Biochem. J. (1985) **228**, 707–711
Printed in Great Britain

Sidedness studies of thylakoid phosphatidylglycerol in higher plants

Mark D. UNITT and John L. HARWOOD
Department of Biochemistry, University College, P.O. Box 78, Cardiff CF1 1XL, Wales, U.K.

(*Received 7 January 1985/15 February 1985; accepted 25 February 1985*)

The transmembrane distribution of phosphatidylglycerol was determined in thylakoids from barley (*Hordeum vulgare*), lettuce (*Lactuca sativa*) and pea (*Pisum sativum*) chloroplasts. Phospholipase A_2 and phospholipase D digestion and chemical-labelling methods were used. Phosphatidylglycerol was preferentially localized in the outer (stromal) leaflet. The proportion of the phospholipid in this leaflet ranged from about 66% in pea to about 75% for barley and lettuce thylakoids. One of the main fatty acids, *trans*-Δ^3-hexadecenoic acid, was exclusively located in the outer leaflet in all three plant types. The data are discussed in relation to suggested roles for phosphatidylglycerol in thylakoid function.

The specific distribution of acyl lipids within membranes from higher-plant chloroplasts has received attention recently. There are several reasons for this. First, the photosynthetic membranes of oxygen-evolving organisms are the most prevalent type of membranes in Nature. Secondly, they have a lipid composition that is highly conserved (Harwood, 1980) and their major lipids are seldom found in other membranes (e.g. Gounaris & Barber, 1983; Harwood & Russell, 1984). Thirdly, studies with developing chloroplasts (cf. Harwood, 1983), incubation of thylakoids with lipases (e.g. Jordan *et al.*, 1983), reconstitution experiments (e.g. Gounaris *et al.*, 1983*b*; Remy *et al.*, 1982) and nutrient-limited growth of *Chlorella* (e.g. Sinensky, 1977) have indicated that photosynthetic membrane lipids may play rather specific roles in efficient photosynthesis.

The major phospholipid component of thylakoid membranes (in cyanobacterial thylakoids, the only phospholipid) is phosphatidylglycerol (Harwood, 1980). In photosynthetically active eukaryotes, phosphatidylglycerol contains an unusual fatty acid, *trans*-Δ^3-hexadecenoic acid. This acid is exclusively esterified to the *sn*-2 position of the glycerol backbone, is not found in other phospho- or glyco-lipids and, with the exception of storage seeds of some Compositae, is never found outside the chloroplast (Harwood, 1980; Hitchcock & Nichols, 1971).

The lateral heterogeneity of lipids in thylakoids has been examined by the use of various subthylakoid preparations (Chapman *et al.*, 1984; Gounaris *et al.*, 1983*a*; Murphy & Woodrow, 1983). However, no general statements can be made about phosphatidylglycerol distribution from these studies because of the different nature of the preparations examined. In addition, the sided distribution of phosphatidylglycerol has been examined in spinach (*Spinacia oleracea*) thylakoids, where it is preferentially located in the outer (stromal) leaflet (Rawyler & Siegenthaler, 1981). Spinach is a dicotyledonous plant and belongs to the so-called '16:3 group' of plants (those containing the $C_{16:3}$ hexadecatrienoic acid; cf. Heinz, 1977). In order to be able to make some general statements about the sided distribution of phosphatidylglycerol we have examined its transmembrane location in other types of plants. Moreover, we have studied the distribution by several methods. In addition, because of the particular interest of the fatty acid composition of phosphatidylglycerol, we have determined the acyl groups esterified to the phospholipid in the two leaflets. Preliminary reports of some of the results have been made (Unitt & Harwood, 1982*a*,*b*).

Materials and methods

Materials

Lettuce (*Lactuca sativa*) was obtained from the local market. Pea (*Pisum sativum* cv. Feltham First) seeds were obtained from Asmer Seeds, Leicester, U.K., and were germinated in moist vermiculite at 20°C with 200 μmol/s per m^2 illumination. Barley (*Hordeum vulgare* cv. Maris Otter) seeds were generously given by Dr. T.

Galliard (RHM Research, High Wycombe, Bucks., U.K.) and were germinated under similar conditions to those used for pea. Leaves were harvested from pea and barley seeds after 10–14 days growth.

Phospholipase A_2 (porcine pancreas), and phospholipase D (cabbage, *Brassica oleracea*) were purchased from Boehringer Corporation (London) Ltd., Lewes, East Sussex, U.K. Fatty acid standards for g.l.c. were from Nu-Chek Prep., P.O. Box 172, Elysian, MN 56028, U.S.A., and NaB^3H_4 (340GBq/mmol) was from Amersham International.

Isolation of chloroplast thylakoids

Chloroplasts were isolated from lettuce leaves as described by Jones & Harwood (1980). Chloroplasts were also isolated from lettuce, pea and barley leaves after homogenization with a Polytron blender (three 2s bursts) in 50mM-sodium phosphate buffer (pH6.5)/10mM-NaCl/330mM-sorbitol. The homogenate was filtered through six layers of Miracloth (Calbiochem, La Jolla, CA 92037, U.S.A.) and centrifuged by acceleration to 4500*g* in a MSE 8 × 50ml angle head, followed by immediate deceleration. The supernatant was decanted and the pellet gently resuspended in the homogenization buffer adjusted to pH7.4. The centrifugation and resuspension were repeated three times. Chloroplasts prepared by this procedure were free from all detectable non-chloroplast membranes or organelles as judged by enzyme marker distribution (cf. Unitt & Harwood, 1982*a*). The purified chloroplasts were ruptured by osmotic shock with the homogenization buffer, pH7.4, diluted 20-fold. The thylakoids were then sedimented at 14000*g* for 5min, the pellet resuspended in the diluted buffer and re-sedimented at 14000*g*. The final pellet was resuspended in the homogenization buffer, pH7.4, at a concentration of 0.7–1.0mg of chlorophyll/ml. All the above procedures were carried out at 0°C by cooling vessels and solutions with an ice/salt mixture.

Right-side-out and inside-out thylakoid vesicles were prepared from lettuce chloroplasts and characterized by the method of Andersson & Akerlund (1978) as detailed by Unitt & Harwood (1982*a*).

No endogenous acyl hydrolase activity was present in any of the thylakoid preparations.

Determination of lipid sidedness

Incubation of thylakoid membranes was carried out with phospholipase A_2 (porcine pancreas) under conditions described by Rawyler & Siegenthaler (1981). Hydrolysis with phospholipase D (cabbage) was performed in 100mM-sodium acetate/acetic acid buffer (pH5.8)/50mM-$CaCl_2$. The phospholipase D preparation was shown to be free from lipid and endogenous proteinase activity. Incubations with the phospholipases were carried out at temperatures between 5 and 20°C as detailed in the Results and discussion section for up to 2h. For control incubations where thylakoid vesicles were disrupted, the preparations were bath-sonicated for 2min in the presence of the appropriate enzyme at the incubation temperature before hydrolysis was initiated by the addition of $CaCl_2$. The sonication was repeated for 10s every 10min in control incubations.

Chemical labelling was carried out on the thylakoid preparations by allowing phosphatidylglycerol to chemically react with the diazonium salt of sulphanilic acid, followed by reduction in the presence of NaB^3H_4 (Schafer *et al.*, 1974). Standard conditions used 2mg of sulphanilic acid diazonium salt/mg of chlorophyll and 0.5mM-NaB^3H_4 (27.7μCi), with the oxidation and reduction reactions taking place for 15min and 8h respectively at 0°C. Control thylakoids were disrupted with 0.2% (w/v) sodium dodecyl sulphate. The labelled phosphatidylglycerol was extracted from the incubations and separated by t.l.c. as below. Bands corresponding to modified phosphatidylglycerol were scraped off the t.l.c. plate and counted for radioactivity, in a scintillant consisting of PCS (Amersham–Searle)/xylene (2:1, v/v), with a LKB Racbeta scintillation counter. Quench corrections were made by the external-standard channels-ratio method.

Lipids were extracted from the incubation mixtures by the method of Garbus *et al.* (1963). A portion of the chloroform phase was separated by t.l.c. on silica-gel G plates impregnated with 0.15 M-$(NH_4)_2SO_4$, with chloroform/methanol/acetic acid/water (170:30:20:7, by vol.) as solvent. Bands were located by spraying with 0.01% (w/v) methanolic 8-anilino-4-sulphonic acid, followed by exposure to u.v. light. Bands corresponding to phosphatidylglycerol and monoacylphosphatidylglycerol were scraped off the plate, derivatized by transmethylation and the fatty acids quantified by g.l.c. by using an internal standard of methyl pentadecanoate as described by Russell & Harwood (1979). In some experiments the phospholipids were quantified by phosphate determination (Bartlett, 1959). Digestion with phospholipase D was estimated by quantifying the phosphatidic acid and phosphatidylglycerol bands.

Chlorophyll was determined by the method of Arnon (1949), and correction was made, where appropriate, for errors in sampling volumes by determining the total chlorophyll in the chloroform extracts and in residues from samples used to spot t.l.c. plates.

Results and discussion

When membrane vesicles are treated with phospholipases, there are generally two phases of phospholipid hydrolysis. A rapid phase, which is not particularly temperature-dependent, is due to hydrolysis of phosphatidylglycerols in the outer leaflet. A temperature-dependent phase, which at low temperatures is much slower than the first phase, is due to transbilayer movement and subsequent hydrolysis of phospholipids from the inner leaflet (op den Kamp *et al.*, 1980). These types of results are seen clearly in Fig. 1, where data from the phospholipase A_2 treatment of lettuce thylakoid vesicles are shown. The maximum extent of hydrolysis in the rapid phase is approximately similar for the different digestion temperatures and, after correction for transbilayer movement, shows that about 74% of the total phosphatidylglycerol was present in the outer leaflet of lettuce thylakoids.

In studies of sidedness distribution using phospholipases it is important that certain criteria are fulfilled (Siegenthaler, 1982). It has to be shown that no lysis of thylakoids occurs when the enzyme acts on the outside of the vesicles; otherwise, the transmembrane distribution will be invalidated. Secondly, it has to be shown that total phospholipid digestion is possible in control experiments where both sides of the membrane are attacked, i.e. that there is no inaccessible phospholipid. Thirdly, the lipolytic treatment has to be shown not to modify the actual distribution of lipids. The latter point is most usually answered by using low-temperature digestion, where transbilayer movement is negligible.

Table 1 shows that in experiments with thylakoids obtained from three different types of plants, total digestion of phosphatidylglycerol was at least 97%, showing that only small or insignificant amounts of inaccessible phospholipid were present. It was of considerable interest that the major acyl moieties of phosphatidylglycerol were unevenly distributed. The major molecular species of phosphatidylglycerol in barley, lettuce and pea are the 1-linolenoyl-, 2-palmitoyl- and 1-linolenoyl-2-*trans*-Δ^3-hexadecanoyl species. The three major fatty acids are distributed with a fairly even distribution for palmitate and with 63–82% of the linolenate in the outer leaflet. The enrichment of linolenate in the outer (stromal) leaflet is due to its association with *trans*-Δ^3-hexadecenoate in one of the two major molecular species. Indeed, it is noticeable that *trans*-Δ^3-hexadecenoate appears to be virtually exclusively located in the outer leaflet in these three plant species. Although there have been no previous studies on the sided distribution of phosphatidylglycerol fatty acids, Duval *et al.* (1980) did show that *trans*-Δ^3-hexadecenoate was particularly rapidly hydrolysed during studies of the effect of phospholipase A_2 treatment of spinach thylakoids on chlorophyll fluorescence of the light-harvesting complex. Such a result is in keeping with the present data.

The exclusive localization of *trans*-Δ^3-hexadecenoate-containing phosphatidylglycerol in the outer leaflet is unusual and must surely be related to the function of the acid in thylakoids. Were the acid to be involved in granal stacking (Tuquet *et al.*, 1977), then such a distribution would make good sense. However, *trans*-Δ^3-hexadecenoate is found in stromal lamellae to approximately the same extent as in grana lamellae (Chapman *et al.*, 1984) and work with mutants (Bolton *et al.*, 1978) and other tissues (Percival *et al.*, 1979) have shown that there is often no correlation between stacking and *trans*-Δ^3-hexadecenoate content. The same problems of reconciling distribution with a func-

Table 1. *The sided distribution of phosphatidylglycerol fatty acids in chloroplast thylakoids from lettuce, pea and barley leaves*

Distributions were determined by digestions with phospholipase A_2 (porcine pancreas) as described in the Materials and methods section. Results are the means ± S.D. for independent experiments, the numbers of which are indicated in parentheses. For simplicity only the major fatty acids are shown. Abbreviations used: PG, phosphatidylglycerol, $C_{16:0}$, palmitic acid; *t*-$C_{16:1}$, *trans*-Δ^3-hexadecenoic acid; $C_{18:3}$, α-linolenic acid; n.d., none detected.

Plant		Fatty acid (% of total PG fatty acids)		
		$C_{16:0}$	*t*-$C_{16:1}$	$C_{18:3}$
Lettuce	Outer leaflet	12.5 ± 0.7 (4)	29.0 ± 2.1 (4)	37.7 ± 3.0 (4)
	Inner leaflet	11.5 ± 0.7 (4)	n.d. (4)	8.3 ± 0.6 (4)
	Total digestion in disrupted thylakoids (%)	98	100	98
Barley	Outer leaflet	10.3 ± 1.3 (2)	27.0 ± 1.7 (2)	23.9 ± 4.1 (2)
	Inner leaflet	7.7 ± 0.9 (2)	n.d. (2)	14.1 ± 1.9 (2)
	Total digestion in disrupted thylakoids (%)	96	99	97
Pea	Outer leaflet	17.7 ± 1.5 (4)	15.0 ± 1.0 (4)	20.1 ± 1.9 (4)
	Inner leaflet	16.3 ± 1.4 (4)	n.d. (4)	9.9 ± 0.8 (4)
	Total digestion in disrupted thylakoids (%)	98	100	99

tion in the oligomeric form of the light-harvesting complex (Remy *et al.*, 1982) also apply. At present, we do not have an accepted theory for the function of *trans*-Δ^3-hexadecenoate in thylakoids, although other suggestions have been made (Harwood, 1984).

Studies on lipid distribution which depend exclusively on phospholipase A_2 digestion can be criticized because of doubts concerning the exclusiveness of hydrolysis of outer-leaflet components (see above). These problems are not helped by the production of a lytic monoacylphospholipid product. Accordingly, we have checked the distribution of phosphatidylglycerol in thylakoid membranes by the use of chemical labelling and phospholipase D digestion.

We used the method of Schafer *et al.* (1974) to label phosphatidylglycerol chemically. This technique involves the oxidation of the terminal hydroxy group of the head-group glycerol with the diazonium salt of sulphanilic acid. The oxidized phosphatidylglycerol can then be reduced with NaB^3H_4. Chemical reaction in the presence of sodium dodecyl sulphate, when the thylakoids are disrupted, provides the control. In two experiments carried out with excess diazonium salt the amount of labelling of phosphatidylglycerol in intact lettuce thylakoids averaged 79% of the control (Table 2). This was a slightly higher value than the amount of phosphatidylglycerol estimated to be in the outer leaflet by digestion with phospholipase A_2 (Fig. 1 and Table 3). However, we did notice small amounts of labelling of the fatty acyl groups, which indicated that NaB^3H_4 penetrated the hydrophobic membrane region to some extent and may, in consequence, have labelled small quantities of inner-leaflet phosphatidylglycerol.

We also attempted to check the distribution of phosphatidylglycerol in thylakoids by using inside-out thylakoid vesicles. Such vesicles were prepared from lettuce and pea thylakoids by the method of Andersson & Akerlund (1978). However, in our hands we were unable to obtain preparations that were free from right-side-out vesicles. The best preparations were merely slightly enriched in inside-out vesicles as judged by light-driven proton movement (cf. Unitt & Harwood, 1982*a*) and could not, therefore, be used for meaningful sidedness studies.

Table 3 shows a summary of the distribution of phosphatidylglycerol in thylakoids from three

Table 2. *Trans-membrane distribution of phosphatidylglycerol in lettuce thylakoids as determined by chemical labelling*

Phosphatidylglycerol was modified with sulphanilic acid diazonium salt essentially by the method of Schafer *et al.* (1974) as described in the Materials and methods section. Labelling was performed at concentrations of 0.02–2.0mg of sulphanilic acid/mg of chlorophyll and the numbers of replicates are shown in parentheses. Results are expressed as means ± S.D.

	Phosphatidylglycerol labelling (c.p.m./μg of chlorophyll)	
	Expt. 1	Expt. 2
(*A*) Thylakoids	466 ± 48 (9)	230 ± 12 (5)
(*B*) Thylakoids + detergent	603 ± 53 (9)	285 ± 19 (6)
Localisation in outer leaflet ($A/B \times 100$)	77	81

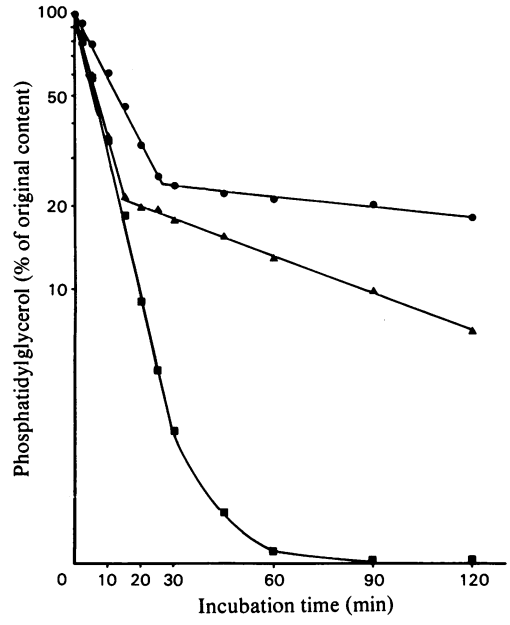

Fig. 1. *Time course of phosphatidylglycerol hydrolysis in lettuce thylakoids*

Incubations were carried out in duplicate at all time intervals with phospholipase A_2 (porcine pancreas) and thylakoid membranes at different temperatures as described in the Materials and methods section. Control thylakoids were disrupted initially by bath sonication in the incubation mixture containing phospholipase A_2 but no added NaCl or $CaCl_2$. Hydrolysis was started by the addition of $CaCl_2$. Control preparations were bath-sonicated at regular intervals (see the Materials and methods section). Estimation of hydrolysis was made by lipid extraction, t.l.c. and quantitative g.l.c. of the phosphatidylglycerol and monoacylphosphatidylglycerol fatty acids (see the Materials and methods section). ●, Incubation at 10°C; ▲, incubation at 20°C; ■, disrupted vesicles incubated at 20°C.

Table 3. *Transmembrane distribution of phosphatidylglycerol in thylakoids from various plants as determined by different methods*

For details, see the Materials and methods section. Results are expressed as means ± S.D. for the number of independent experiments shown in parentheses.

	Localization in outer leaflet (% of total PG)		
Plant	Digestion with phospholipase A_2	Digestion with phospholipase D	Chemical labelling
Lettuce	70 ± 3 (4)	82 ± 4 (2)	79 ± 3 (2)
Barley	73 ± 2 (2)	75 (1)	–
Pea	66 ± 6 (4)	67 (1)	–

plant species as determined by digestion with phospholipase A_2 and phospholipase D and by chemical labelling. There was reasonably good agreement between the three methods. All the three plant species showed that most (66–75%) of their thylakoid phosphatidylglycerol was localized in the outer (stromal) leaflet. This is comparable with the value of approx. 70% estimated by Rawyler & Siegenthaler (1981) for spinach thylakoids. Thus it seems to be a general characteristic of plant thylakoids that phosphatidylglycerol is located predominantly in the outer leaflet. In addition, the exclusive location of the 1-linolenoyl-2-*trans*-Δ^3-hexadecanoyl molecular species in the outer leaflet is a particularly interesting feature that requires a functional explanation.

The financial support of the Science and Engineering Research Council is gratefully acknowledge.

References

Andersson, B. & Akerlund, H.-E. (1978) *Biochim. Biophys. Acta* **503**, 462–472

Arnon, D. I. (1949) *Plant Physiol* **24**, 1–15

Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468

Bolton, P., Wharfe, J. M. & Harwood, J. L. (1978) *Biochem. J.* **174**, 67–72

Chapman, D. J., de Felice, J. & Barber, J. (1984) in *Structure, Function and Metabolism of Plant Lipids* (Siegenthaler, P.-A. & Eichenberger, W., eds.), pp. 457–464, Elsevier, Amsterdam

Duval, J. C., Dubacq, J.-P. & Tremolieres, A. (1980) in *Biogenesis and Function of Plant Lipids* (Mazliak, P., Benveniste, P., Costes, C. & Douce, R., eds.), pp. 91–94, Elsevier, Amsterdam

Garbus, J., de Luca, H. F., Loomans, M. E. & Strong, F. M. (1963) *J. Biol. Chem.* **238**, 59–63

Gounaris, K. & Barber, J. (1983) *Trends Biochem. Sci.* **8**, 378–381

Gounaris, K., Sundby, C., Andersson, B. & Barber, J. (1983*a*) *FEBS Lett.* **156**, 170–174

Gounaris, K., Whitford, D. & Barber, J. (1983*b*) *FEBS Lett.* **163**, 230–234

Harwood, J. L. (1980) in *Biochemistry of Plants* (Stumpf, P. K. & Conn, E. E., eds.), vol. 4, pp. 1–55, Academic Press, New York

Harwood, J. L. (1983) *Biochem. Soc. Trans.* **11**, 343–346

Harwood, J. L. (1984) in *Structure, Function and Metabolism of Plant Lipids* (Siegenthaler, P.-A. & Eichenberger, W., eds.), pp. 543–550, Elsevier, Amsterdam

Harwood, J. L. & Russell, N. J. (1984) *Lipids in Plants and Microbes*, Allen and Unwin, Hemel Hempstead

Heinz, E. (1977) in *Lipids and Lipid Polymers in Higher Plants* (Tevini, H. & Lichtenthaler, H. K., ed.), pp. 102–120, Springer-Verlag, Berlin

Hitchcock, C. & Nichols, B. W. (1971) *Plant Lipid Biochemistry*, Academic Press, London

Jones, V. H. M. & Harwood, J. L. (1980) *Biochem. J.* **190**, 251–254

Jordan, B. R., Chow, W. R. & Baker, A. J. (1983) *Biochim. Biophys. Acta* **725**, 77–86

op den Kamp, J. A. F., Kauerz, T. & van Meer, G. (1980) in *Biogenesis and Function of Plant Lipids* (Mazliak, P., Benveniste, P., Costes, C. & Douce, R., eds.), pp. 153–160, Elsevier, Amsterdam

Murphy, D. J. & Woodrow, I. E. (1983) *Biochim. Biophys. Acta* **725**, 104–112

Percival, M. P., Wharfe, J., Bolton, P., Davies, A. O., Jeffcoat, R., James, A. T. & Harwood, J. L. (1979) in *Advances in the Biochemistry and Physiology of Plant Lipids* (Appelqvist, L. A. & Liljenberg, C., eds.), pp. 219–224. Elsevier, Amsterdam

Rawyler, A. & Siegenthaler, P.-A. (1981) *Biochim. Biophys. Acta* **635**, 348–358

Remy, R., Tremolieres, A., Duval, J. C., Ambard-Bretteville, F. & Dubacq, J.-P. (1982) *FEBS Lett.* **137**, 271–275

Russell, N. J. & Harwood, J. L. (1979) *Biochem. J.* **181**, 339–345

Schafer, R., Hinnen, R. & Franklin, R. M. (1974) *Eur. J. Biochem.* **50**, 15–27

Siegenthaler, P.-A. (1982) in *Biochemistry and Metabolism of Plant Lipids* (Wintermans, J. F. G. M. & Kuiper, P. J. C., eds.), pp. 351–358, Elsevier, Amsterdam

Sinensky, M. (1977) *J. Bacteriol.* **129**, 516–524

Tuquet, C., Guillot-Salomon, T., de Lubac, M. & Signol, M. (1977) *Plant Sci. Lett.* **8**, 59–64

Unitt, M. D. & Harwood, J. L. (1982*a*) *Biochem. Soc. Trans.* **10**, 249–250

Unitt, M. D. & Harwood, J. L. (1982*b*) in *Biochemistry and Metabolism of Plant Lipids* (Wintermans, J. F. G. M. & Kuiper, P. J. C., eds.), pp. 359–362, Elsevier, Amsterdam