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The transmembrane distribution of galactolipids in spinach thylakoid inside-out vesicles is opposite to that found in intact thylakoids

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The transmembrane distribution of galactolipids has been determined in spinach thylakoid inside-out vesicles obtained by fragmentation in a Yeda Press followed by partition in an aqueous dextran-polyethylene glycol two-phase system. Using the lipase from *Rhizopus arrhizus* to digest selectively the galactolipids in the outer monolayer of the membrane, we have found the molar outside/inside distribution to be $42 \pm 1/58 \pm 1$ for monogalactosyldiacylglycerol (MGDG) and $82 \pm 0.2/18 \pm 0.2$ for digalactosyldiacylglycerol (DGDG). As expected, the transmembrane distribution of galactolipids in inside-out vesicles was the opposite to that found in intact thylakoids which, under similar conditions, was found to be 62/38 for MGDG and 20/80 for DGDG. The reliability of the enzymatic approach to determine the transmembrane distribution and the relative arrangement of acyl lipids in the thylakoid membrane are discussed.

Thylakoid membrane; Thylakoid inside-out vesicle; Lipase; Galactolipid distribution; Galactolipid asymmetry; (Spinach, *Rhizopus arrhizus*)

1. INTRODUCTION

The asymmetric distribution of proteins within the thylakoid membrane, both laterally and transverally, confers vectorial properties to this particular type of membrane [1]. A transversal heterogeneity also exists for acyl lipids [2]. The first report, showing that phospholipids are asymmetrically distributed across the thylakoid membrane from spinach, appeared in 1981 [3]. The outer monolayer was found to be enriched in phosphatidylglycerol whereas the inner one enriched in phosphatidylcholine. Since then, these results have been confirmed for other plant species thylakoids [4,5]. Galactolipids, which are the major acyl lipids in photosynthetic membranes, present an analogous transveral heterogeneity in a wide variety of temperate climate plants [6–11]. Using an enzymatic approach [10,12], we have found an outside/inside distribution of about 65/35 (mol%) and MGDG and 15/85 for DGDG in spinach thylakoids. Although there is general agreement concerning the above distribution for MGDG, the localization of DGDG is still controversial [9–11]. Some of the differences observed may be attributed to technical difficulties encountered in the galactose oxidase labelling technique, i.e. the contribution of non-specific labelling in the acyl chains which may significantly alter the distribution of DGDG, as discussed [11].

Although the hydrolysis kinetics in the presence of lipolytic enzymes have been monitored very carefully under a variety of conditions (e.g. [10]), there is still the possibility that some unknown factors may have distorted the resulting acyl lipid distribution. Thus, it appears that the use of thylakoid inside-out vesicles, which are now ob-

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Abbreviations: DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; F, fluorescence

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tainable in rather pure form with the aqueous partition polymer two-phase system [13] may provide an answer to the above controversy. Indeed, if the distribution of galactolipids in intact spinach thylakoids is correct, one should expect that the transversal distribution of these lipids will be the opposite in inside-out vesicles. In this study, we show that this is the case.

2. MATERIALS AND METHODS

Thylakoids from spinach leaves (*Spinacia oleracea* L., var. Nobel) were prepared according to [10]. After the final centrifugation, the thylakoid preparation was suspended in 10 mM sodium phosphate buffer (pH 6.5), 5 mM NaCl, 5 mM $MgCl_2$ and 100 mM sucrose, then adjusted to 0.4 mg chlorophyll/ml. Inside-out thylakoid vesicles were obtained by following exactly the procedure described by Andersson [14] which consisted of: (i) two passages through a Yeda press (100 kg/cm^2 and 6 ml/min) followed by an addition of 10 mM EDTA to destack the thylakoids and by two more passages through the press; (ii) two centrifugation steps to remove residual unfragmented thylakoids and starch and to concentrate the vesicle suspension to 4 mg chlorophyll/ml; (iii) a series of partition steps in an aqueous dextran-polyethylene glycol two-phase system allowing the separation of rightside-out (T1 fraction) and inside-out vesicles (B7 fraction); (iv) a final centrifugation at 100000 × *g* for 30 min of both vesicle types. The pellets were resuspended in the desired medium depending on their experimental fate.

Light-induced reversible proton extrusion by inside-out vesicles and proton uptake by rightside-out vesicles were measured according to Andersson et al. [15], except that 2,5-dichloro-*p*-benzoquinone was used as the photosystem II electron acceptor. Fluorescence emission spectra at 77 K were obtained with a fluorimeter made of Bausch and Lomb monochromators (excitation at 480 nm; emission at 660–780 nm), a light-chopper and a PAR lock-in amplifier (model 128A) components. Chlorophyll concentration was determined according to Bruinsma [16] and chlorophyll *a* and *b* were estimated as described by McKinney [17].

In order to determine the membrane galactolipid distribution, thylakoid membranes or thylakoid inside-out vesicles were incubated with the lipase in darkness at 2, 10 or 20°C as indicated in the legends of the figures. The incubation medium contained 100 mM sorbitol, 10 mM NaCl, 10 mM $MgCl_2$, 5 mM 4-morpholinopropane sulfonic acid-KOH (pH 7.6), the lipase from *Rhizopus arrhizus* (30 Boehringer units per mg chlorophyll) and the thylakoid preparation (0.5 mg chlorophyll/ml). After sampling a zero time control, the lipase was added to initiate the hydrolysis of galactolipids. Then, aliquots (140 μl) of the above medium were taken at various times for the determination of galactolipids remaining in the membrane. Lipid extraction was carried out according to a modification [18] of the Bligh and Dyer procedure [19]. We have checked that solvent extraction (chloroform/methanol; 53:37, v/v) immediately stopped the enzyme activity. The whole lower phase was spotted on type 60 silica gel-coated glass plates using an automatic sample applicator fitted with a Hamilton syringe (Linomat III from Camag, Switzerland). Plates were developed in chloroform/methanol/acetic acid/water (85:15:10:3, v/v) and briefly dried. After slight staining with primuline, lipid zones corresponding to MGDG and DGDG were scraped into test-tubes and their sugar content was determined with a microassay derived from the method of Dubois et al. [20], using galactose as standard. The hydrolysis kinetics were expressed as the amount of remaining undegraded acyl lipids (in % of its initial amount), plotted on a log scale versus incubation time.

3. RESULTS

3.1. *Characterization of thylakoid inside-out vesicles*

Before determining galactolipid distribution in inside-out vesicles, it was essential to verify that our vesicles displayed characteristics similar to those reported in [15,21–24]. The ratio inside-out (fraction B7)/rightside-out (fraction T1) vesicles, expressed on a chlorophyll basis was 59/41. This high yield is similar to that found by other authors [14,23]. The chlorophyll *a/b* weight ratio was found to be 1.5 and 2.5 for inside-out and rightside-out vesicles, respectively. The former ratio which was lower than the value (2.3) reported earlier [21] reflects better the enrichment of inside-out vesicles in photosystem II. This is in agreement with our fluorescence data showing that the 77 K F_{698}/F_{735} ratio (= 2.06) is greater than that (0.9) reported in [21]. In comparison, this ratio value was much smaller in the rightside-out vesicles (0.98). The light-induced extrusion of protons in inside-out vesicles and the light-induced uptake of protons in rightside-out vesicles corresponded to 138 and 109 nmol H^+/mg chlorophyll, respectively. These values are greater than those reported earlier [15] but in the same range as those obtained by Akerlund and Andersson [24]. These analyses show that our thylakoid inside-out vesicles present characteristics which are quite similar to those reported in the literature [13–15, 21–24].

3.2. *Transversal distribution of acyl lipids in inside-out vesicles*

The rationale of the enzymatic approach used for the determination of acyl lipid transmembrane distribution has been discussed elsewhere [10,12]. Among the prerequisites necessary for the success of this approach four are of particular importance in this study. Firstly, biological structures under investigation should form closed vesicles.

Microscopic studies have shown that thylakoid inside-out vesicles appear as closed vesicular structures [21,22,25]. Secondly, each type of structure (thylakoids or thylakoid inside-out vesicles) must have, within the same population, an identical sidedness. Freeze-fracture electron microscopy confirms that the vesicles obtained by the Yeda press treatment followed by the separation in a polymer two-phase system exist in an inside-out orientation, i.e., with the lumenal membrane surfaces exposed to the medium [22]. Thirdly, the inside-out vesicle fraction should contain the lowest amount of rightside-out vesicles. It has been found that the B_3 fraction contains 74% inside-out and 26% rightside-out vesicles [22]. We can assume that further purification steps (i.e. from B_3 to B_7 fraction, see [14]) increase to a great extent the ratio inside-out/rightside-out vesicles. This assumption is verified by the greater extrusion of protons (on a chlorophyll basis) found in the B_7 fraction (this study) than in the B_3 fraction [22]. Finally, the enzymatic approach requires that total galactolipid digestion occurs in control experiments where both sides of the membrane are attacked by the enzyme, i.e., that there are no inaccessible galactolipids under our experimental conditions. We have verified that when thylakoid inside-out vesicles were disrupted by sonication in the presence of the enzyme, more than 95% of both MGDG and DGDG were quickly hydrolyzed (not shown).

Fig.1 illustrates hydrolysis kinetics of both galactolipids, expressed as semi-log plots of the percentage of residual galactolipids in the membrane. The justification of this representation is discussed elsewhere [10,26]. The experiments have been carried out under conditions (salt concentrations in the reaction mixture, temperature ranging from 2 to 20°C) which were shown to be optimal for this kind of study [10]. Fig.1A shows typical hydrolysis kinetics of MGDG and DGDG in inside-out vesicles. These kinetics displayed several pools of different reactivity (expressed by the slope of the curves) in the outer monolayer. The diminution of the hydrolysis rates occurring during the first 10 min of incubation with the lipase (see fig. 1) may be due to the accumulation of hydrolysis products (free fatty acids and lyso-MGDG-(-DGDG)) causing an increase in the membrane packing pressure. The presence of these different

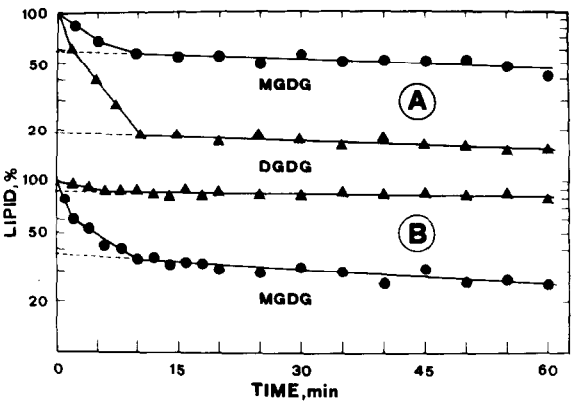

Fig.1. Time course of galactolipid hydrolysis at 10°C for thylakoid inside-out vesicles (A) and at 2°C for intact thylakoids (B) treated with the lipase from *Rhizopus arrhizus*. (●), MGDG; (▲), DGDG. The 100% values corresponded to 286 ± 31 ($n = 9$) and to 155 ± 18 ($n = 9$) nmol/mg chlorophyll for MGDG and DGDG, respectively, in inside-out vesicles (A) and to 1375 and 675 nmol/mg chlorophyll for MGDG and DGDG, respectively, in intact thylakoids (B).

pools has been discussed recently as well as the extrapolation to zero time of the last reactive pool allowing the estimation of the transversal distribution of galactolipids [10]. The molar outside/inside ratio ($n = 5$) was $42 \pm 1/58 \pm 1$ for MGDG and $82 \pm 0.2/18 \pm 0.2$ for DGDG. This distribution was independent of the hydrolysis rate; for instance, at 20°C (not shown), where the rate was greater, the galactolipid distribution was similar ($42 \pm 4.5/58 \pm 4.5$ ($n = 4$) for MGDG and $75 \pm 2/25 \pm 2$ ($n = 3$) for DGDG).

The transmembrane distribution of galactolipids in inside-out vesicles has been compared with that of intact thylakoids (see fig.1B). The hydrolysis kinetics also displayed several pools of different reactivity before reaching the inner topological pool. The molar outside/inside ratio in this particular experiment was 62/38 for MGDG and 20/80 for DGDG, in agreement with the values reported recently [10].

4. DISCUSSION

The results of this investigation show several interesting features. It is most remarkable that the transmembrane distribution of both MGDG and DGDG in inside-out vesicles is just the opposite of that found in intact thylakoids. The small dif-

ference between the MGDG distribution in these two vesicle types may be attributed to a slight contamination of the B_7 fraction by rightside-out vesicles.

Although these results were expected, they are of great importance in view of the controversy concerning the relevance of using the lipolytic approach [27,28] to determine the transmembrane distribution of acyl lipids in thylakoids. Basically, the main objections are: (i) lipases do not have ready access to substrates which are localized in the outer monolayer of two adjacent stacked thylakoid membranes; (ii) fatty acids and lyso-derivatives released by lipase activity may influence the rate and amplitude of lipid hydrolysis and, consequently, may lead to a wrong estimation of the real transmembrane distribution; (iii) the greater affinity of the lipase from *Rhizopus arrhizus* for MGDG than for DGDG may bias the transmembrane distribution of galactolipids [27]; (iv) the membrane bilayer integrity does not remain intact during lipid hydrolysis; (v) changes in one lipid class can result in a reorganization of other lipid classes which are not substrates for the enzyme used [28].

Objections (i) and (ii) have been refuted and discussed elsewhere [10]. From objection (iii), one may conclude that the amount of MGDG and DGDG degraded in the outer leaflet only reflects the specificity of the lipase. However, one should realize that a low degradation of a given lipid (e.g. DGDG) can also result from a strong enrichment of this lipid in the inner monolayer. This view is supported by the fact that in inside-out vesicles, DGDG is even a better substrate for the lipase from *Rhizopus arrhizus* than MGDG and that the distribution of both galactolipids is opposite to that found in intact thylakoids (fig.1). A low degradability of phosphatidylserine in the outer monolayer due to its strong asymmetric distribution has also been observed in erythrocyte membranes [29]. Concerning the maintenance of membrane integrity (objection iv) and reorganization of membrane constituents (objection v) during lipid hydrolysis which may affect galactolipid distribution, it is now clear that these objections are no longer valid since our results show unambiguously that the transmembrane distribution of both MGDG and DGDG in intact thylakoids and in inside-out vesicles are opposite. These distributions are illustrated in fig.2 where both galactolipids are expressed either as mol% per lipid class (upper part) or as mol% per total acyl lipids (lower part). As also expected, the molar MGDG/DGDG ratio was about 0.95 in the outer monolayer of the inside-out vesicles as well as in the inner monolayer of intact thylakoids and about 6.0 in the other homologous leaflets. The possible physiological significance of such a difference has been considered elsewhere [11].

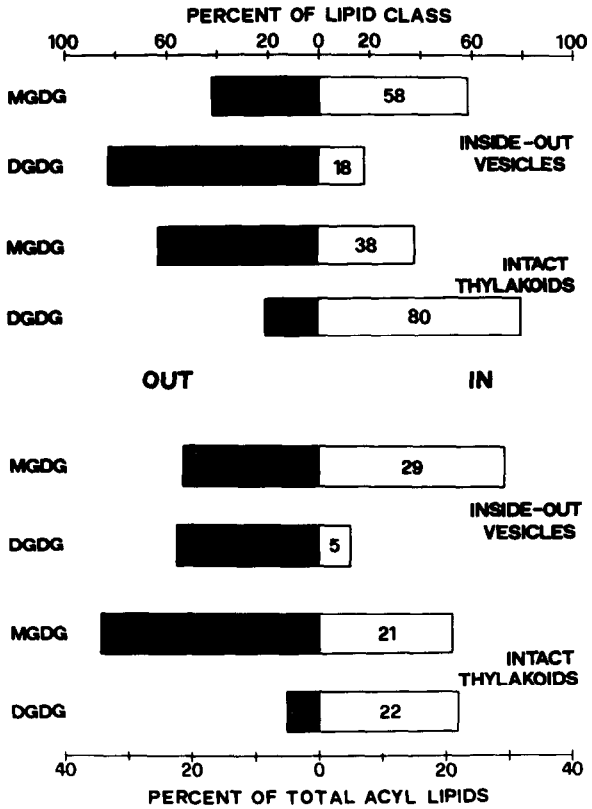

Fig. 2. Distribution of acyl lipids in the two monolayers of thylakoid inside-out vesicles and of intact thylakoids from spinach. Results are expressed either in mol% for each class of lipids or in mol% for total acyl lipids. Total acyl lipids were 573 and 2500 nmol/mg chlorophyll in inside-out vesicles and in intact thylakoids, respectively.

The last interesting feature of this study concerns the strong decrease of acyl lipids to chlorophyll ratio in inside-out vesicles (see legends to figs 1 and 2). This diminution has been also observed by other authors [30–32] and seems to be related to the number of transfer steps in the phase partition procedure. The essentially lipidic nature of the margins as suggested by Murphy [1,33]

might explain this decrease. However, the fact that the transmembrane distribution of both galactolipids is opposite in inside-out vesicles and in intact thylakoids, does not support any significant lateral heterogeneity of these lipids. These results shed some doubt on the use of the geometrical shape of these lipid molecules to predict their arrangement in the thylakoid membrane.

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