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LIPID DISTRIBUTION IN *ACHOLEPLASMA LAIDLAWII* MEMBRANE

A STUDY USING THE LACTOPEROXIDASE-MEDIATED IODINATION

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Summary

The lactoperoxidase-mediated radioiodination has been applied to study the transbilayer distribution of phospho- and glycolipids in *Acholeplasma laidlawii* membranes. After radioiodination, about 5% of the ^{125}I-iodine was found in membrane lipids. A comparison of the labeling intensities of the various lipid species between iodinated intact cells and isolated membranes revealed that the glycolipids monoglucosyldiglyceride and diglucosyldiglyceride are located almost exclusively in the outer half of the bilayer, whereas the phospholipids phosphatidylglycerol and diphosphatidylglycerol as well as the phosphoglycolipids glycerophosphoryl-diglucosyldiglyceride and glycerophosphoryl-monoglucosyldiglyceride are almost equally distributed in the outer and inner halves of *A. laidlawii* membranes.

Information on the transbilayer distribution of components in a biological membrane is required in order to gain insight into the molecular organization and function of the membrane. Although *Acholeplasma laidlawii* cells have most useful properties for membrane studies [1], the transbilayer distribution of *A. laidlawii* membrane lipids has not been sufficiently investigated.

It was recently found that the phosphatidylglycerol in *A. laidlawii* membranes is vulnerable to phospholipase A_2, so that this enzyme can be used for localization studies [2]. Nevertheless, phosphatidylglycerol constitutes only 30% of *A. laidlawii* membrane polar lipids, the rest being glycolipids and phosphoglycolipids, which are vulnerable neither to phospholipase nor to specific labeling agents [1,3]. Based on the finding that the amount of lectins bound to intact *A. laidlawii* cells was almost the same as that bound to isolated

membranes, Kahane and Tully [4] suggested that essentially all the carbohydrate-containing membrane lipids of *A. laidlawii* are exposed on the outer half of the cell membrane. However, the finding that glycolipids and phosphoglycolipids constitute more than 60% of total membrane lipids in this organism appears to oppose this notion [1]. In a previous study the lactoperoxidase-mediated radioiodination procedure was successfully applied to determine polypeptide asymmetry in *A. laidlawii* membranes [5]. Although the iodine predominantly bound to membrane polypeptides, apparently through its binding to exposed tyrosine residues, about 10% of the label was found in membrane lipids [5]. Recently the efficient labeling of lipids of chick embryo cells by the lactoperoxidase-mediated radioiodination was also described [6]. In the present communication we applied the lactoperoxidase-mediated iodination technique to the study of transbilayer distribution of the various polar lipid species of *A. laidlawii* membranes.

A. laidlawii (oral strain) was grown in a modified Edward medium [7]. The pH of the medium was adjusted to 8.5. To label membrane lipids, 1 μCi of [1-^{14}C]-palmitic acid (56 Ci/mol. The Radiochemical Centre, Amersham, UK) was added to each liter of the growth medium. The organisms were harvested after 16 to 20 h of incubation at 37°C and were washed once with 0.25 M NaCl. Cell membranes were isolated by osmotic lysis of the organisms [7], and collected by centrifugation at 34 000 $\times$ *g* for 30 min. Protein in the membrane suspensions was determined according to Lowry et al. [8].

Lactoperoxidase-mediated iodination was carried out by a modification of the procedure of Amar et al. [9]. Commercially available lactoperoxidase (EC 1.11.1.7) and glucose oxidase (EC 1.1.3.4) were used without further purification (Calbiochem and Miles Laboratories, Kankakee, IL, respectively). The reaction mixture, in a final volume of 2 ml, consisted of 0.05 M phosphate buffer, pH 7.5 in 0.2 M NaCl, washed cells or isolated membranes (1 mg membrane protein), 100 μg lactoperoxidase, 200 μCi of Na^{125}I (The Radiochemical Centre, Amersham, U.K.) in 10 μM unlabeled KI, and 10 units of glucose oxidase. Glucose (100 μg) was added to start the reaction, and another portion of 100 μg glucose was added 2 min later. To label membrane lipids by ^{125}I the same reaction mixture was used, but with 200 μCi of $^{125}I_2$ (The Radiochemical Centre, Amersham, U.K.) instead of Na^{125}I. At room temperature the incorporation of the iodine label into membrane lipids was linear for the first 4 min of incubation. In all experiments described the reaction mixtures were incubated for 10 min to assure that all labeling sites exposed to the radioiodination system were iodinated. After the incubation, the reaction was terminated by the addition of 0.1 ml of 0.1 M sodium azide. After thorough mixing, 8 ml of 0.25 M NaCl was added and the test tubes were immediately centrifuged. Membranes were then isolated from the iodinated cells as described above. Lipids were extracted from the membrane preparations by the method of Bligh and Dyer [10]. The chloroform layer was washed 6 times with 1 mM KI, then evaporated to dryness under a stream of nitrogen, redissolved in 1 ml chloroform, and applied to a carboxymethyl-cellulose column (5 by 150 mm, Ref. 11) and neutral lipid and traces of free iodine were eluted from the column with 150 ml chloroform. The polar lipids were eluted with 200 ml of chloroform/methanol (1:1, v/v) followed by 50 ml

methanol. The lipid fractions were dried under a stream of nitrogen and redissolved in 0.2 ml of chloroform/methanol (2:1, v/v). Neutral lipids were chromatographed on Silica gel G plates as described previously [7]. Polar lipids were chromatographed on Silica gel H plates (0.25 mm thick). The plates were developed with light petroleum (b.P. 40—60°C)/acetone (3:1, v/v), then with chloroform/methanol/water (65:25:4, v/v/v). Lipid spots were detected by iodine vapor, phospholipid spots by the molybdate spray reagent [12], α-glycol containing lipids by the periodate/Schiff reagent [13] and glycolipids by the anthrone reagent [14]. To determine phosphorous in phospholipid and phosphoglycolipid spots, the spots were scraped off the plate into test tubes, digested with 0.5 ml of ethanolic-Mg $(NO_3)_2$ solution and phosphorous was determined as described by Ames [15]. Glycerol was determined according to Bublitz and Kennedy [16] and sugar content as described by Wieslander and Rilfors [3]. The reaction of the lipid spots with the spraying reagents revealed two glycolipids, two phospholipids and two phosphoglycolipids. The phosphate-glycerol-fatty acids ratio of the phospholipids, the phosphate-glucose -fatty acid ratio of the phosphoglycolipids and the glucose-fatty acid ratio of the glycolipids were in accord with the previous identification of *A. laidlawii* polar lipids [3]. Accordingly, the two phospholipids are phosphatidylglycerol and diphosphatidylglycerol; the phosphoglycolipids, glycerophosphoryldiglucosyldiglyceride and glycerophosphorylmonoglucosyldiglyceride; and the glycolipids, monoglucosyldiglyceride and diglucosyldiglyceride. In the one-dimensional thin layer chromatography system used, the R_f of the iodinated phospho-, glyco- and phosphoglycolipids was the same as that of non-iodinated lipids. The lipid spots were then scraped off the plate into vials. ^{125}I was measured in a Packard Auto Gamma spectrometer. For measuring ^{14}C-labeled lipids, 5 ml of toluene-based scintillation liquid was added and the radioactivity was measured in a Packard Tri-Carb scintillation spectrometer.

Methyl esters were prepared from the various polar lipit species by heating the lipid samples for 15 min at 80°C in boron trifluoride/methanol solution (BF_3 Sigma, St. Louis, MO). The methyl esters were analyzed by gas liquid chromatography as previously described [17] and the fatty acid content of each of the lipids was determined using an internal standard of methyl pentadecanoate [17]. Since the percentage of palmitic acid in each of the lipid species was approximately the same (32 to 40 mol %), in some experiments the incorporation of [^{14}C]palmitate into the lipid species served as a measure for the fatty acid content of the lipids. A good correlation was always found between [^{14}C] palmitate incorporated and the fatty acid content (see Table II).

Table I shows that when *A laidlawii* membranes were iodinated by the lactoperoxidase-mediated procedure, about 5% of the radioactivity was found in membrane lipids. The labeling of membrane lipids depended on the presence of the lactoperoxidase; thus omission of the enzyme reduced the label by over 98% (not shown in the table). Likewise, in a reaction mixture that contained $^{125}I_2$ instead of $Na^{125}I$, the labeling intensity of membrane lipids was reduced by about 95%. After the lipid extraction, the organic phase still contained free ^{125}I and intensive washing with KI was required. Most of the

TABLE I

LABELING OF *ACHOLEPLASMA LAIDLAWII* MEMBRANE LIPIDS BY THE LACTOPEROXIDASE-MEDIATED ^{125}I IODINATION

Fraction	Radioactivity	
	cpm × 10^{-6}/mg membrane protein	% of total*
Isolated membranes	45.0	100.0
Lipid extract**	3.5	7.7
Washed lipid extract***	2.1	4.7
Neutral lipids	0.8	1.7
Polar lipids	1.3	2.9

*Compared to that of isolated membranes
**Lipids from membranes containing 1 mg membrane protein
***The lipids were washed 6 times with 1 mM KI.

free iodine was removed by six subsequent washings; the residue was eluted from the carboxymethylcellulose column by chloroform together with the neutral lipids. As is also shown in Table I, 40% of the radioactivity of the washed lipid extract was found in the neutral lipid fraction eluted from the carboxymethylcellulose by chloroform. Thin layer chromatography of the neutral lipid fraction revealed that most of the label is associated with the di- and monoglyceride spots. Very little, if any, was found in cholesterol, free fatty acids and carotenoids. The polar lipid fraction, eluted from the column with chloroform/methanol followed by methanol, contained 60% of the bound ^{125}I. The label was found in all the major phospholipid, glycolipid and phosphoglycolipid species constituting the polar lipid fraction of *A. laidlawii* [3].

Throughout the lactoperoxidase-mediated radioiodination procedure, *A. laidlawii* cells remained intact [5] and isolated membrane fragments unsealed [1]. Thus, lipid distribution was studied by comparing the labeling intensities of lipids of radioiodinated intact cells and isolated membranes, assuming that the disposition of membrane lipids in isolated membranes does not significantly differ from that in membranes of intact cells. When intact cells are treated, the labeling agent will have access to lipids in the outer half of the lipid bilayer, whereas when isolated membranes are treated, the labeling agent will have access to lipids in both outer and inner halves of the lipid bilayer. Table II shows that when isolated membranes were radioiodinated, the labeling intensities of the phospholipids and phosphoglycolipids were approximately two-fold higher than the labeling intensities obtained with radioiodinated intact cells. These results suggest that the phospholipids (Phosphatidylglycerol and diphosphatidylglycerol) and the phosphoglycolipids (glycerophosphoryl-diglucosyldiglyceride and glycerophosphroyl-monoglucosyldiglyceride) are almost equally distributed between the outer and inner halves of the bilayer. However, the labeling intensities of the two major glycolipids were almost the same in intact cells and isolated membranes, suggesting that the glycolipids are preferentially located in the outer half of the bilayer. The distribution of iodine-label between lipids of intact cells and isolated membrane preparation as well as among the various lipid species was almost the same when the radioiodination was carried out at 0, 22, or 37° C.

The fatty acid content of each of the major lipid species is also shown in Table II. This can be taken as a measure of the relative content of the hy-

TABLE II

TRANSBILAYER DISTRIBUTION OF THE MAJOR LIPID SPECIES OF *ACHOLEPLASMA LAIDLAWII* AS REVEALED BY THE LACTOPEROXIDASE—MEDIATED RADIOIODINATION

Lipid	Fatty acid content* (% of total fatty acids in polar lipids)	[^{14}C]palmitate incorporated (% of total cpm in polar lipids)	^{125}I-bound** (cpm × 10^{-4}/mg membrane protein)		Lipid distribution*** Outer half	Inner half
			Membranes	Cells	(% of total)	
Glycerophosphoryl-diglucosyldiglyceride	14.0	8.0	6.8	3.8	56	44
Glycerophosphoryl-monoglucosyldiglyceride	24.6	23.0	10.8	4.2	39	61
Phosphatidylglycerol	12.0	13.6	9.5	5.5	58	42
Diphosphatidylglycerol	22.5	25.7	16.8	10.0	60	40
Diglucosyldiglyceride	10.5	13.0	5.8	5.8	100	0
Monoglucosyldiglyceride	16.2	15.6	5.9	5.8	98	2

*The fatty acid content was determined from gas liquid chromatographs as described in the text.
**Radioactivity in lipids from membranes containing 1 mg membrane protein
***The lipid distribution was calculated from the labeling intensity. The labeling of intact cells represent lipids located in the outer half. Substracting the labeling of the intact cells from that of the isolated membranes represents labeling in lipids located in the inner half.

drophobic moiety of each of the lipid species in the bilayer. Combining these results with those of the distribution of the various lipid species between the inner and outer halves of the bilayer, the lipids in the outer half of *A. laidlawii* membranes were calculated to be twice as much as in the inner half. The lower lipid content in the inner half appears likely in light of the finding that two thirds of *A. laidlawii* membrane proteins face the cytoplasm (5). This suggests that the high protein content in the inner side of the membrane impedes lipid incorporation by decreasing the area available for lipids in the inner half of the bilayer.

The lactoperoxidase-mediated iodination procedure used provided information on the distribution of the major lipid species of *A. laidlawii* membranes. Several other techniques were utilized for lipid asymmetry studies in biological membranes, primarily, the use of labeling agents which react with primary amines and phospholipase treatment [18]. While the former cannot be used with *A. laidlawii* which completely lacks aminophospholipids [1,3] the latter provides information on membrane phospholipids which constitute only about 30% of *A. laidlawii* polar lipids [2,3]. The results on the distribution of phosphatidylglycerol obtained by the radioiodination technique are in accordance with the results of Bevers et al. [2], who by phospholipase A_2 treatment at 5°C found that phosphatidylglycerol is distributed in both halves of the lipid bilayer. At 37°C however, phospholipase A_2 completely hydrolyzed phosphatidylglycerol of intact cells, suggesting a rapid translocation mechanism which occurs at 37°C [2]. Using the radioiodination technique, no differences were found between cells labeled at 37°C or 0°C, suggesting a low rate of phosphatidylglycerol translocation. This finding supports the notion of Bevers et al. [2] that the rapid translocation at 37°C is triggerred by the depletion of phosphatidylglycerol from the outer half of the bilayer as a result of phospholipase A_2 activity. It can be assumed that as no such depletion occurs upon radioiodination, translocation rates during the radioiodination procedure remained low even at 37°C.

The nature of the iodine binding site is not yet known. The iodine may be bound to the lipid either by its addition to the double bonds or through the iodination of the α-position of a carbonyl compound. Since the increase in the palmitate content of *A. laidlawii* membrane polar lipid up to 80 mol%

of the total fatty acids by growing the cells in a medium containing albumin and palmitic acid [7] did not markedly affect the levels of ^{125}I bound to membrane lipids, it appears that α-substitution rather than iodine addition to double bonds is the major labeling site. This notion is further supported by showing that among the neutral lipids, iodine was found in the glycerides, rather than in cholesterol or in the highly unsaturated carotenoids of *A. laidlawii* [1].

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