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The topology of phosphatidylglycerol populations is essential for sustaining photosynthetic electron flow activities in thylakoid membranes

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Abstract

The transmembrane distribution of phosphatidylglycerol (PG) was determined in rightside-out (RO) and inside-out vesicles (IO) obtained by fragmentation of spinach thylakoids in a Yeda press, followed by partition in an aqueous dextran-polyethyleneglycol two-phase system. Using the phospholipase A_2 from porcine pancreas to digest selectively PG molecules in the outer monolayer (exposed to the incubation medium) of the membrane, we found the molar outside/inside distribution to be $70/30 \pm 5$ in RO and $40/60 \pm 3$ in IO. The transmembrane distribution of PG in IO was the opposite of that in intact thylakoids (molar ratio $58/42 \pm 3$). The phospholipid population which sustained most of the uncoupled photosystem II electron flow activity was localized in the inner monolayer (exposed to the thylakoid lumen) of both thylakoid and RO membranes. In contrast, the activity in IO membranes was highly dependent on the PG population located in the outer monolayer. This finding brings the first direct demonstration of the dependence of the photosynthetic electron flow activity on the integrity of the inner topological pool of PG in the thylakoid membrane.

Keywords: Lipid transmembrane distribution; Thylakoid monolayer, outer/inner; Phosphatidylglycerol; Photosynthetic electron flow; *Spinacia oleracea* L.; Thylakoid inside-out vesicle

1. Introduction

The distribution of proteins within the thylakoid membrane has been extensively studied. Their asymmetric distribution, both laterally and transversally, confers vectorial properties to this membrane [1,2]. Transversal heterogeneity was also reported for acyl lipids [3] and has been mainly studied by selectively digesting lipids with specific lipases. The outer monolayer of the thylakoid membrane was found to be enriched in MGDG [4] and PG [5], whereas the inner monolayer is highly enriched in DGDG [4]. These results have been confirmed in thylakoids of several other plant species [6,7]. The transmembrane distribution of galactolipids in spinach thylakoids has been verified by using thylakoid inside-out vesicles and found to be the opposite of that found in intact thylakoids [8]. Such a demonstration has not been reported for PG.

Phosphatidylglycerol is an important lipid because it is involved in several structural and functional properties of the thylakoid membrane, e.g. stabiliza-

Abbreviations: DGDG, digalactosyldiacylglycerol; IO, inside-out vesicles; MGDG, monogalactosyldiacylglycerol; PLA_2, phospholipase A_2; PG, phosphatidylglycerol; PpBQ, phenyl-*p*-benzoquinone; PSII/I, photosystem II/I; RO, rightside-out vesicles; TM, thylakoid membranes

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tion of the PSII components, biogenesis and structural organization of the light-harvesting chlorophyll *a/b* protein complex (LHCII), trimerization of LHCII monomers and grana stacking (for a review, refer to [9]). Certain PG molecule species were also found to be involved in the process of chilling injury in higher plants [10] and cyanobacteria [11]. In addition, the phospholipid population which sustains the uncoupled non-cyclic electron flow activity was reported to be localized in the inner monolayer of the thylakoid membrane [5]. In order to get a direct demonstration that the inner pool of PG is crucial to support the electron flow activity, we used thylakoid inside-out vesicles to directly attack the lipid in the outer monolayer (exposed to the incubation medium) of the vesicles which, in fact, corresponds to the inner monolayer of the intact thylakoid membrane. Preliminary results of this investigation were presented elsewhere [12,13].

2. Materials and methods

2.1. Preparation of thylakoid vesicles

Spinach leaves (*Spinacia oleracea* L.) were purchased on the local market. Thylakoids were prepared according to [14]. After the final centrifugation, the thylakoid preparation was suspended in 10 mM sodium phosphate buffer (pH 7.4), 5 mM NaCl, 5 mM $MgCl_2$ and 100 mM sucrose, then adjusted to 4 mg chlorophyll/ml. Inside-out and rightside-out thylakoid vesicles were obtained by following exactly the procedure described in [15] which consisted of: (1) two passages through a Yeda press (100 kg/cm^2 and 6 ml/min) followed by an addition of 5 mM EDTA (final concentration) to chelate the magnesium and destack the thylakoids and by two more passages through the press; (2) one low-speed centrifugation step ($1000\times g$ for 10 min) to remove residual unfragmented thylakoids and starch; (3) a series of three partition steps in an aqueous dextran-polyethyleneglycol (5.7%) two-phase system allowing the separation of rightside-out (RO fraction) and inside-out vesicles (IO fraction); and (4) a dilution (4–5 times) and two final centrifugations at $100\,000\times g$ for 30 min of both vesicle types. The pellets were resuspended in the desired medium depending on their experimental fate.

2.2. Enzymatic treatment

In order to determine the transmembrane distribution of PG, thylakoid membranes, thylakoid rightside-out or inside-out vesicles (1 mg Chl/ml) were incubated in darkness at 2°C for 60–70 min followed by a second incubation at 20°C for 70–90 min in 25 mM Tricine (pH 7.8), 10 mM NaCl, 300 mM sorbitol, 5 mM $MgCl_2$, 1 mM $CaCl_2 \pm$ phospholipase A_2 from porcine pancreas (4 Boehringer units/mg Chl). At various times (including a zero-time control without phospholipase A_2), aliquots (240 μl) 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 [16]. After incubation on ice for 10 min, the samples were diluted 20-fold with a bovine serum albumin-free medium containing 25 mM Tricine (pH 7.8), 300 mM sorbitol, 5 mM $MgCl_2$ and 2 mM EGTA for thylakoid membranes, 10 mM sodium phosphate buffer (pH 7.4), 100 mM sucrose and 5 mM NaCl for thylakoid vesicles. After centrifugation at $10\,000\times g$ for 6 min, the resulting pellet was resuspended in 240 μl of a medium containing 10 mM Tricine (pH 7.8), 100 mM KCl and 1 mM EGTA for the thylakoid membranes and 100 mM sucrose and 5 mM NaCl for thylakoid vesicles. This final preparation was used for the determination of phospholipid content, chlorophyll, proton pumping, fluorescence emission spectra and electron transport activity.

2.3. Chemical analyses

Total lipids were extracted by adding 4 ml chloroform/methanol (53:37, v/v) and 2 ml 0.5 M KCl to the thylakoid suspension (150 μl). This resulted in a two-phase system. The lipids of the lower phase were separated by TLC, then the PG was hydrolyzed and esterified with 5% H_2SO_4 in methanol for 1 h at 85°C. The resulting fatty acid methyl esters were separated and identified by GLC according to [17]. Chlorophyll concentration was determined according

to [18] and chlorophyll *a* and *b* were estimated as described in [19].

2.4. Photosynthetic activities

Light-induced reversible proton extrusion by inside-out vesicles and proton uptake by rightside-out vesicles and thylakoids were measured according to [20], except that *N*-methylphenazonium methosulfate (20 μM) was used as electron carrier and the thylakoid material corresponded to 100 μg Chl/ml. Fluorescence emission spectra at 77 K of thylakoid preparations were determined according to [8]. Uncoupled PSII electron flow was measured in a medium containing 0.2 mM phenyl-*p*-benzoquinone (PpBQ), 30 mM sodium phosphate buffer (pH 6.5), 60 mM sucrose, 3 mM NaCl, 2 mM NH_4Cl and thylakoid material corresponding to 20 μg Chl/ml, using a Clark-type oxygen electrode.

3. Results and discussion

3.1. Characterization of thylakoid vesicles

First, it was essential to verify that our vesicles displayed characteristics similar to those reported in the literature [8,15,20–22]. The chlorophyll *a*/*b* weight ratio was found to be 2.6, 2.4 and 2.1 for intact thylakoids, rightside-out and inside-out vesicles, respectively. The values obtained for thylakoids and inside-out vesicles corresponded roughly to those reported in [22]. The chlorophyll *a*/*b* weight ratio for the rightside-out vesicles was much smaller than that found by Juhler et al. [22] and Akerlund and Andersson [15]. This discrepancy is difficult to explain, all the more so since our fluorescence data show that the 77 K F_{698}/F_{740} (PSII/PSI) ratio was much greater in inside-out vesicles (3.9) than in rightside-out vesicles (1.0). In addition, the value of the ratio F_{698}/F_{740} in intact thylakoids was twice as high as that in rightside-out vesicles. These results are in accordance with those reported in [21]. Thus, our fluorescence data show that the inside-out vesicles were enriched in PSII components, including Chl *b*, whereas rightside-out vesicles contained higher amounts of PSI than PSII components. On the other hand, the light-induced extrusion of protons in inside-out vesicles and the light-induced uptake of protons in rightside-out vesicles and thylakoids corresponded to 216, 150 and 303 nmol H^+/mg Chl, respectively. These values were much greater than those reported by Andersson et al. [20], but the uptake of protons was found by these authors to be greater in rightside-out vesicles than in thylakoids. Altogether, our results clearly show that the orientation of the membrane components was the same in intact thylakoids and rightside-out vesicles, but opposite in inside-out vesicles.

3.2. Transmembrane distribution of phosphatidylglycerol

The rationale of the enzymatic approach used for the determination of acyl lipid transmembrane distribution has been reported elsewhere [4,5,8,23]. Relevant to the present study are the following prerequisites: (1) biological structures under investigation should form closed vesicles; (ii) during PG hydrolysis, the membrane integrity has to be preserved and the access of phospholipase A_2 must be restricted to the outer surface of thylakoid vesicles. This has been verified in thylakoids by measuring the osmotic responsiveness towards sorbitol, a molecule having a much smaller size than phospholipase A_2 [5]; (e) each type of structure (thylakoids, thylakoids rightside-out and inside-out vesicles) must have, within the same population, an identical sidedness; and (4) the inside-out and rightside-out vesicle fractions should contain the lowest amount of the other category of vesicles. Andersson and Akerlund [24] found that the inside-out fraction contains a contamination of 26% of rightside-out vesicles. The cross-contamination between these two fractions might explain the low chlorophyll *a*/*b* ratio value in rightside-out vesicles as well as the lower uptake of protons in rightside-out vesicles than in intact thylakoids.

The hydrolysis kinetics of PG in the presence of PLA_2, expressed as semi-log plots of the percentage of residual PG in the membrane, was employed to determine the transmembrane distribution of PG. The justification of this representation is discussed elsewhere [4,25]. We found that the molar outer/inner distribution of PG was $58/42 \pm 3$ in the thylakoid membrane, $70/30 \pm 5$ and $40/60 \pm 3$ in rightside-out and inside-out vesicles, respectively. These results

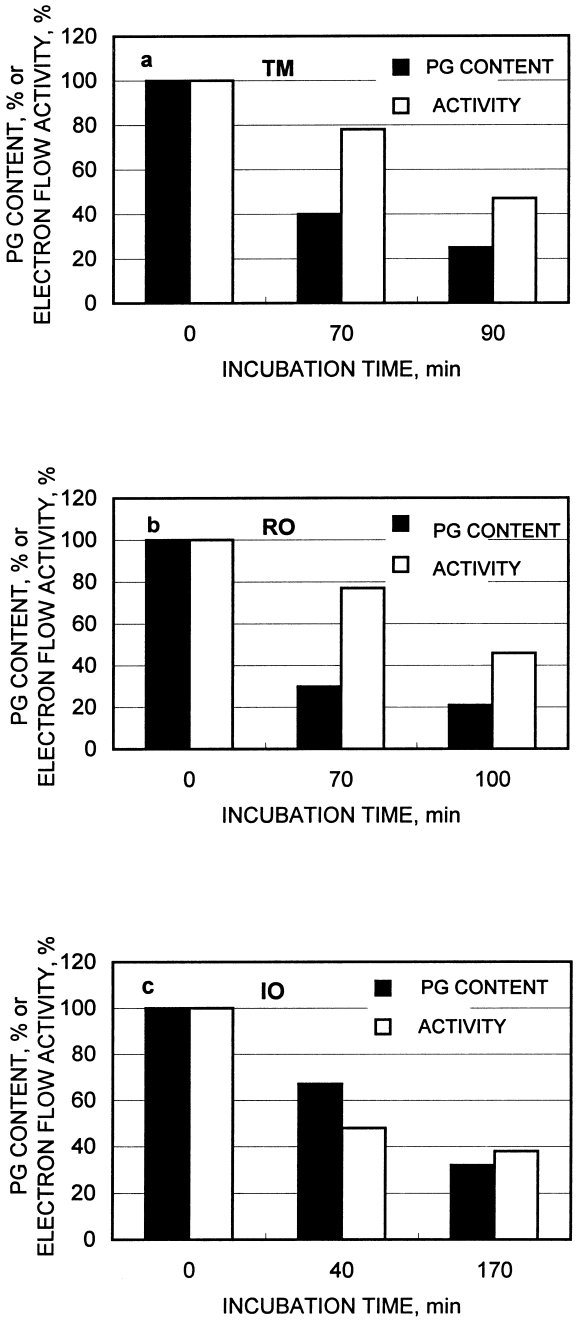

Fig. 1. Relationship between the extent of the depletion of PG and the uncoupled PSII electron flow activity in thylakoids (TM), rightside-out (RO) and inside-out thylakoid vesicles (IO). The histograms at the center of each figure correspond to the nearly complete depletion of PG in the outer monolayer of each membrane vesicle, whereas those at the righthand-side correspond to the partial depletion of PG in the inner monolayer. The 100% values for PG content were 264 ± 15, 274 ± 51 and 302 ± 58 nmol/mg Chl and for PSII activities 90 ± 9, 39 ± 12 and 130 ± 9 μmol $O_2 \times$mg Chl^{-1} h^{-1} for TM, RO and IO, respectively. For all experiments, $n = 5$.

show that, as far as PG is concerned, the sidedness of rightside-out and inside-out vesicles was opposite.

3.3. Depletion of phosphatidylglycerol and electron flow activity

Fig. 1 illustrates the relationship between the extent of the depletion of PG and the electron flow activity at three moments of the hydrolysis kinetics of PG in the presence of phospholipase A_2. At zero time, the two bars represent the initial content of PG and the activity in the control samples (Fig. 1a–c). After 70 min, 60 mol% of PG in thylakoids and 70 mol% in rightside-out vesicles were removed (Fig. 1a,b). In both cases, this amount corresponded to the depletion of the whole outer population of PG that was revealed by the extrapolation to zero time of the plateau of PG hydrolysis obtained at 2°C (for further details, refer to [5]). Interestingly, this extent of PG depletion was responsible for only about 20% inhibition of the PSII uncoupled electron flow activity. In contrast, a removal of 33% of PG in the outer monolayer of the inside-out vesicles (Fig. 1c) caused a more pronounced decrease in the electron flow activity (52%). The third set of bars represents the results obtained after 90 (Fig. 1a), 100 (Fig. 1b) and 170 (Fig. 1c) min of incubation at 20°C in the presence of PLA_2. Under these conditions, part of the PG population located in the inner monolayer of thylakoids and vesicles was removed. In thylakoid membranes (Fig. 1a), a further small decrease in the content of inner PG (15%) resulted in a relatively great decrease in the activity (31%). A similar effect occurred in the rightside-out vesicles (Fig. 1b). By contrast, in inside-out vesicles, a 35% removal of inner PG induced only a 10% decrease in the activity. Since, in the inside-out vesicles, the outer monolayer corresponds to the inner monolayer of intact thylakoids and rightside-out vesicles, one can conclude that, in intact thylakoid membranes, the inner population of PG sustains most of the PSII electron flow activity. To the best of our knowledge, it is the first direct demonstration of a close relationship between topologically defined pools of PG and the electron flow activity. In an early report, this was indirectly demonstrated with PLA_2-treated thylakoids [5]. First, the depletion of PG in the outer monolayer was carried out at 2°C and resulted in only a slight

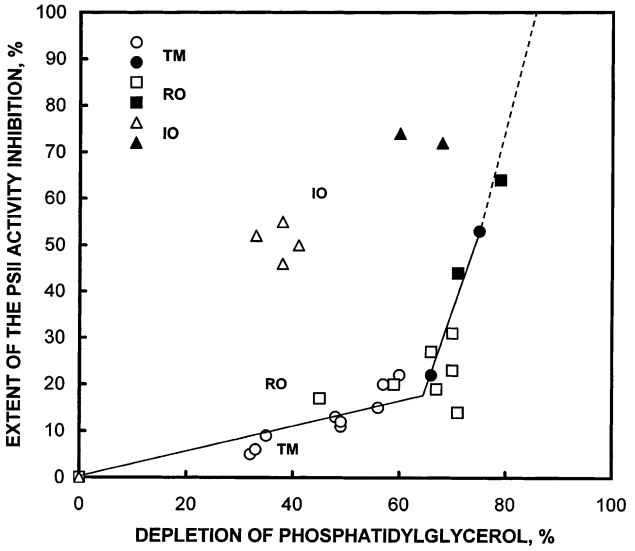

Fig. 2. Phosphatidylglycerol-dependency of uncoupled PSII electron flow activity (H_2O to PpBQ). The extent of activity inhibition was calculated with reference to the control values obtained for each time of incubation in the absence of phospholipase A_2. The 100% values corresponded to those indicated in Fig. 1. Thylakoid membranes (TM) correspond to ○ and ●, rightside-out vesicles (RO) to □ and ■ and inside-out vesicles to △ and ▲. The open symbols correspond to the depletion of PG during the incubation at 2°C (i.e. in the outer monolayer), the closed ones to that at 20°C (i.e. in the inner monolayer). The straight lines refer only to the thylakoid membrane samples and the dotted line to the extrapolation to 100% of the activity inhibition. For all experiments, $n = 5$.

inhibition of the activity, whilst, after a further incubation at 20°C in the presence of PLA_2, depletion of the inner PG was achieved by transmembrane movement that, finally, obliterated almost completely the activity.

A convenient way of identifying the PG populations which sustain the uncoupled PSII electron flow activity is presented in Fig. 2, where the extent of activity inhibition is plotted versus PG depletion. Three PG populations can be distinguished in intact thylakoids and rightside-out vesicles. The first one is entirely located in the outer monolayer. Depletion of about 60 mol% in thylakoid membranes and 70 mol% in rightside-out vesicles resulted in about 20% inhibition of the electron transport activity. The second population (about 30 mol% in thylakoid membranes and 20 mol% in rightside-out vesicles) was confined to the inner monolayer. Contrary to the first pool, a small depletion in the second PG population caused a great inhibition of the activity in both thylakoid membranes and rightside-out vesicles. In addition, a third pool of PG (about 5 mol%) can be seen in the graph which, under our experimental conditions, was not accessible to phospholipase A_2.

Due to the great difficulty of obtaining enough material for simultaneous lipid analyses and photosynthetic activity measurements, only six points are presented in the figure for thylakoid inside-out vesicles. Obviously, the points did not fit the two straight lines characterizing the thylakoid membranes and the rightside-out vesicles. A depletion of phosphatidylglycerol of 40 mol% in the outer monolayer of inside-out vesicles had a much bigger effect on the electron flow activity (about 50% inhibition) than in thylakoid membranes and rightside-out vesicles (about 10% inhibition). This difference is also true when comparing a 60 mol% depletion of PG in the two categories of vesicles.

In conclusion, these results substantiate, in a direct way, the finding that the inner populations of PG mostly sustain the PSII electron flow activity in the thylakoid membrane [5].

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