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].

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

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

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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

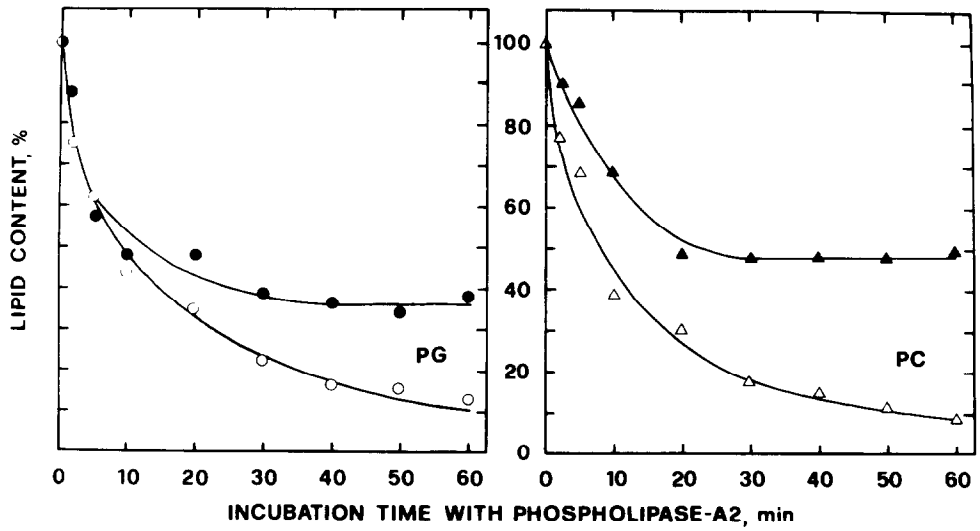

Fig.1. Time course of phospholipid hydrolysis at 5°C of normal (●, ▲) and disrupted (○, △) oat prothylakoids treated with pancreatic phospholipase A_2. Normal prothylakoids were treated as described in section 2. Disrupted prothylakoids were sonicated in the presence of phospholipase A_2 and 0.1 mM EDTA, 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.

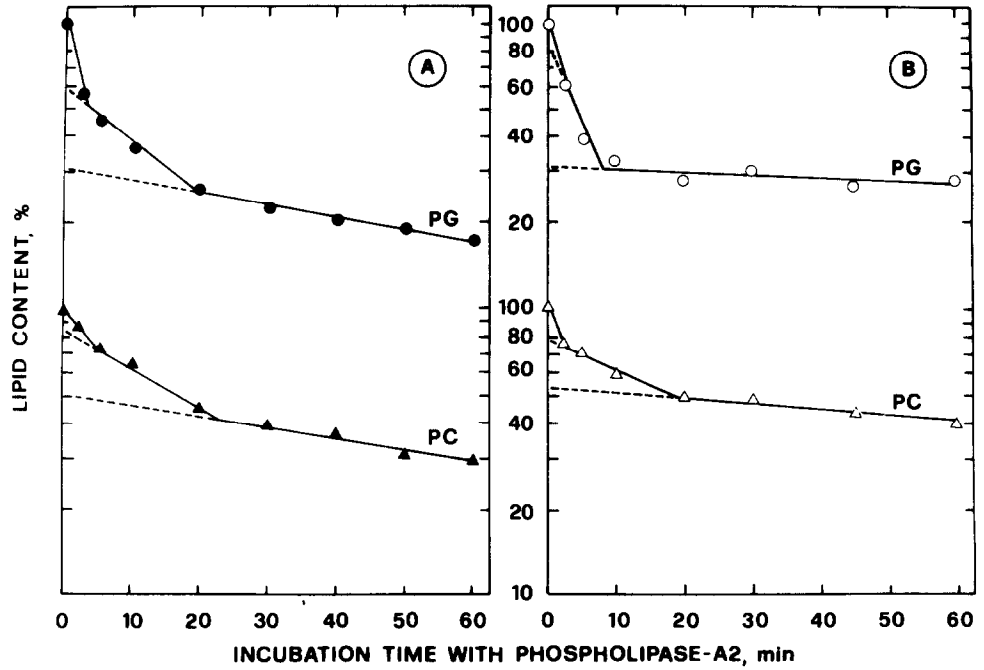

Fig.2. Time course of phospholipid hydrolysis at 4°C of oat prothylakoids treated with either pancreatic (A) or *V. russelli* (B) phospholipase A_2, expressed as semilog plots. In A, the reaction mixture contained 120 mM sorbitil, 5 mM Tricine-NaOH (pH 8), 4 mM $CaCl_2$, 0.8 Sigma units pancreatic phospholipase 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.

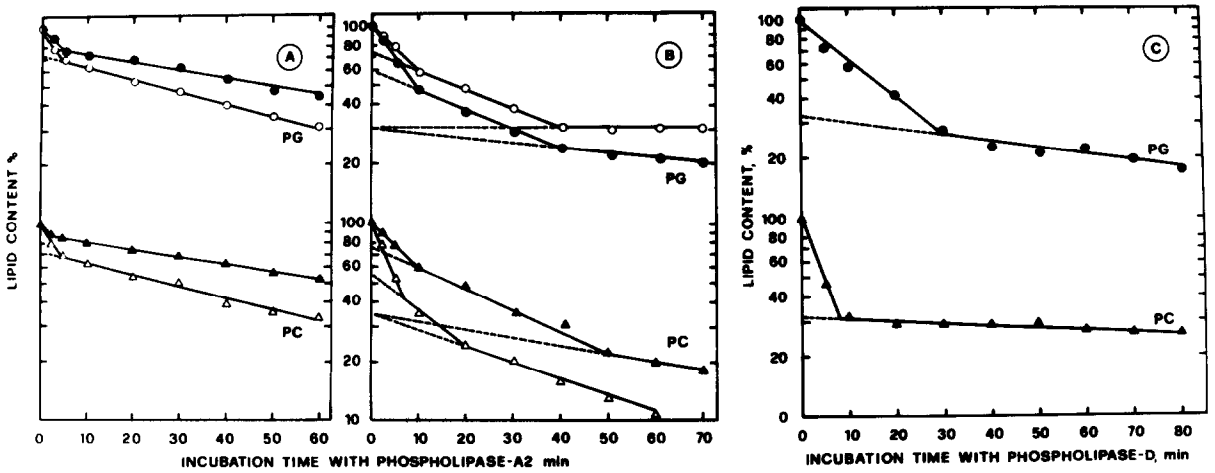

Fig.3. Time course of phospholipid hydrolysis, expressed as semilog plots of oat thylakoids treated with pancreatic phospholipase A_2 (A,B) or with phospholipase D from *S. chromofuscus* (C) under different conditions of temperature and ionic environment. In A and B, the reaction mixture contained 200 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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