PHOSPHOLIPID ASYMMETRY IN MIXED LIPOSOMES DETECTED BY ENZYMATIC RADIO-IODINATION

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1. Introduction

The lactoperoxidase-catalyzed introduction of iodine into aromatic amino acid residues and the localization of this reaction at the cell membrane is an established method for studying cell surface proteins [1–5]. It has been shown, however, that in some cases a fraction of the membrane-bound radioactivity could be extracted by organic solvents according to [6], which implied a lipid nature for these radioactive substances [7–9]. Further investigations have confirmed this assumption and have revealed that at least the phospholipids undergo enzymatic iodination both as purified substances and as cell membrane constitutents [10]. This was accomplished by the introduction of iodine to their acyl moiety (preferentially into the chain of the fatty acids attached to the β position of the glycerol) probably via addition and/or substitution reactions [10].

Anticipating possible applications of the enzymatic labeling of lipids to the study of the lipid component of the cell membrane, additional investigations seemed necessary, especially those concerning the arrangement of membrane phospholipids.

This work utilizes artificial phospholipid membranes to determine whether the arrangement of phospholipids into internal or external leaflets within the membrane bilayers may be discerned by means of lactoperoxidase-catalyzed iodination. In other words, can the membrane lipid asymmetry be established in that way?

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2. Materials and methods

2.1. *Preparation of mixed liposomes*

Egg yolk phosphatidylcholine (3 mM, Sigma, Tel Aviv) and 2 mM phosphatidylethanolamine (Sigma) were sonicated in a Ca^{2+},Mg^{2+}-free phosphate buffer, NaCl solution (isotonic PBS, pH 7.4) using a Braun Sonic 300 Homogenizer. The suspension was centrifuged for 20 min at 40 000 × *g* and the supernatant was retained and analyzed. By this method suspensions of mixed liposomes, total conc 7 mM phospholipids at a molar ratio of 2.5:1.0 phosphatidylcholine to phosphatidylethanolamine, were obtained.

2.2. *Preparation of liposomes with entrapped iodination system*

Mixed liposomes which contain 0.35 μmol phospholipids at a molar ratio of 2.5:1.0 phosphatidylcholine to phosphatidylethanolamine, 100 μCi Na-^{125}I (The Radiochemical Centre, Amersham) 1.25 μg lactoperoxidase (EC 1.11.1.7, Sigma) and 300 μU glucose oxidase, (EC 1.1.3.4, Sigma) trapped inside for radioiodination were prepared as follows: to a PBS solution containing 3 mCi $^{125}I^-$ were added 30 μmol phosphatidylcholine; 2 μmol phosphatidylethanolamine; 500 μg lactoperoxidase and 120 mU glucose oxidase. The final volume was 1 ml. Following sonication and centrifugation as described in section 2.1, a 50 μl aliquot was applied to a Sepharose 6B column (bed vol. 0.3 ml) and the liposomes eluted in the void volume.

2.3. *Iodination procedure*

(a) External iodination. To 50 μl mixed liposomes in Ca^{2+},Mg^{2+}-free PBS reagents were added in the following order: 1.25 μg lactoperoxidase; 100 μCi

carrier free $^{125}I^-$; 300 μU glucose oxidase and 250 nmol D-glucose; PBS was added to 0.35 ml final vol.

(b) Internal iodination. Liposomes (50 μl) with the trapped iodination system were added to a mixture containing 300 μl 0.83 mM D-glucose solution in Ca^{2+},Mg^{2+}-free PBS.

2.4. *Incubation and analyses*

The reaction mixtures were incubated for 10 min at room temperature, then 1 μmol $Na_2S_2O_3$ in 10 μl H_2O was added to reduce the different forms of iodine to iodide (I^-). The water was evaporated under a stream of N_2 and the residue was taken in a small volume of $CHCl_3$:CH_3OH:CH_3COOH:H_2O (25:15:4:2, by vol.), applied to a column of Sephadex LH-20 (Pharmacia, Uppsala) column with 0.3 ml bed vol. pre-equilibrated with the same solvent mixture. Iodinated phospholipids were eluted and subsequently separated by chromatography on a column of silica gel H (Merck, Darmstadt) prepared and eluted with the above solvent mixture (cf. details in [10]).

2.5. *Auto radio-iodination of lactoperoxidase and glucose oxidase*

Lactoperoxidase (10 μg) and 120 mU glucose oxidase, 100 μC_i $^{125}I^-$ and 250 nmol D-glucose in 250 μl final vol. Ca^{2+},Mg^{2+}-free PBS were incubated for 10 min at room temperature. The iodinated proteins were purified by chromatography on Sephadex G-25.

2.6. *Exclusion chromatography*

Following entrapment by liposomes (cf. section 2.2) of either $^{125}I^-$ or iodinated proteins, an aliquot was chromatographed on a Sepharose 6B column having 0.3 ml bed vol.

3. Results and discussion

Interactions between iodide and the polar head groups of phosphatidylcholine [11], might lead to a shift in the distribution between free iodide and that entrapped by liposomes, in the favor of the latter. Therefore, special precautions were taken to ensure the same iodide/enzymes ratio for external and internal iodinations.

Figure 1 shows distribution between liposome-trapped (tr.) and untrapped (untr.) iodide with a ratio

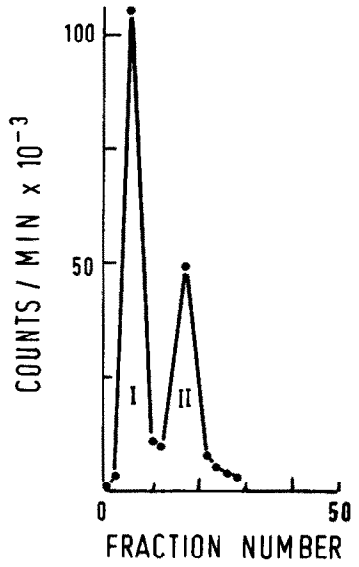

Fig.1. Iodide trapping inside mixed liposomes. $^{125}I^-$ (100 μCi) was sonicated with phosphatidyl choline:phosphatidylethanolamine mixed liposomes at a molar ratio of 2.5:1, which were then chromatographed on a calibrated Sepharose 6B column. I, V_o; $^{125}I^-$-containing liposomes. II, free $^{125}I^-$.

I^-(tr.)/I^-(untr.) of 1:1.5. When such a distribution was obtained for the $^{125}I^-$-labeled enzyme (fig.2), the ratio between trapped and untrapped enzyme was lower by an order of magnitude; i.e., $E_{tr.}/E_{untr.}$ =

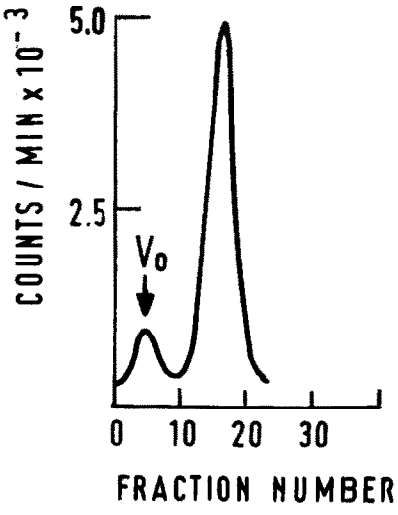

Fig.2. Sepharose 6B chromatography of liposome-trapped, ^{125}I-labeled enzymes and free $^{125}I^-$. The liposomes were eluted in V_o.

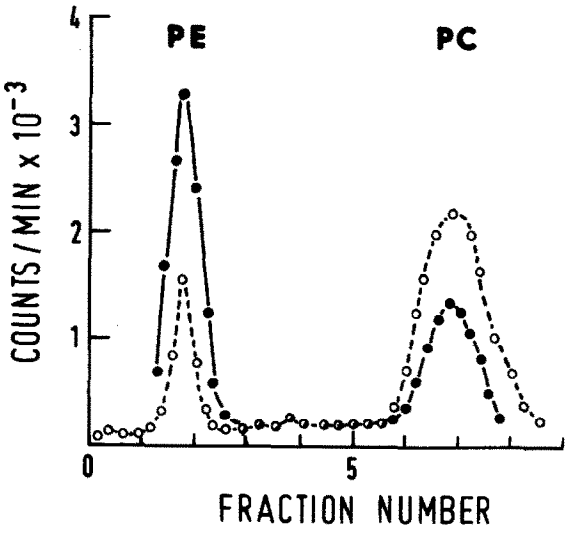

Fig.3. Silica gel H chromatography of externally or internally labeled phospholipids in mixed liposomes. Details of labeling and chromatography are given in the text. (o–o) Outside; (•–•) inside.

1:15. This indicates a lack of uniformity of the distribution of different constituents of the iodination reaction mixture. Considering this data, the experimental protocol was altered, yielding a uniform ratio between reaction components for external and internal iodination.

Figure 3 shows the labeling pattern of phosphatidylethanolamine and phosphatidylcholine in mixed liposomes following inside and outside iodinations. Interestingly enough, phosphatidylethanolamine and phosphatidylcholine are apparently not identical substrates for enzymatic iodination. Whereas the phosphatidylethanolamine to phosphatidylcholine molar ratio in mixed liposomes was 0.4, the respective ratio between their total radioactivity on both sides of the membrane, i.e., $(PE_{ext.} + PE_{int.})/(PC_{ext.} + PC_{int.})$, was 0.75. This indicates a preferential labeling of phosphatidylethanolamine by this iodination system which apparently need not be dependent upon the topography of the labeling process. Figure 3 also demonstrates that phosphatidylcholine was predominantly labeled upon outside iodination whereas phosphatidylethanolamine became primarily labeled with the internally trapped iodination system. These observations suggest asymmetric arrangement for phospholipids in the liposome membranes.

Specific reagents for amino group determinations, i.e., trinitrobenzene sulfonate (TNBS) or fluorescamine, were used [12,13] to elucidate the distribution of phosphatidylethanolamine between external and internal surfaces in mixed phosphatidylcholine (PC), phosphatidylethanolamine (PE) liposomes. They reached similar conclusions, i.e., phosphatidylethanolamine is preferentially located at the inner leaflet of the membrane. However, their values for the ratio $PE_{out}/PE_{total} = 0.56$ [12] was higher than the ratio obtained in the present study (i.e., 0.35). This discrepancy, although not contradictory to the pattern of phosphatidylethanolamine distribution, might originate either from contamination with multibilayered liposomes or, alternatively, due to differences in the experimental design.

However relevant this contamination might be, especially for the determination of the ratio ext./total, for a single membrane component (i.e., PE), it seems to us that its significance diminishes when such a determination takes place for more than one component and with reverse patterns of distribution. Actually the reverse ratio between externally and internally labeled PE and PC (PE_{out}/PE_{in} was ~0.43 and PC_{out}/PC_{in} was ~1.9) may not be explained satisfactorily in terms of contamination, thus reinforcing the contention that the differences in phospholipid labeling are due to their asymmetric arrangement.

We believe that enzymatic iodination may be a method of choice for determination of membrane asymmetry. Especially, when taking into consideration that most methods used for such studies are based on modifications of a particular group (e.g., amino groups), this method identifies most phospholipids by their common acyl moiety [10], thus permitting the simultaneous study of the arrangement of several phospholipids in complex structures such as biological membranes.

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