome f, la photooxydation de ce composé et sa réduction à l'obscurité étant diminuées à la fois en vitesse et en amplitude. L'activité liée au PS-II ne pouvant être restaurée par l'addition de donneurs artificiels, l'inhibition ne devrait pas se situer au niveau du système de dégagement de l'oxygène. L'examen des paramètres de fluorescence indique que la distribution de l'énergie entre les deux photosystèmes est altérée et ceci, au profit du PS-I. L'inhibition de l'augmentation de fluorescence induite par les cations divalents suggère quant à elle que l'hydrolyse du MGDG a pour effet de réduire la mobilité latérale des complexes dans la membrane.

La dégradation du MGDG appartenant aux complexes prothylacoïdes/corps prolamellaires des étioplastes réduit l'activité de la protochlorophyllide-réductase, en particulier sa vitesse maximale. Son affinité pour le NADPH diminue alors que celle pour le protochlorophyllide augmente.

Si certains lipides interagissent spécifiquement avec des complexes chlorophylle-protéine de la membrane thylacoïdale, alors l'hydrolyse sélective de ces lipides devrait perturber l'organisation supramoléculaire de ces complexes. En effet, l'hydrolyse sélective du MGDG par la lipase de Rhizopus arrhizus a permis de montrer que ce lipide pourrait interagir avec le LHCP en stabilisant la strucure quaternaire de ce dernier.

Lorsque les lipides des thylacoïdes sont hydrolysés par la lipase de Rhizopus arrhizus, les différentes activités photochimiques, l'organisation et la mobilité des complexes chlorophylle-protéine sont perturbées. Ces effets peuvent être la conséquence soit de l'hydrolyse des lipides-parents, soit de la libération dans la membrane des produits d'hydrolyse. Afin d'évaluer le potentiel des composés "lyso" à désorganiser la membrane, nous avons mené une étude comparative des pouvoirs solubilisants du lyso-MGDG et du lyso-DGDG. Celui du premier est très faible alors que le second détergent parvient à extraire des thylacoïdes le facteur de couplage et le centre réactionnel du PS-II. Une incubation des thylacoïdes en présence de lyso-DGDG a pour conséquence de réduire leur teneur en DGDG, vraisemblablement par un effet de substitution.

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