Biochimica et Biophysica Acta, 975 (1989) 104–111
Elsevier

BBA 43012

The phospholipid population which sustains the uncoupled non-cyclic electron flow activity is localized in the inner monolayer of the thylakoid membrane

Paul-André Siegenthaler, André Rawyler and Jana Smutny

Laboratoire de Physiologie végétale, Université de Neuchâtel, Neuchâtel (Switzerland)

(Received 23 September 1988)
(Revised manuscript received 16 January 1989)

Key words: Phospholipid distribution; Photosynthetic electron flow; Thylakoid membrane; Phosphatidylcholine; Phosphatidylglycerol; (Spinach chloroplast)

When spinach thylakoids were treated with pancreatic phospholipase A_2, phospholipids were hydrolyzed and the uncoupled non-cyclic electron flow activity (from H_2O to $NADP^+$) was progressively inhibited. By removing, under controlled conditions, hydrolysis products (free fatty acids and lysophospholipids) with bovine serum albumin, it was possible to monitor the inhibition of electron flow activity as a function of the extent of phospholipid depletion. Phospholipid depletion in the outer and inner thylakoid membrane was achieved in a stepwise fashion by incubating thylakoids, first at 2°C for 60 min then at 20°C for 70 min. Complete phospholipid depletion (i.e., 56% of phosphatidylcholine and 74% of phosphatidylglycerol or 70% of the total phospholipids) in the outer monolayer led to less than 20% inhibition of the electron flow activity. In contrast, the depletion of the phospholipid populations (i.e., 44% of phosphatidylcholine and 16% of phosphatidylglycerol or 24% of total phospholipids) localized in the inner monolayer caused more than 80% inhibition of the activity. Finally, a third phosphatidylglycerol population (10%), which is presumably localized in the inner monolayer, was not involved in the electron flow activity. During phospholipid hydrolysis, it was verified that the osmotic responsiveness of thylakoids towards sorbitol remained intact, attesting that the access of phospholipase A_2 was restricted to the outer surface of thylakoid vesicles. These results point to the nonequivalent role of phospholipids in the inner and outer monolayers of the thylakoid membrane in supporting the uncoupled non-cyclic electron flow activity. Furthermore, it was shown that after complete depletion of both phospholipids in the outer monolayer, a stepwise delocalization of inner phospholipid molecules through transbilayer movement inhibited the activity in a nonlinear fashion. This suggests the occurrence of interactions having different types and strengths between these inner lipids, more particularly between phosphatidylglycerol and other membrane components.

Introduction

Several questions can be asked about the role of acyl lipids in the thylakoid membrane function: (a) what is the role of the different lipid classes or molecular species and their overall unsaturation level; (b) what are the extents of lipid asymmetry and the role of inner and outer lipids in the thylakoid membrane function? A number of fruitful attempts have been made to answer to the first question, mainly by using reconstitution and catalytic hydrogenation procedures. For instance, energy transfer [1], CF_0-CF_1 ATP synthetase activity [2], cytochrome b_6/f [3] and cytochrome *b*-559 [4] redox properties, oxygen evolution in subchloroplast particles [5,6], illustrate the requirement for specific lipid classes or species. Fatty acid unsaturation not only controls the polymorphic character of the acyl lipids [7] but reducing its level in a native membrane significantly alters several photosynthetic functions [8,9]. Concerning the next question, thylakoid membranes are characterized by an asymmetric distribution of both phospholipids [10,11] and galactolipids [12–15], the latter having been confirmed recently in thylakoid inside-out vesicles [16]. A direct consequence of these asymmetries is that the inner and outer monolayers of the thylakoid membrane have a very different composition in acyl lipids. This is

Abbreviations: Mops, 4-morpholinepropanesulphonic acid; PG, phosphatidylglycerol; PC, phosphatidylcholine; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Correspondence: P.A. Siegenthaler, Laboratoire de Physiologie végétale, Université de Neuchâtel, 20 Chemin de Chantemerle, CH-2000 Neuchâtel, Switzerland.

mainly due to the fact that the major thylakoid lipid, monogalactosyldiacylglycerol, represents 73% of the total lipids present in the outer monolayer but only 38% of the total lipids present in the inner monolayer [14]. Since the native monogalactosyldiacylglycerol is a nonbilayer-forming lipid, and since total lipids are almost equally distributed over the two monolayers, the ability of the outer lipids to form lamellar structures should be much lower than that of the inner lipids [14,15].

Thus, one can expect that, due to their different composition in acyl lipids, the two monolayers will be assigned different roles in the thylakoid membrane function. The purpose of this study was to investigate the respective contribution of outer and inner phospholipids in sustaining uncoupled non-cyclic electron flow activity and to see whether particular phospholipid of the outer and/or inner monolayers are differentially involved in this respect. To this aim, we have designed experimental conditions under which phospholipid depletion in the thylakoid membrane occurs sequentially, first in the outer, then in the inner monolayer. Here, we demonstrate unambiguously that the phospholipid population which is located in the inner monolayer of the thylakoid membrane is of great importance to sustain the uncoupled non-cyclic electron flow activity.

Materials and Methods

Enzymes and chemicals. Phospholipase A_2 from porcine pancreas was purchased from Sigma and used without further purification. Bovine serum albumin was purchased from Fluka and washed several times in acetone and ethanol before use as described elsewhere [17]. These repeated washings were necessary to remove free fatty acids and lysophospholipids which contaminated the original batch of bovine serum albumin.

Preparation of thylakoid membranes. Spinacia oleracea (var. Nobel) was purchased on the local market or grown in a growth chamber and the leaves were harvested after 14 weeks of hydroponic culture [18]. Intact chloroplasts were prepared according to Nakatani and Barber [19] in a medium containing 330 mM sorbitol/20 mM Tricine/0.2 mM $MgCl_2$ adjusted to pH 8 with KOH. Intact chloroplasts were washed once in 330 mM sorbitol adjusted to pH 8 with Tricine (centrifugation: $2200 \times g$ for 30 s). The sedimented intact chloroplasts were shocked osmotically in 15 ml H_2O for at least 30 s then an equal volume containing 20 mM Mops (pH 8)/100 mM sorbitol/100 mM NaCl (medium I) was added. After centrifugation for 3 min at $4300 \times g$, the resulting pellet was resuspended in the above medium (H_2O/medium I: 1/1). Thylakoids were washed twice, resedimented and resuspended in the medium I to a concentration of 2 mg chlorophyll/ml. During these sedimentations, the molar ratio PG/PC increased to reach a constant value of about 2.5 as already shown [20], thus indicating that the phospholipids measured in our thylakoid preparations were representative of these membranes and could not be attributed to envelope contaminations. Chlorophyll was determined as described earlier [21].

Enzymatic treatments. The incubation of control- and phospholipase A_2-treated thylakoid membranes (1.0 mg chlorophyll/ml) was carried out in darkness at 2°C for 60 min followed by a second incubation at 20°C for 70–90 min in 50 mM Tricine/10 mM Mops (pH 8)/85 mM NaCl/50 mM sorbitol/1 mM $CaCl_2$ ± phospholipase A_2 (2–4 units/mg chlorophyll). At various times (including a zero-time control without phospholipase A_2), aliquots (≤ 1 ml) of the above medium were withdrawn and supplemented with 2 mM EGTA (pH 7.8) to stop the enzymatic reaction and with 20 mg/ml of bovine serum albumin to remove free fatty acids and lysophospholipids from the thylakoid membranes. After incubation on ice for 10 min, the samples were diluted 7-fold with a bovine serum albumin-free medium (50 mM Tricine (pH 8)/100 mM NaCl) and spun at $6000 \times g$ for 3 min. The resulting pellet was washed once more as above, then resuspended and used for the determination of electron transport activity and phospholipid content. Under these conditions, it has been shown that thylakoid membranes do not contain any free fatty acids and lysophospholipids [17].

Determination of electron flow activity. Uncoupled non-cyclic electron flow activity (Photosystem II + I) was measured at 20°C by the photoreduction of $NADP^+$ (340 nm) from H_2O in a reaction mixture containing 50 mM Tricine (pH 8.4)/35 mM NaCl/2 mM $CaCl_2$/2 mM $NADP^+$/70 μg ferredoxin/ml/2 mM NH_4Cl and thylakoids (100 μg chlorophyll/ml). The intensity of actinic light after filtration through a Calflex and DT-red filter was about 29 $mW \cdot cm^{-2}$ at the level of the cuvette (2 mm pathway) and was found to be saturating for the electron transport activity.

Lipid analysis. Thylakoid suspensions (280 μl) were extracted by 0.5 ml chloroform/methanol (53 : 37) resulting in a two-phase system. The lower phase was then spotted on silicagel 60-coated plates, which were developed in acetone/toluene/methanol/water (90 : 25 : 7 : 12) as described earlier [17]. The phospholipid content of control and phospholipase A_2-treated thylakoid membranes was determined by phosphate analysis according to Rouser et al. [22].

Packed volume. The packed volume of thylakoids were measured in the dark as described earlier [23]. Aliquots of thylakoid suspension were washed once in 50 mM Tris-HCl (pH 8)/35 mM NaCl, then transferred in Eppendorf tubes containing 200 μl of sorbitol at various concentrations (see Fig. 3). The resulting sus-

pension was introduced in capillary tubes which were centrifuged for 3 min in a Christ microcentrifuge (23 000 × *g*). The packed volume was estimated by the ratio of the height of the thylakoid sediment to the total height of the solution in the capillary tube. When bovine serum albumin was used, it was added to the thylakoid suspension aliquots for 10 min at 2 °C then the suspension was washed in the same above medium and introduced in Eppendorf tubes containing the different sorbitol concentrations.

Results

First, we have designed conditions under which phospholipid depletion occurred only in the outer monolayer of the thylakoid membrane. This was achieved by an incubation of membranes with phospholipase A_2 for 60 min at 2 °C, a temperature at which negligible transbilayer movement of the inner lipids takes place. Fig. 1 shows that, at 2 °C, the hydrolysis of PC and PG proceeded in a stepwise fashion and reached a (quasi) horizontal 'plateau' after 40 min, indicating that under our conditions the transbilayer movement of the internal phospholipids was indeed insignificant. This degradation corresponds, as shown previously [10,11] to the hydrolysis of all phospholipids in the outer leaflet. In this particular experiment, the outer/inner molar ratio was 74/26 for PG and 56/44 for PC. These values are in the range of those obtained earlier [10,11]. When the temperature was raised to 20 °C (e.g., after the first 60 min of incubation), the degradation of both phospholipids resumed very rapidly.

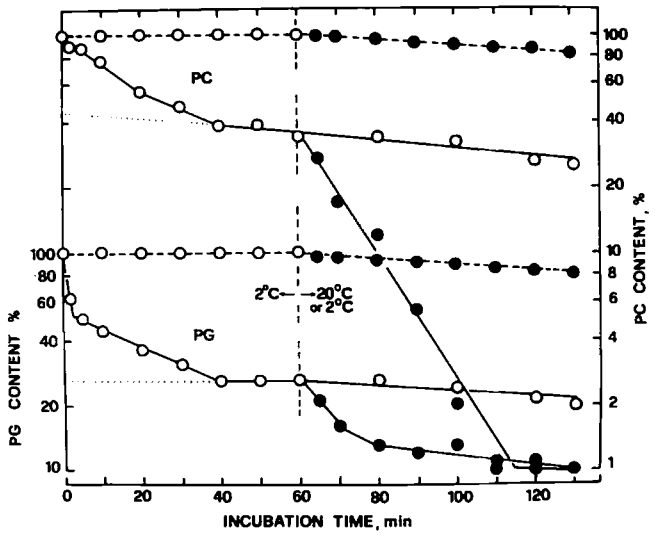

Fig. 1. Time-course of PC and PG hydrolysis at 2 °C (○) and at 20 °C (●) of spinach thylakoids in the presence (———) or absence (— — —) of pancreatic phospholipase A_2. Results are expressed as semilog plots. The 100% values corresponded to 98 and 251 nmol/mg chlorophyll for PC and PG, respectively. Extrapolation (·····) to zero time of the plateau levels reached at 2 °C after 60 min indicates the outer/inner molar ratio for each phospholipid.

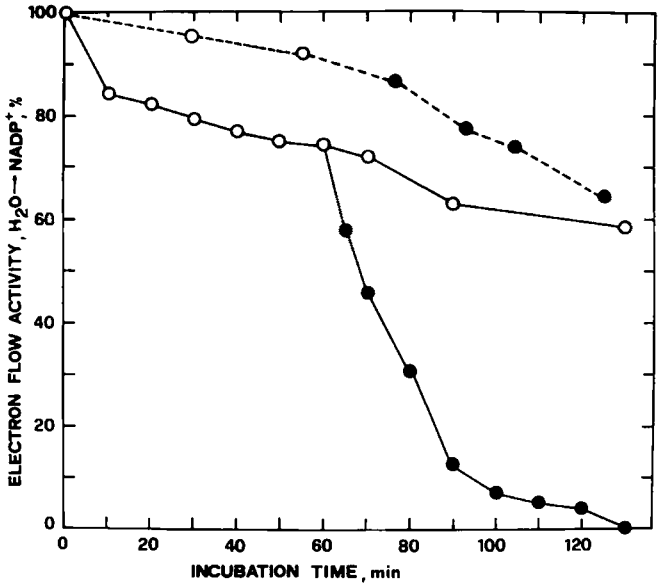

Fig. 2. Time-course of $NADP^+$ photoreduction rates (from H_2O) of spinach thylakoids in the presence (———) and absence (— — —) of pancreatic phospholipase A_2. Incubations were carried out at 2 °C (○) and 20 °C (●). The 100% value corresponded to 135 μmol $NADP^+$ reduced/(mg chlorophyll) per h.

After 130 min, no more PC and only about 10% of PG remained in the membrane. In contrast, at 2 °C, the 'plateaus' reached after 40 min of incubation remained almost at a constant level up to 130 min, thus confirming the occurrence of transbilayer movement of inner phospholipid molecules at 20 °C. It is noteworthy that, at 2 °C, initial rates of hydrolysis reflect the known preference of the pancreatic phospholipase A_2 for anionic lipids, whereas the corresponding rates at 20 °C merely express the difference in the transbilayer movement rates of these two phospholipids. The PC and PG content of control membranes did not change at 2 °C, whilst it diminished very slowly at 20 °C (Fig. 1). We must emphasize here that, under the above conditions, bovine serum albumin used during the washing step removed all hydrolysis products (free fatty acids and lysophospholipids) as shown recently [17]. Thus, the hydrolysis curves express also the progressive phospholipid depletion in thylakoid membranes.

The changes of uncoupled electron flow activities in the same phospholipase A_2-treated thylakoid preparation are shown in Fig. 2. At 2 °C, after a rapid but limited decrease, the activity diminished progressively in a way parallel to that of the control up to 130 min. In contrast, when the temperature was raised to 20 °C (after 60 min of incubation), the inhibition of activity resumed very rapidly and was completely after 130 min. In control samples, the activity diminished progressively to reach 70% of the initial value after 130 min. Since the strong inhibition of the electron flow activity observed at 20 °C in phospholipase A_2-treated membranes (see Fig. 2) might be caused by the access of enzyme mole-

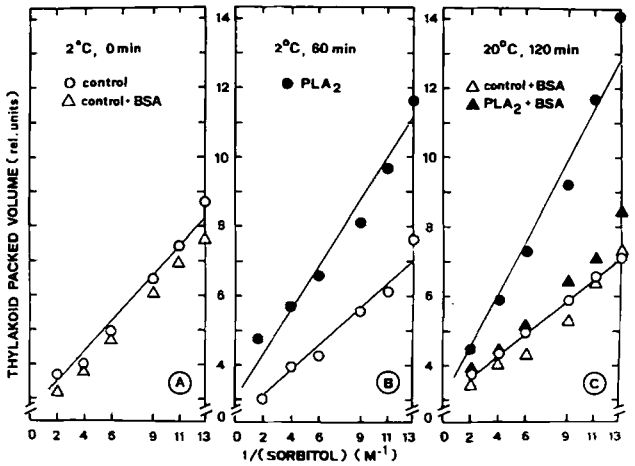

Fig. 3. Osmotic volume responses of spinach thylakoids as expressed by the thylakoid packed volume versus the reciprocal of osmolarities (essentially sorbitol and salts). Experiments were performed at three critical steps during the hydrolysis kinetics shown in Figs. 1 and 2: (A) at the beginning of the experiment (2°C, 0 min); (B) after 60 min at 2°C and (C) after 120 min at 20°C. BSA, bovine serum albumin; PLA_2, pancreatic phospholipase A_2.

cules to the lumenal compartment, it was necessary to appreciate the thylakoid membrane integrity during the hydrolysis process. To this end, we have tested the osmotic responsiveness of thylakoids towards sorbitol, a molecule having a much smaller size than phospholipase A_2. Thylakoid packed volumes were plotted versus the reciprocal of the external osmolarities under three critical conditions: (1) at the beginning of the experiment (Fig. 3A); (2) after 60 min at 2°C in the presence of phospholipase A_2, corresponding to the hydrolysis of all phospholipids in the outer monolayer (Fig. 3B) and (3) after 120 min at 20°C in the presence of phospholipase A_2, corresponding to a complete phospholipid hydrolysis (or depletion in the presence of bovine serum albumin) in the thylakoid membrane (Fig. 3C) (see also Fig. 1). Two parameters are important here: the slope of the straight line which expresses the Boyle-Van't Hoff relation [24] and the intercept on the ordinate corresponding to the thylakoid volume (V_∞) that would be obtained at an infinite external osmotic pressure. Under the three above conditions, the slopes and V_∞ were not significantly different from control samples. The packed volume, but not V_∞, of phospholipase A_2-treated thylakoids was increased in the absence of bovine serum albumin (Figs. 3B and C). This could be ascribed to the presence of end-products of phospholipid hydrolysis [25], since after the washing procedure with bovine serum albumin the slope was identical to that observed for control thylakoids.

A convenient way to identify the phospholipid populations which sustain the uncoupled non-cyclic electron flow activity is presented in Fig. 4. Based on the results shown in Figs. 1 and 2, the extent of activity inhibition has been plotted versus PG (left panel) and PC (right panel of Fig. 4) depletion. Three phospholipid populations can be distinguished. The first one was entirely located in the outer monolayer. Depletion of 74% of PG and 56% of PC and (corresponding to about 70% of total phospholipids) resulted only in less than 20% inhibition of electron flow activity. The second population was confined to the inner monolayer. It is noteworthy that further depletion of 16% of PG and 44% of PC (corresponding to 24% of the total phospholipids) led to complete inhibition of the activity. In addition, a third pool of PG (about 10%), which under our experimental conditions was not accessible to phospholipase A_2, was obviously not required for this type of activity.

A definitive way to show the crucial importance of inner phospholipid populations in supporting the electron flow activity, would be to demonstrate that not only depletion but a simple delocalization of these populations is sufficient in itself to impair the activity. This has been achieved in the following fashion: (1) the outer monolayer of the thylakoid was completely depleted in both phospholipids (at 2°C as in Fig. 1), the electron flow activity being only slightly affected under these conditions (see Figs. 2 and 4); (2) the phospholipase A_2 activity was blocked by addition of EGTA and the thylakoid membranes were post-incubated at 20°C for another 90 min, adequate controls being run simultaneously (2°C with EGTA, 20°C without EGTA, as shown in Fig. 5); (3) the uncoupled non-cyclic electron flow activity and the total content of phospholipids were measured in parallel. Results of such experiments as well as their interpretation in terms of phospholipid (de)localization are illustrated in Fig. 5. At 2°C in the presence of EGTA, conditions under which phospholipid hydrolysis was completely stopped, both phospholipid levels remained constant (Figs. 5A and B) whilst the activity decreased slightly (Fig. 5C). In contrast, at 20°C and in the absence of EGTA, both phospholipid depletion (Figs. 5A and B) and activity inhibition (Fig. 5C) were complete. However, at 20°C in the presence of EGTA, conditions under which phos-

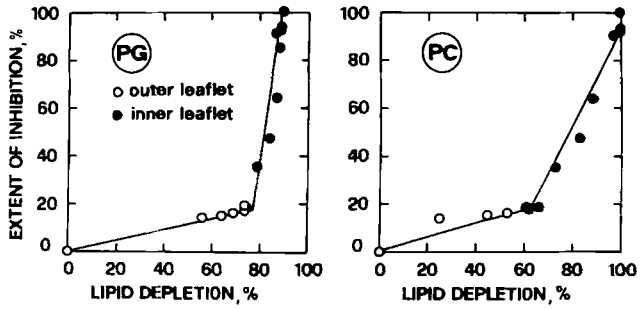

Fig. 4. Phospholipid-dependency of uncoupled non-cyclic electron flow activity (H_2O/$NADP^+$). The extent of activity inhibition and of PG and PC depletion are computed from the data of Figs. 1 and 2 and calculated with reference to the control values obtained for each time in both kinetics.

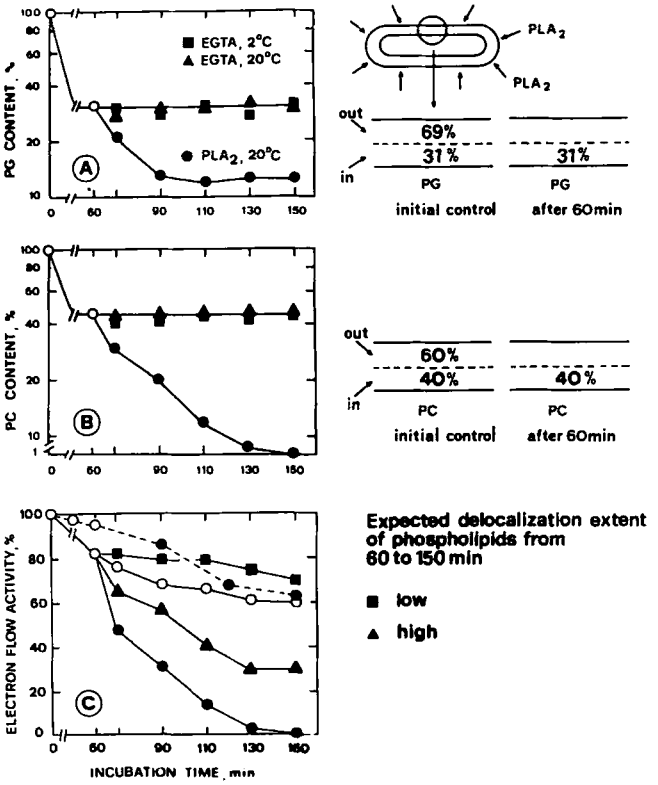

Fig. 5. Discrimination between the effects of phospholipid depletion and delocalization in the thylakoid inner monolayer on the uncoupled non-cyclic electron flow activity. During the first part of the experiment (0–60 min) the outer monolayer has been depleted in phospholipids under the conditions described in Fig. 1 (2° C). Then phospholipase A_2-treated thylakoids were post-incubated at 20° C without EGTA (●), 20° C with EGTA (▲) or at 2° C with EGTA (■) for 90 min. PG (in A), PC (in B) content and the electron transport activity (in C) were determined (see also explanations in the text). In C, control activities correspond to (○———○) for PLA_2-treated thylakoids at 2° C, to (○— — —○) for thylakoids without PLA_2 at 2° C and to (●— — —●) for thylakoids without PLA_2 at 20° C. The 100% values were similar to those given in Figs. 1 and 2. In the right panel, the transmembrane distribution of phospholipids is schematically represented and the expected delocalization of phospholipids due to outwards transbilayer movement is indicated for the above post-incubation conditions for two EGTA-containing samples.

pholipid hydrolysis was blocked, the phospholipid content did not change (Figs. 5A and B), whereas the activity decreased and eventually stabilized at about half of the (2° C with EGTA)-sample level (Fig. 5C).

The decrease in activity found under these last conditions may be interpreted in terms of transbilayer movement of phospholipids which are delocalized from the inner to the outer monolayer of the thylakoid membrane. Indeed, during the 60–150 min incubation period, new phospholipid molecules should become available in the outer monolayer which was previously completely depleted in phospholipids. Therefore, the hydrolysis pattern of PG obtained upon addition of fresh phospholipase A_2 at 150 min should indicate how much of the inner PG has been translocated to the outer monolayer during the 60–150 min incubation periods. To this end, thylakoids obtained after 150 min treatment (end of incubation in Fig. 5) were washed twice as described in the legend to Table I, then incubated again in the presence of phospholipase A_2 at 2° C. The hydrolysis kinetics of PG presented a rapid degradation phase followed by a quasi-horizontal plateau (data not shown), from which the delocalized amount of PG could be determined. Due to the very low amount of PC remaining in the membrane after 150 min, the behaviour of this lipid could not be investigated with sufficient accuracy. Table I shows that the total amount of PG remained constant only when phospholipase A_2 was blocked by the addition of EGTA (see also Figs. 5A and B). As expected (see Fig. 5), the delocalization of PG occurred when phospholipase A_2 was inactivated by EGTA and its extent was increased when the temperature was raised from 2 to 20° C. In contrast, when phospholipase A_2 was active during the same period (20° C, without EGTA), the 12% of PG remaining in the membrane kept their inaccessibility. When the delocalization extent was expressed as percent of PG remaining in the thylakoid membrane after 150 min, it is seen that this phospholipid did not redistribute equally between the two monolayers, especially at 2° C.

Discussion

Methodological considerations

It is now well established that the enzymatic approach allows the determination of acyl lipid transmembrane distribution in thylakoids [26,27]. Indeed, the same transversal distribution of galactolipids has been found when two lipases (lipolytic acyl hydrolase from potato tubers and the lipase from *Rhizopus arrhizus*) differing in their specificity and mode of action were used [12,14,15]. This is also true for phospholipid distribution when using either the pancreatic or the *Vipera russelli* venom phospholipase A_2 and the phospholipase D from *Streptomyces chromofuscus* [10]. The reliability of this technique has been recently confirmed by the demonstration that the transmembrane distributions of galactolipids in thylakoids and thylakoid inside-out vesicles are opposite [16].

The enzymatic approach is also very useful for determining the involvement of various lipid classes in the thylakoid membrane function [27,28]. However, one has to ascertain that the breakdown products (which are by themselves strongly inhibitory) released in the membrane by the enzyme action are efficiently and totally removed from the membrane. Only under these conditions will it be possible to establish a direct correlation between the parent lipid depletion per se and the occurrence of functional alterations. In the present investigation, these conditions have been fulfilled, as described previously [17]. Another important requirement is that the barrier properties of the thylakoid should remain

TABLE I

Delocalization of PG from the inner to the outer monolayer estimated by the availability of this lipid in the outer monolayer of the thylakoid membrane

Fresh thylakoids were treated for 60 min at 2° C with phospholipase A_2 then divided into three samples which were incubated from 60 to 150 min at (a) 2° C with EGTA; (b) 20° C without EGTA and (c) 20° C with EGTA. At the end of the incubation, thylakoids were washed twice: firstly, in 50 mM Tricine (pH 7.8)/100 mM NaCl/bovine serum albumin (20 mg/ml); secondly, in the same washing medium, but without bovine serum albumin. At this stage ($t = 150$ min), thylakoids were supplemented with fresh phospholipase A_2 at 2° C, under the conditions described in Materials and Methods, in order to determine the amount of PG which is now available in the outer monolayer. The 100% value corresponded to 240 ± 13 ($n = 9$) nmol PG/mg chlorophyll. For all other experiments, $n = 4$.

Incubation		Amount of PG remaining in thylakoid membranes (% lipid class)	Availability of PG in the outer monolayer (% lipid class)	
time (min)	conditions			
0	–	100	69 ±7 [a]	–
60	2° C	31 ± 7	0 [a]	–
150	2° C, + EGTA	30 ± 2	3.0 ± 2.1 [a]	10 ± 7 [b]
150	20° C, − EGTA	12 ± 3	0.2 ± 0.2 [a]	2 ± 2 [b]
150	20° C, + EGTA	27 ± 2	6.2 ± 1.3 [a]	23 ± 5 [b]

[a] Calculated from the total amount of PG in the initial control samples (= 100%).
[b] Calculated from the amount of PG remaining in the thylakoid membrane after 150 min which is taken as 100%.

intact upon phospholipid depletion. The results of Fig. 3 show that these properties towards sorbitol were unchanged even after complete phospholipid hydrolysis, provided that breakdown products were removed by bovine serum albumin. If sorbitol cannot readily cross the thylakoid membrane under these conditions, it can be safely concluded that the much larger phospholipase A_2 molecule has no access to the lumenal thylakoid face.

The molar ratio PG/PC in thylakoids being constant after three sedimentations (see Materials and Methods), we have taken this observation as a reliable indication that the small amount of PC in thylakoids is not due to envelope contamination. At least two observations support this conclusion: (a) the double-bond index of PC is about 1.4-times higher in thylakoid than in envelope membranes [29]; (b) diacylglycerol is always present in conventionally isolated envelope membranes whilst it is absent or in trace amounts in thylakoids [30].

Involvement of outer and inner phospholipid populations in the electron flow activity: effect of phospholipid depletion

In a previous study, we have found that distinct phospholipid populations are involved, although with various efficiencies, in supporting electron flow activity [17]. However, these populations could not be localized with accuracy in the thylakoid membrane because the temperature (20° C) at which hydrolysis was carried out favoured the transbilayer movement of internal phospholipids, thereby making less clear-cut the distinction between outer and inner phospholipids. In contrast, we show here that the transbilayer movement was negligible when depletion occurred at 2° C (see Fig. 1). This allows us to distinguish clearly between outer and inner phospholipid populations and to determine the extent to which they sustain electron flow activity (see Fig. 4). The fact that the removal of all phospholipids in the outer leaflet (i.e., 74% of PG and 56% of PC) resulted in less than 20% electron flow activity inhibition indicates that the two monolayers have certainly quite different roles to play in this type of function. Indeed, it is noteworthy that the simultaneous depletion of only 16% of PG and 44% of PC in the inner monolayer caused the inhibition of the 80% remnant activity. To evaluate the relative functional importance of the two monolayers, it can be calculated that the efficiency of outer/inner phospholipids to support electron flow activity is about 1/12. Although it is the first report of a topologically defined lipid requirement of a plant membrane activity, similar results have been found for two ATPases in the erythrocyte membrane [31].

The stringent requirement of non-cyclic electron flow activity for inner phospholipids indicates that this activity relies essentially upon that part of the thylakoid membrane which exhibits the strongest bilayer-forming potential, namely the inner monolayer. We cannot therefore agree with the views of Thomas et al. [32], who attributed the phospholipase A_2-induced inhibition of non-cyclic electron flow to the subsequent formation of monogalactosyldiacylglycerol-enriched cylindrical inverted micelles rather than to phospholipid hydrolysis per se. Although we acknowledge the fact that phospholipase A_2 treatment of pea thylakoid membranes resulted in the formation of non-bilayer structures, we believe that the mere presence of high amounts of both lysophospholipids and free fatty acids, which were not removed from the membranes by these authors [32], may well be responsible for the formation of such nonbilayer structures. Indeed, aging in vitro of thylakoid membranes, during which endogenous lipolytic activities generate both lysolipids and free fatty acids [33], is

TABLE II

Relative efficiency of PG molecules which were delocalized from the inner monolayer in sustaining uncoupled non-cyclic electron flow activity

Successive delocalizations of PG molecules occurring during the 60–150 min period have been achieved as described in Fig. 5. The amount of delocalized PG and the corresponding extent of inhibition are calculated from Table I and Fig. 5, respectively.

Delocalization conditions	Amount of delocalized PG (%)	Corresponding extent of inhibition (%)	Relative efficiency
(2°C, +EGTA)	3.0	12	4
(20°C, +EGTA)−(2°C +EGTA)	3.2	40	12.5
(20°C, −EGTA)−(20°C +EGTA)	12.8	30	2.3

known to promote the appearance of these structures [34]. However, clarification of this issue requires further studies.

Involvement of inner phospholipid (sub)populations in the electron flow activity: effect of phospholipid delocalization

Removal of phospholipids in the inner monolayer is the result of two closely related phenomena: phospholipids move from the inner to the outer monolayer (delocalization) where they are hydrolyzed by the phospholipase A_2, the breakdown products being subsequently removed by bovine serum albumin (depletion). One may wonder which of the two phenomena, delocalization per se or/and delocalization plus depletion, is responsible for the complete inhibition of the activity. The results of Fig. 5 and Table I show clearly that delocalization is the important phenomenon, at least for PG. This emphasizes again that the presence of this phospholipid in the inner monolayer is compulsory to sustain electron flow activity. In addition, it can be calculated that the extent of inhibition of the activity does not depend linearly on the amount of PG which has been delocalized from the inner monolayer as indicated by the relative efficiencies in Table II. Inner PG molecules may consist of several subpopulations which sustain to quite different extent the electron flow activity. The results of Tables I and II, obtained under particular experimental conditions suggest the occurrence of four such subpopulations, including the nondelocalizable one (12%), which interact increasingly stronger with other components of the inner monolayer.

Another aspect of our results deserves some comments. When a given lipid is partially or totally removed from the outer monolayer of a membrane by a nonlytic treatment with a lipase, an outward transmembrane movement of inner lipid molecules can occur, the extent of which is strongly temperature-dependent. This diffusional process is generally considered as an attempt to correct the lipase-induced desequilibrium in the transmembrane (asymmetric) distribution of the lipid, at the expense of the lipid content of the inner monolayer [35]. When phospholipase A_2 was active, 6 and 19% of the total thylakoid PG were delocalized from the inner monolayer at 2 and 20°C, respectively, whereas upon inhibition of phospholipase A_2 by EGTA, the respective delocalization extents were 3 and 6.2% only (Table III). These unexpected low values can be explained as follows. The continuous PG hydrolysis in the outer monolayer, followed by the removal of breakdown products by bovine serum albumin, maintains the above-mentioned desequilibrium in the transmembrane distribution, so that a maximum of delocalizable PG molecules will eventually leave the inner monolayer at a given temperature. On the other hand, when phospholipase A_2 is inactive, no hydrolysis will occur in the outer monolayer, the desequilibrium will be decreased and the translocation of inner PG molecules will be significantly reduced. Moreover, since all delocalization processes studied here were carried out over a similar time-period (from 60 to 150 min), any change in the delocalization extent should be reflected by an antiparallel change in the delocalization half-time. As shown in Table III, this was indeed the case, thereby confirming our interpretation. Thus, it appears that some of the interactions occurring between the inner PG population and other membrane components are in fact dedicated mechanisms for maintaining the native localization of this population, so as to meet the requirement of electron flow activity for inner PG. One may hypothesize

TABLE III

Influence of temperature and of EGTA on the extent and the rate of outward transbilayer movement (delocalization) of inner PG

Data from Tables I and II and from Fig. 1 were used to calculate the amount of PG delocalized from the inner monolayer and the half-time value of the corresponding transbilayer movement which occurred during the 60–150 min incubation period.

Conditions	Amount of delocalized PG (% of total PG)	Half-time (min)
2°C, +EGTA [a]	3.0	260
2°C, −EGTA [b]	6.0	110
20°C, +EGTA [a]	6.2	110
20°C, −EGTA [b]	19.0	10

[a] PLA_2 inhibited (see Fig. 5A).
[b] PLA_2 active (see Figs. 1 and 5A).

that the inner monolayer, because of the high bilayer-forming potential of its lipid complement [14,15], is implied in the stabilization of the bilayer configuration of thylakoid membranes and, more particularly, in the 'lipid-dependency' of electron transport activities. On the other hand, the outer monolayer, through the polymorphic character of its lipid moiety, may rather be involved in as yet unknown 'fine tuning' mechanisms of membrane function.

In conclusion, we believe that the use of lipolytic enzymes, as described in the present report, offers two definite advantages with respect to other approaches for studying the lipid-dependence of thylakoid membrane electron flow activities (as reviewed recently in Ref. 28). First, lipid depletion is not only quantitatively controlled (in contrast to solvent or detergent extraction) but it is also selective with respect to lipid classes through the appropriate choice of lipolytic enzymes. Second, the progress of lipid depletion is completely defined in terms of transmembrane distribution of lipids; indeed, the enzymatic approach enables one to know whether depletion actually occurs in the outer or in the inner monolayer of the thylakoid membrane. Attempts to restore the inhibited activity by back-additions of phospholipids, although desirable, may suffer from the fact that addition of anionic lipids, such as PG or sulphoquinovosyldiacylglycerol, was reported to inhibit strongly – rather than to reactivate – Photosystem II-associated electron flow activities [4,35,36].

Acknowledgements

This work was supported in part by the Swiss National Science Foundation (Grant No. 3.346.0.86 to P.A.S.). The authors thank Dr. L. Vigh for reading the manuscript.

References

1 Siefermann-Harms, D., Ninnemann, H. and Yamamoto, H.Y. (1987) Biochim. Biophys. Acta 892, 303–313.
2 Pick, U., Weiss, M., Gounaris, K. and Barber, J. (1987) Biochim. Biophys. Acta 891, 28–39.
3 Chain, P.K. (1985) FEBS Lett. 180, 321–325.
4 Matsuda, H. and Butler, W.L. (1983) Biochim. Biophys. Acta 724, 123–127.
5 Gounaris, K., Whitford, D. and Barber, J. (1983) FEBS Lett. 163, 230–234.
6 Murphy, D.J., Crowther, D. and Woodrow, I.E. (1984) FEBS Lett. 165, 151–155.
7 Horvath, I., Mansourian, A.R., Vigh, L., Thomas, P.G., Joo, F. and Quinn, P.J. (1986) Chem. Phys. Lipids 39, 251–264.
8 Vigh, L., Joo, F., Droppa, M., Horvath, L.I. and Horvath, G. (1985) Eur. J. Biochem. 147, 477–481.
9 Horvath, G., Droppa, M., Szito, T., Mustardy, L.A., Horvath, L.I. and Vigh, L. (1986) Biochim. Biophys. Acta 849, 325–336.
10 Siegenthaler, P.A. and Giroud, C. (1986) FEBS Lett. 201, 215–220.
11 Unitt, M.D. and Harwood, J.L. (1985) Biochem. J. 228, 707–711.
12 Rawyler, A. and Siegenthaler, P.A. (1985) Biochim. Biophys. Acta 815, 287–298.
13 Sundby, C. and Larsson, C. (1985) Biochim. Biophys. Acta 813, 61–67.
14 Rawyler, A., Unitt, M.D., Giroud, C., Davies, H., Mayor, J.-P., Harwood, J.L. and Siegenthaler, P.A. (1987) Photosynth. Res. 11, 3–13.
15 Giroud, C. and Siegenthaler, P.A. (1988) Plant Physiol. 88, 412–417.
16 Siegenthaler, P.A., Sutter, J. and Rawyler, A. (1988) FEBS Lett. 228, 94–98.
17 Siegenthaler, P.A., Smutny, J. and Rawyler, A. (1987) Biochim. Biophys. Acta 891, 85–93.
18 Siegenthaler, P.A. and Depéry, F. (1976) Eur. J. Biochem. 61, 573–580.
19 Nakatani, H.-Y. and Barber, J. (1977) Biochim. Biophys. Acta 461, 510–512.
20 Giroud, C. and Siegenthaler, P.A. (1984) in Structure, Function and Metabolism of Plant Lipids (Siegenthaler, P.A. and Eichenberger, W., eds.), pp. 413–416, Elsevier, Amsterdam.
21 Bruinsma, J. (1961) Biochim. Biophys. Acta 53, 576–578.
22 Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494–496.
23 Siegenthaler, P.A. (1968) Bull. Soc. Bot. Suisse 78, 202–209.
24 Nobel, P.S. (1970) in Plant Cell Physiology. A Physico-Chemical Approach, pp. 54–57, W.H. Freeman, San Francisco.
25 Siegenthaler, P.A. (1972) Biochim. Biophys. Acta 275, 182–191.
26 Siegenthaler, P.A. (1982) in Biochemistry and Metabolism of Plant Lipids (Wintermans, J.F.G.M. and Kuiper, P.J.C., eds.), pp. 351–358, Elsevier Biomedical Press, Amsterdam.
27 Siegenthaler, P.A., Rawyler, A. and Giroud, C. (1987) in The Metabolism, Structure and Function of Plant Lipids (Stumpf, P.K., Mudd, J.B. and Nes, W.D., eds.), pp. 161–168, Plenum, New York.
28 Siegenthaler, P.A. and Rawyler, A. (1986) in Encyclopedia of Plant Physiology (Staehelin, L.A. and Arntzen, C., eds.), New Series, Vol. 19, pp. 693–705, Springer Verlag, Berlin.
29 Douce, R., Holz, R.B. and Benson, A.A. (1973) J. Biol. Chem. 248, 7215–7222.
30 Siebertz, H.P., Heinz, E., Linscheid, M., Joyard, J. and Douce, R. (1979) Eur. J. Biochem. 101, 429–438.
31 Roelofsen, B., Van Meer, G. and Op den Kamp, J.A.F. (1981) Scand. J. Clin. Lab. Invest. 41 (Suppl. 156), 111–115.
32 Thomas, P.G., Brain, A.P.R., Quinn, P.J. and Williams, W.P. (1985) FEBS Lett. 183, 161–166.
33 Henry, L.E.A., Strasser, R.J. and Siegenthaler, P.A. (1982) Plant Physiol. 69, 531–536.
34 Gounaris, K., Brain, A.P.R., Quinn, P.J. and Williams, W.P. (1983) FEBS Lett. 153, 47–51.
35 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71.
36 Rochester, C.P. and Andersson, B. (1986) Physiol. Plant. 68, 136–140.