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Transbilayer organization of the thylakoid galactolipids

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The distribution across the thylakoid membrane of its major polar lipid constituents, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), was investigated by tritium labelling of membrane vesicles of opposite sidedness. Tritium was introduced into the galactose headgroups of the lipids by oxidation with galactose oxidase, or sodium periodate, and subsequent reduction with tritiated sodium borohydride. Rightside-out and inside-out vesicles with the same overall composition were prepared from randomized thylakoids. The results indicate, that there is an asymmetric distribution of the two galactolipids across the thylakoid membrane. About 60% of both galactolipids are found in the outer and 40% in the inner half of the bilayer. Since MGDG and DGDG together comprise 75% of the matrix forming lipids, it is suggested that the remaining matrix forming lipids, in particular the anionic phosphatidylglycerol and sulphoquinovosyldiacylglycerol, are mainly distributed to the inner leaflet of the membrane.

Introduction

Membrane lipids have the capacity to play a dynamic role in the regulation of membrane functions. During the last decade detailed information concerning the behaviour of different lipids in controlled model systems has been provided [1], including data on the lipids that build up the photosynthetic membrane [2]. However, while studies on the thylakoid membrane now have provided insight into the distribution and role of several protein complexes, our understanding of the lipid components is still very poor. Thus, the role of the lipids in the bioenergetic events remains unclear. A prerequisite for the correlation of available data on structural and physical properties of the different forms of polar lipids with their in vivo function, is to determine their distribution within the membrane both laterally (along the plane of the membrane) and transversally (across the membrane). For most thylakoid polypeptides, such basic data are already at hand.

The two predominant matrix forming thylakoid lipids, MGDG and DGDG, were recently found to be asymmetrically distributed along the thylakoid membrane. Both galactolipids are more abundant in the non-appressed regions, and MGDG is enriched relative to DGDG in the appressed regions [3,4]. In the present study, we have investigated the transverse distribution of MGDG and DGDG by tritium labelling of inside-out and rightside-out thylakoid vesicles. The results demonstrate a partial asymmetry in the distribution across the membrane of the two galactolipids, both being more abundant in the outer half of the bilayer.

Materials and Methods

Preparation of thylakoid vesicles. Spinach (*Spinacea oleracea*) was grown hydroponically [5]

Abbreviations: DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; SQDG, sulphoquinovosyldiacylglycerol.

and leaves were harvested when 6-weeks old. Thylakoid vesicles with the same overall composition but of normal and reversed sidedness, respectively, were prepared from washed and randomized thylakoids according to Ref. 6.

Tritium labelling of vesicles. Tritium labelling of the terminal galactose groups of the galactolipids in the outer leaflet of the vesicles was accomplished by oxidation of the C_6-alcohol to aldehyde [7] with either galactose oxidase [8], or sodium periodate mainly as in Ref. 8, and subsequent reduction of the aldehyde with tritiated sodium borohydride. Each sample consisted of membrane vesicles corresponding to 25–50 μg chlorophyll suspended in 200 μl of 5 mM sodium phosphate (pH 8.0). Prior to enzymic oxidation each sample was preincubated for 15 min with unlabelled $NaBH_4$ (1 mM) to reduce non-specific labelling. Labelling by oxidation with galactose oxidase (EC 1.1.3.9) and subsequent NaB^3H_4 reduction was performed at room temperature for 1 h (or, in the case of time dependency studies (Fig. 2), for the indicated time) after addition of 1 unit of enzyme in 200 μl of 50 mM sodium phosphate (pH 8.5) and 10 μl of 1 mM NaOH containing NaB^3H_4 to yield a final borohydride concentration of 1.5 mM. Oxidation with periodate prior to reduction was performed without preincubation with unlabelled $NaBH_4$. Firstly, 70 μl of 10 mM sodium phosphate (pH 7.4) containing sodium periodate was added to the samples to yield a periodate concentration of 0.5 mM and oxidation was carried out on ice for 10 min. Thereafter, excess sodium arsenite in 200 μl of 50 mM sodium phosphate (pH 8.5) was added to yield a concentration of 2 mM, and NaB^3H_4 was added as above. The reduction was performed at room temperature for 15 min. Control samples contained no enzyme or periodate. All incubations were stopped by the addition of 1.0 ml of 5 mM sodium phosphate (pH 8.0), followed by immediate lipid extraction.

Galactose oxidase from *Dactylium dendroides* (155 units/mg protein, M_r 42000) was obtained from Sigma (St. Louis, MO, U.S.A.) and tritiated sodium borohydride (specific activity 341 Ci/mol) from NEN (Albany St. Boston, U.S.A.).

Extraction and separation of lipids. Total lipids were extracted [9] with 0.2 M KCl in the water phase. Separation was performed by thin-layer chromatography on Silica gel H, activated at 120°C for 1 h, in a system containing chloroform/methanol/acetic acid/water (170 : 30 : 20 : 7, v/v). Spots were visualized by iodine spraying. MGDG and DGDG were scraped off the plate, and eluted from the silica with chloroform/methanol (2 : 1, v/v) into scintillation vials and counted with 55% efficiency.

Results

In the present study, we have used rightside-out and inside-out vesicles prepared from randomized thylakoids [6] for determination of the transbilayer distribution of MGDG and DGDG by tritium labelling of the vesicles. The inside-out and rightside-out vesicles had the same overall composition, as judged from their polypeptide patterns (Fig. 1).

MGDG and DGDG were tritium labelled after galactose oxidase oxidation of rightside-out and inside-out vesicles (Table I). The labelling without

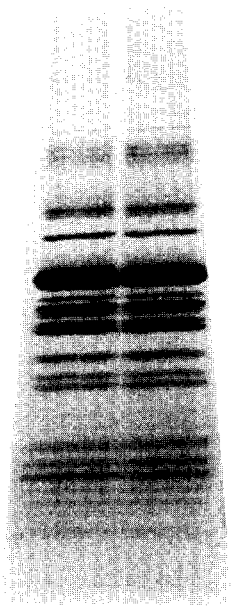

Fig. 1. The polypeptide pattern of rightside-out (left) and inside-out (right) vesicles prepared from randomized thylakoids. Sodium dodecylsulphate polyacrylamide gel electrophoresis was run in the buffer system of Laemmli [32] using 4 M urea and a 12–22.5% polyacrylamide gradient. The gels were stained in Coomassie Brilliant Blue R-250.

TABLE I

LABELLING OF MGDG AND DGDG WITH NaB^3H_4 AFTER OXIDATION WITH GALACTOSE OXIDASE

Data are the mean of six independent experiments. Control labelling was subtracted and thereafter the dpm values for the rightside-out and inside-out vesicles expressed as percentage of total labelling.

		MGDG		DGDG	
		Control	With enzyme	Control	With enzyme
Rightside-out vesicles	dpm	2066	2239	1293	12228
	distribution (%)	–		63 ± 7(6)	
Inside-out vesicles	dpm	1930	1826	1285	7021
	distribution (%)	–		37 ± 7(6)	

enzyme for right-side-out and inside-out vesicles were similar. This background labelling is presumably due to reduction of double bonds in fatty acids [8]. Labelling with 0.05 M galactose plus enzyme gave essentially the same results as without enzyme (not shown), indicating that the enzyme-catalyzed increase in labelling was specific for galactose. For DGDG, a 9-fold increase in label was achieved for rightside-out vesicles, and a 5-fold increase for inside-out vesicles, when the enzyme was present during incubation. From these data the distribution of DGDG across the thylakoid membrane could be calculated (Table I). The results suggest that DGDG is asymmetrically distributed across the membrane, with 63% in the outer and 37% in the inner half of the bilayer. The time dependency of the enzymic oxidation was investigated over a period from 15 min up to 4 h (Fig. 2) and showed that labelling of DGDG was optimal after about 1 h incubation and then decreased slowly. This decrease could be due to lipase activity. Nevertheless, calculation of the distribution of DGDG across the membrane gave the same result over the whole time range (67/33, 64/36, 67/33 and 65/35% outside/inside), indicating that no flip-flop or other structural rearrangements was evoked by the lipid labelling, or by the long incubation time. In contrast to DGDG, MGDG was labelled to the same extent whether galactose oxidase was present or not (Table I). The

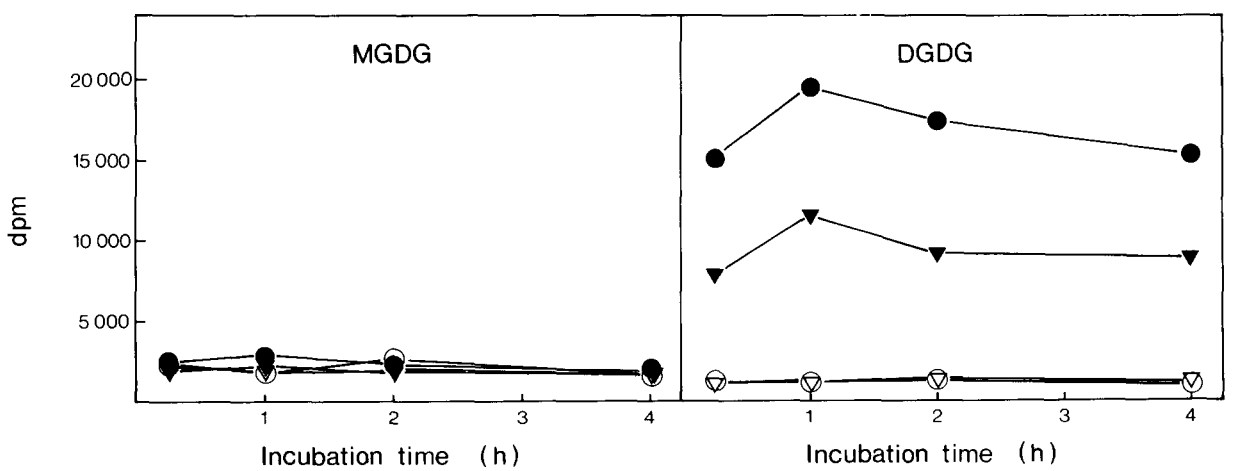

Fig. 2. Tritium labelling of galactolipids in rightside-out and inside-out vesicles as a function of incubation time in the presence of galactose oxidase and NaB^3H_4. ●, Rightside-out vesicles; ▼, inside-out vesicles. Open symbols (○, ▽) are the corresponding controls without enzyme.

lack of labelling of MGDG in the thylakoid vesicles is in agreement with results obtained with pure lipids hydrophobically attached to methylated bovine serum albumin. With these galactolipid-protein complexes there was a marked increase in labelling of DGDG after oxidation with the enzyme, but no increase for MGDG (not shown). The poor labelling of MGDG was not merely the result of slower kinetics, since it was not increased by prolonged incubation (Fig. 2). Obviously, MGDG is a very poor substrate for galactose oxidase. This was also noted by Sundler and coworkers (Sundler, R., personal communication), after labelling liposomes containing MGDG and DGDG with galactose oxidase. It is also consistent with the observation that the longer the sequence of sugar moieties of a glycolipid, the better a substrate for the enzyme [8].

Thus, it was not possible to use the enzyme labelling technique to estimate the distribution of MGDG. We therefore turned to the use of a chemical oxidant, namely sodium periodate. At low temperatures, high pH, and short exposure times the negatively charged periodate ion does not penetrate the erythrocyte membrane [10]. Furthermore, with glycosphingolipid liposomes, periodate influx is negligble at pH 8.4 during a 4 h exposure whilst appreciable at pH 5.5 [11]. When the complexes of pure galactolipids and methylated bovine serum albumin used above were first oxidized with periodate and then reduced with tritiated sodium borohydride there was a substantial increase in label compared with controls for both MGDG and DGDG (not shown). With the same procedure applied to the vesicles it was possible to tritium label also MGDG to a degree sufficient to permit calculations of the transverse distribution (Table II). For MGDG, there was a 4-fold increase in label with rightside-out vesicles, and a more than 2-fold with inside-out vesicles, when periodate was present. These data suggest a transverse asymmetry of MGDG with 62% in the outer, and 38% in the inner leaflet of the bilayer. Furthermore, for DGDG periodate caused a more than 4-fold increase in label with rightside-out vesicles and a 3-fold increase with inside-out vesicles (Table II) which points to an asymmetric distribution with 61% in the outer and 39% in the inner leaflet of the membrane. This result for DGDG is strikingly similar to that obtained after oxidation with galactose oxidase (Table I) and strongly supports the assumption that periodate, under the applied conditions, did not permeate the thylakoid membrane.

Thus, the two polar lipids that together comprise 75% of the matrix forming lipids in the photosynthetic membrane both show an asymmetric distribution with a surplus in the outer leaflet of the bilayer.

Discussion

The introduction of a method for isolation of inside-out thylakoid vesicles [5] was a breakthrough for investigations on the transbilayer organisation of thylakoid polypeptides. The location of several polypeptides, such as plastocyanin [12] and components of the oxygen evolving complex [13], could be ascertained to the inner thylakoid surface. In the present study we have taken advantage of the

TABLE II

LABELLING OF MGDG AND DGDG WITH NaB^3H_4 AFTER OXIDATION WITH PERIODATE

Data are the mean of five independent experiments. Calculations as in Table I.

		MGDG		DGDG	
		Control	With periodate	Control	With periodate
Rightside-out vesicles	dpm	4172	16502	1531	6934
	distribution (%)	62 ± 8(5)		61 ± 7(5)	
Inside-out vesicles	dpm	4597	12220	1563	4741
	distribution (%)	38 ± 8(5)		39 ± 7(5)	

possibility to use sealed vesicles of normal and reversed sidedness for direct probing of the galactolipids of the outer and inner thylakoid surface. That MGDG and DGDG both are distributed with 60% in the outer bilayer half (Table II) is consistent with some recent observations made with lipase treatment of thylakoids. Rawyler and Siegentaler [14] found that about half of both MGDG and DGDG was hydrolysed when spinach thylakoids of normal sidedness were treated with a lipase isolated from potato tubers. Unitt and Harwood [15] used a lipase from *Rhizopus arrhizus* with *Lactuca sativa* thylakoids and reported that about half of MGDG was readily hydrolyzed by their lipase when used with intact thylakoid vesicles. However, in a later report [16] using the same lipase, they concluded from hydrolysis rates with and without the detergent glycocholic acid and with corrections made for flip-flop induced by the lipase treatment, that 80% of MGDG and 55% DGDG was located in the inner thylakoid leaflet. However, the use of detergents in this context is not without problems since the membrane lipids are incorporated into lipid-detergent micelles when the membrane is disrupted [17]. Thus, the accessibility for various probes might change drastically, and hence strongly affect the kinetics. We noted such a change in lipid accessibility resulting in a very high labelling of the control samples when we used 0.01% Triton X-100 for labelling of the outer and inner surface simultaneously (results not shown). Similar results were experienced by Michaelsson et al. [18] when using detergents and TNBS labelling for the determination of the transverse phospholipid distribution in synaptic vesicles.

Radunz [19], using antibodies and *Antirrhinum majus* chloroplast material, suggested that all galactolipids are located in the inner half of the bilayer. An uncharacterized heavy fraction of sonicated thylakoid fragments was used as 'inner surface exposed', which in our experience would contain a mixture of rightside-out and inside-out material, the latter originating from the appressed region solely. Initially, we also tried to use polyclonal antibodies for determination of the transverse distribution of galactolipids. In our hands, however, this approach did not work out well, in spite of using ^{125}I-labelled IgG to more accurately quantify the binding. A problem was that all rabbits that were tested had, already before immunization, antibodies that cross-reacted with the galactose headgroups of the galactolipids. Thus, we always had problems with high and varying backgrounds.

The technique of tritium labelling of membrane components by galactose oxidase oxidation and subsequent NaB^3H_4 reduction used in the present study, has been used extensively for glycolipid (and glycoprotein) labelling in erythrocytes [8,20] and for labelling of various glycolipids prepared from brain tissue [7]. The main problem with this method is that generally only a minor proportion of the lipids are actually labelled [8]. Thus, the specific activity for DGDG was 264 and 136 mCi/mol with galactose oxidase and periodate oxidation, respectively, and for MGDG 161 mCi/mol with periodate oxidation (calculated from the data in Tables I and II). This corresponds to a labelling of 0.2–0.5% of the galactolipids. The specific activity varied between experiments and the maximal degree of labelling we obtained in one experiment was 1%. With the erythrocyte membrane, a similar low labelling of 0.1–1% of the galactose plus *N*-acetylgalactosamine in glycoproteins and glycolipids was observed [8]. For calculations of the transverse distribution one therefore has to assume that the labelling is statistically representative. That this really is the case for the thylakoid membrane, is supported by the findings that the transverse distribution obtained for DGDG is independent of the oxidant used (Tables I and II), and also independent of incubation time and degree of labelling, i.e. specific activity (Fig. 2). Further support is lent by the observation that the total amount of label yielded in MGDG is twice the amount obtained in DGDG, in accordance with the facts that the membrane contains twice as much MGDG as DGDG, and that only the terminal galactose group in DGDG is oxidized.

Our data on lipid transverse distribution is a mean value for the appressed and the non-appressed region [21] since the vesicles used are prepared from randomized thylakoids [6], in which the lateral heterogeneity is abolished and the components of the two separate regions are fully intermixed. Thus, the rightside-out and the inside-out vesicles have the same polypeptide patterns, representative for whole thylakoids (Fig. 1). It is likely

that the lipids are randomized along with the polypeptides. Since the ratio of MGDG to DGDG differs significantly between the two regions [3] it would be interesting to know whether the transbilayer distribution of the two lipids is the same in the two regions. However, the results (not shown) we obtained using vesicles derived from both regions [22] were not reproducible from time to time, possibly explained by transverse rearrangements of lipids in these vesicles, due to their small size and the use of sonication in the preparation procedure.

For most membranes, conclusions on the transverse distribution of lipids are contradictory [23], but for erythrocytes, clear evidences have emerged for an asymmetric lipid distribution across the membrane. Of the predominant matrix-forming lipids the choline-containing lipids phosphatidylcholine and sphingomyelin dominate the outer half of the bilayer leaflet and the amino phospholipids phosphatidylserine and phosphatidylethanolamine the inner, cytoplasmic half [24]. For thylakoids, it has been suggested that MGDG and DGDG in a similar manner could be asymmetrically and oppositely arranged with one of them preferentially in the outer, and the other preferentially in the inner half of the bilayer [25]. Murphy [26] recently made a specified suggestion of an excess of MGDG at the inner surface, which should be the result of an enrichment of MGDG in the highly curved concave inner thylakoid margins. Our results do not support these suggestions since we find that both lipids are asymmetrically distributed to the outer half of the bilayer.

The transbilayer asymmetry of MGDG and DGDG was only partial (60 to 40, Tables I and II). A partial asymmetry is typical for lipids [27], in contrast to the absolute asymmetry that is characteristic for proteins. Also, if one considers the different physical properties of the two lipids [2] rendering them different roles in the bilayer [28] one should not expect any of the two to be exclusively present on one side since they should be complementary and probably both needed in the outer as well as in the inner leaflet. However, since MGDG and DGDG comprise 47 and 24%, respectively, of the polar lipids [3], already a small deviation from a 50/50 distribution such as 60/40 leaves quite a large gap in the inner leaflet. If one assumes that a planar bilayer requires a 50/50 distribution of the polar lipids between the two bilayer leaflets the remaining 29% matrix-forming lipids should be distributed preferentially to the inner surface. PG and SQDG constitute 22% of the remaining 29% matrix forming lipids [3]. A 20/80 distribution of these two negatively charged lipids to the inner bilayer half would counterbalance the asymmetric distribution of the uncharged galactolipids. Thus, we predict the two anionic lipids, PG and SQDG, to be asymmetrically distributed in a way opposite to MGDG and DGDG; that is, preferentially to the inner leaflet.

Our prediction is supported by the fact that the inner surface of thylakoids has a significantly lower isoelectric point (pH 4.0) than the outer surface (pH 4.7) [29]. A preferential location of PG and SQDG at the inner leaflet would also fit with the suggestion [30] that the negative charges of the outer surface of thylakoids are confined to anionic amino acids rather than anionic lipid headgroups. If exposed internally the latter should not be detected when probed from the outside. The anionic lipids PG and SQDG could, if exposed to the inner surface, act as counterions for the protons that are accumulated in the lumen during electron transport and even act as 'proton-conducting pathways' as recently suggested by Haines [31].

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