Biochimica et Biophysica Acta, 552 (1979) 114—119

BBA 78325

EXCHANGEABILITY AND RATE OF FLIP-FLOP OF PHOSPHATIDYLCHOLINE IN LARGE UNILAMELLAR VESICLES, CHOLATE DIALYSIS VESICLES, AND CYTOCHROME OXIDASE VESICLES

PAUL E. DICORLETO and DONALD B. ZILVERSMIT

Division of Nutritional Sciences, and Section of Biochemistry, Molecular and Cell Biology, Division of Biological Sciences, Cornell University, Ithaca, NY 14853 (U.S.A.)

(Received August 17th, 1978)

Key words: Phospholipid exchange; Flip-flop; Cytochrome oxidase; Cholate dialysis; (Membrane vesicle)

Summary

Three model membrane systems have been characterized in terms of their interaction with phospholipid exchange proteins. Large unilamellar vesicles of phosphatidylcholine prepared by ether vaporization are shown to be homogeneous by gel filtration. Phospholipid exchange proteins from three sources are capable of catalyzing the rapid exchange of approximately half of the phospholipid from these vesicles. The remaining pool of radioactive phospholipid is virtually nonexchangeable ($t_{1/2}$ of several days). Small unilamellar vesicles of phosphatidylcholine prepared by cholate dialysis also exhibit two pools of phospholipid (65% rapidly exchangable, 35% very slowly exchangeable) when incubated with beef liver phospholipid exchange protein. Cytochrome oxidase vesicles prepared both by a cholate dialysis method and by a direct incorporation method have been fractionated on a Ficoll discontinuous gradient, and tested for interaction with beef heart exchange protein. Two pools of phospholipid are once again observed (70% rapidly exchangable, 30% nonexchangeable), even for vesicles which have incorporated the transmembranous enzyme at a phospholipid to protein weight ratio of 2. The size of the rapidly exchangeable pool of phosphatidylcholine for each of the vesicle systems is consistent with the calculated fraction of phospholipid in the outer monolayer. The extremely slow rate of exchange of the second pool of phospholipid reflects the virtual nonexistence of phospholipid flip-flop in any of these model membranes.

Phospholipid exchange proteins will catalyze the transfer of radioactive phospholipid from sonicated phosphatidylcholine vesicles [1,2] and from mixed phospholipid multilamellar vesicles [3], but not from phosphatidylcholine multilamellar vesicles [3]. The exchangeability of the phospholipid in other model membrane sytems has not been reported. The phospholipid distribution across the bilayer and the rate of phospholipid flip-flop has been determined in sonicated phosphatidylcholine vesicles by use of exchange proteins and radioactive phospholipid [4,5]. The rate of flip-flop in these vesicles and also in phagosomal membranes [6] and viral membranes [7] has been found to be much slower ($t_{1/2}$ of days) than the rate of flip-flop in microsomal membranes ($t_{1/2}$ of minutes) [8] or erythrocyte ghost membranes ($t_{1/2}$ of hours) [9], as determined by the exchange protein technique. We report here on the interaction of phospholipid exchange proteins with large unilamellar vesicles, cholate dialysis vesicles, and cytochrome oxidase vesicles. The experiments were chosen to examine the effect of membrane curvature and the presence of a transmembranous enzyme on the exchangeability and rate of flip-flop of phospholipids.

Deamer and Bangham [10] have recently described an ether vaporization method for the preparation of large unilamellar vesicles (1500 Å average diameter). These vesicles have considerably less curvature than sonicated vesicles. The elution profile on Bio-Gel A-50m (Bio-Rad Laboratories, Rockville Center, NY) of ether vaporization vesicles prepared with phosphatidylcholine only is shown in Fig. 1. The recovery of phospholipid from the column was approx. 75%. Multilamellar vesicles of phosphatidylcholine that were eluted from the same column in a separate run (less than 10% of applied sample phospholipid recovered) mark the void volume which was also determined by Blue Dextran *. The ether vaporization vesicles appear quite homogeneous and exhibit a volume of elution that is consistent with vesicles of greater than 1000 Å in diameter as determined by calibration of a comparable column through which sonicated vesicles, sized by electron microscopy, had been eluted.

The exchangeability of the phosphatidylcholine in ether vaporization vesicles was tested with exchange proteins from beef heart (purified according to Johnson and Zilversmit [2]), beef liver (purified according to Kamp and Wirtz [11]), and rat liver (purified according to Bloj and Zilversmit [12]). The three proteins gave similar exchange results. Approx. 50% of the radioactive phosphatidylcholine of these vesicles was transferrable to multilamellar vesicles [3] as is shown for beef liver exchange protein in Fig. 2. The half-time of phosphatidylcholine flip-flop in these vesicles, as calculated from the level portion of the exchange curve [9], is approximately 9 days, or longer than our detection limits. The fact that one-half of the phospholipid is accessible for exchange is consistent with the size of these vesicles if the exchangeable pool is the phosphatidylcholine of the outer monolayer. The bilayer thickness is small compared to the radius of the particle so the inner and outer monolayers should be

* Multilamellar vesicle preparations consistently yield a poor recovery on several types of agarose gel filtration columns including Bio-Gel A-150m, and Sepharose 2B and 4B (Pharmacia). Most of the phospholipid appears to remain at the top of the column.

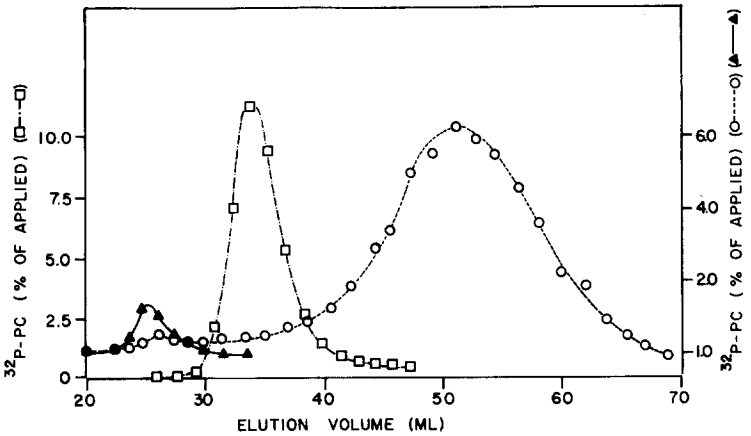

Fig. 1. Gel filtration of phosphatidylcholine multilamellar vesicles (▲), cholate dialysis vesicles (○) and ether vaporization vesicles (□). Each preparation (5 mg [^{32}P]phosphatidylcholine (PC) with a trace of [^{3}H]triolein in 1 ml 50 mM Tris-HCl, pH 7.4, 5 mM Na_2EDTA buffer) was eluted through a Bio-Gel A-50m column (15 X 2.5 cm) at a flow rate of 20 ml/h. The column was preequilibrated with 25 mg egg phosphatidylcholine (Lipid Products Inc., England) by elution in the same buffer. The ^{32}P to ^{3}H ratio through each peak is constant. Recoveries of label are given in the text. Radioactive lipids were obtained and purified as described previously [3]. Cholate dialysis vesicles [14] and ether vaporization vesicles [10] and multilamellar vesicles [3] were prepared according to published procedures.

comparable in surface area, and probably in phospholipid packing. Our results seem to argue against a lack of membrane curvature as the cause for non-exchangeability of phospholipid from phosphatidylcholine multilamellar vesicles [3]. It is possible, however, that the large unilamellar vesicles possess a

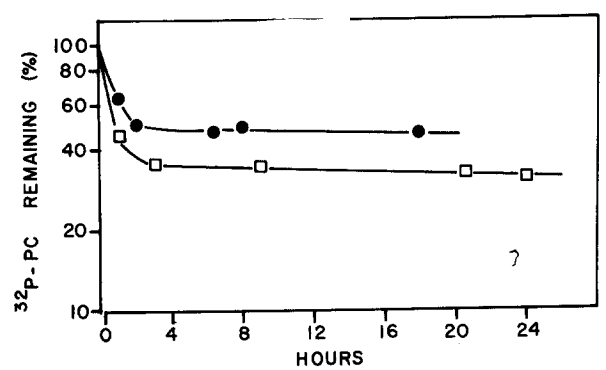

Fig. 2. Transfer of [^{32}P]phosphatidylcholine (PC) from cholate dialysis vesicles (□) and ether vaporization vesicles (●) to multilamellar vesicles. Each donor vesicle preparation (0.025 mg [^{32}P]phosphatidylcholine/ml) was individually incubated with excess phosphatidylcholine : phosphatidylethanolamine : cardiolipin (70 : 25 : 5, v/v) multilamellar vesicles [3] (2.0 mg phospholipid/ml) and beef liver phospholipid exchange protein (10.0 units/ml) in the buffer of Fig. 1 at 37°C. After 1 h the acceptor particle was removed by centrifugation (40 000 X *g* for 15 min) and replaced by fresh acceptor in order to prevent reverse flow of labeled phospholipid during subsequent incubation. Both donor particles contained a trace of [^{3}H]triolein as nontransferrable marker [2]. The calculation of phospholipid transfer and definition of exchange units has been described [3]. The exchange protein is fully active after incubation under the above conditions for 24 h. Nonradioactive phospholipids were obtained from Lipid Products, Inc. (England) and checked for purity as described previously [3].

greater flexibility in the surface such that transient regions of high curvature would exist due to puckering.

The rapid input of ultrasonic energy in the preparation of sonicated vesicles has led some workers to refer to them as kinetic or metastable structures [13]. Multilamellar vesicles, on the other hand, are prepared slowly with a minimum of added energy and may be referred to as equilibrium particles. Small unilamellar vesicles may be prepared without ultrasound in a process that might more closely resemble the formation of multilamellar vesicles. Phospholipids are dispersed in a buffer containing sodium cholate (1.0%) [14]. The cholate is then slowly removed by dialysis to form vesicles which are similar in size to those prepared by sonication as determined by gel filtration (Fig. 1 vs. profiles in ref. 3) and electron microscopy [15]. As shown in Fig. 2, the phosphatidylcholine of cholate dialysis vesicles is readily exchangeable by bovine liver exchange protein. The extent of exchange levels at 65%, which agrees with the fraction of phospholipid in the outer monolayer of a 270 Å diameter vesicle assuming a bilayer thickness of 35 Å. The lack of significant flip-flop in cholate dialysis vesicles is apparent from the lack of slope in the later stage of the exchange curve ($t_{1/2}$ is greater than 4 days).

Mitochondrial cytochrome *c* oxidase can be integrated into the bilayer structure of a liposome by several means. The cholate dialysis method, which was the first to be described [16], yields proton-impermeable vesicles with cytochrome oxidase incorporated transmembranously in a single orientation [17,18]. Alternatively, presonicated phospholipid vesicles of certain compositions will incorporate cytochrome oxidase by simple incubation without added detergent or ultrasound [19]. These two different techniques of preparation yield a similar result: a small subpopulation of vesicles that contain a large proportion of the protein [18]. This subpopulation is separable from the majority of the vesicles by discontinuous Ficoll density gradient ultracentrifugation [18]. These cytochrome oxidase vesicles have a phospholipid to protein ratio of 2 : 1 to 7 : 1 as compared to an initial ratio of greater than 20 : 1. The effect of a transmembranous enzyme in phospholipid vesicles on the exchangeability and flip-flop of phosphatidylcholine was examined by the exchange protein technique.

Vesicles containing very little cytochrome oxidase (lipid to protein weight ratio to greater than 180 : 1), as isolated from an upper layer of the Ficoll gradient, and vesicles containing a great deal of protein (ratio of less than 7 : 1) were subjected to extensive phospholipid exchange in the presence of beef heart exchange protein. In Fig. 3A the results are given for fractionated cytochrome oxidase vesicles prepared by the direct incorporation method [19] with phospholipid vesicles of phosphatidylcholine : phosphatidylethanolamine : phosphatidylinositol (1 : 1 : 1, v/v). The exchange protein is capable of catalyzing the transfer of 70% of the radioactive phosphatidylcholine. The lack of phosphatidylcholine flip-flop is apparent, even for vesicles containing a large amount of cytochrome oxidase. There is no significant difference in the fraction of exchangeable phospholipid or rate of flip-flop in the vesicles containing varying amounts of cytochrome oxidase.

Similar results are obtained when Ficoll-fractionated cytochrome oxidase vesicles, that have been prepared by the cholate dialysis method with phos-

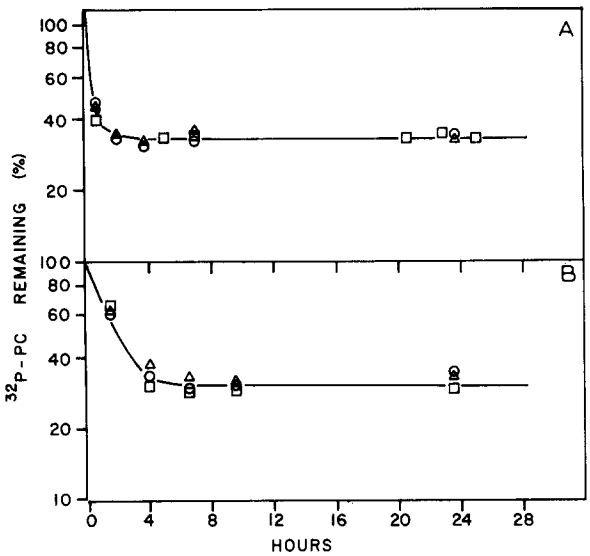

Fig. 3. Transfer of [^{32}P]phosphatidylcholine (PC) from cytochrome oxidase vesicles to multilamellar vesicles. (A) Cytochrome oxidase vesicles were prepared by direct incorporation of protein into liposomes of [^{32}P]phosphatidylcholine : phosphatidylethanolamine : phosphatidylinositol (1 : 1 : 1, v/v) and a trace of [^{3}H]triolein [19]. Unfractionated cytochrome oxidase vesicles (○) with phospholipid to protein ratio of 25 by weight, and fractionated vesicles with ratios of 183 (△) and 2 (□) were incubated individually at a concentration of 0.025 mg phospholipid/ml with excess phosphatidylcholine : phosphatidylethanolamine : cardiolipin (70 : 25 : 5, v/v) multilamellar vesicles (2 mg phospholipid/ml) and beef heart phospholipid exchange protein (12.5 units/ml) in potassium phosphate (50 mM, pH 7.4) buffer at 37°C. After 40 min the acceptor particle was removed by centrifugation (40 000 × *g* for 15 min) and replaced by fresh acceptor. (B) Fractionated cytochrome oxidase vesicles (prepared by cholate dialysis with [^{32}P]-phosphatidylcholine : phosphatidylinositol (70 : 30, v/v) and a trace of [^{3}H]triolein) with phospholipid to protein weight ratios of 180 (△), 23 (○) and 7 (□) were incubated with beef heart exchange protein and multilamellar vesicles as described for A. The cytochrome oxidase assay and Ficoll discontinuous gradient fractionation have been described [18]. Respiratory control ratios [18] were between 4 and 6 for all the cytochrome oxidase vesicles employed in these studies.

phatidylcholine : phosphatidylinositol (70 : 30, v/v), are incubated with beef heart exchange protein (Fig. 3B). The two pools of phosphatidylcholine in these two sets of experiments (rapidly exchangeable and virutally nonexchangeable) most likely represent the phosphatidylcholine of the outer and inner monolayer, respectively. Gel filtration of the two preparations of cytochrome oxidase vesicles yields elution profiles with constant [^{32}P]phosphatidylcholine to [^{3}H]triolein ratio that closely resembles the included volume peak obtained with sonicated or cholate dialysis vesicles without cytochrome oxidase. Indeed, the cytochrome oxidase vesicles prepared by cholate dialysis have been shown by electron microscopy to be only slightly larger than vesicles prepared in the absence of protein [15]. Gel filtration experiments in our laboratory have also demonstrated a constant oxidase activity to phospholipid mass ratio through the elution peak. Recently, it has been reported that the smaller phospholipid vesicles obtained by gel filtration, prior to incubation with protein, are responsible for the incorporation process [20]. Our gel filtration results on the reconstituted vesicles indicate that some events, such as fusion, may occur subsequent to insertion of protein such that the protein-containing subpopula-

tion ultimately assumes a size distribution that matches the majority of the phospholipid vesicles.

The absence of phosphatidylcholine flip-flop in vesicles which possess a phospholipid to cytochrome oxidase ratio of 2 : 1 is in contrast to recent observations on the effect of incorporation of glycophorin, a transmembranous protein of erythrocytes, into liposomes [21]. By the use of ^{13}C nuclear magnetic resonance spectroscopy and phospholipase hydrolysis, an increased flip-flop rate of lysolecithin was observed ($t_{1/2}$ 2—5 h) when glycophorin was incorporated into small unilamellar vesicles. Similarly the rate of dioleoyl phosphatidylcholine transbilayer movement in small vesicles was found to be enhanced greatly by the presence of glycophorin when measured by ^{13}C nuclear magnetic resonance spectroscopy and phospholipid exchange [22].

In summary, large vesicles with little membrane curvature, small vesicles prepared without ultrasound, and vesicles containing a transmembranous enzyme all represent 'substrate' particles for phospholipid exchange proteins. In every system only the outer monolayer phospholipid is available for exchange due to a virtual lack of phospholipid flip-flop.

Acknowledgements

We wish to thank Dr. R. Carroll for the preparation, fractionation, and characterization of cytochrome oxidase vesicles [18] and Dr. E. Racker for many helpful discussions. Thomas Shea provided expert technical assistance. This work was supported by Public Health Service Research Grant HL 10940 from the National Heart, Lung, and Blood Institute, U.S. Public Health Service. D.B.Z. is a Career Investigator of the American Heart Association. P.E.D. is a recipient of a National Institutes of Health predoctoral traineeship supported by Training Grant No. GM 07273 from the United States Public Health Service.

References

1 Zilversmit, D.B. (1971) J. Biol. Chem. 246, 2645—2649
2 Johnson, L.W. and Zilversmit, D.B. (1975) Biochim. Biophys. Acta 375, 165—175
3 DiCorleto, P.E. and Zilversmit, D.B. (1977) Biochemistry 16, 2145—2150
4 Johnson, L.W., Hughes, M.E. and Zilversmit, D.B. (1975) Biochim. Biophys. Acta 375, 176—185
5 Rothman, J.E. and Dawidowicz, E.A. (1975) Biochemistry 14, 2809—2816
6 Sandra, A. and Pagano, R.E. (1978) Biochemistry 17, 332—338
7 Rothman, J.E., Tsai, D.K., Dawidowicz, E.A. and Lenard, J. (1976) Biochemistry 15, 2361—2370
8 Zilvermsit, D.B. and Hughes, M.E. (1977) Biochim. Biophys. Acta 469, 99—110
9 Bloj, B. and Zilversmit, D.B. (1976) Biochemistry 15, 1277—1283
10 Deamer, D. and Bangham, A.D. (1976) Biochim. Biophys. Acta 443, 629—633
11 Kamp, H.H. and Wirtz, K.W.A. (1974) in Methods in Enzymology (Fleisher, S. and Packer, L., eds.), Vol. 32B, pp. 140—146, Academic Press, New York
12 Bloj, B. and Zilversmit, D.B. (1977) J. Biol. Chem. 252, 1613—1619
13 Sheetz, M.P. and Chan, S.I. (1972) Biochemistry 11, 4573—4581
14 Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477—5487
15 Ruben, G.C., Telford, J.N. and Carroll, R.C. (1976) J. Cell Biol. 68, 724—739
16 Hinkle, P.C., Kim, J.J. and Racker, E. (1972) J. Biol. Chem. 247, 1338—1339
17 Racker, E. (1972) J. Membr. Biol. 10, 221—235
18 Carroll, R.C. and Racker, E. (1977) J. Biol. Chem. 252, 6981—6990
19 Eytan, G.D., Matheson, M.J. and Racker, E. (1976) J. Biol. Chem. 251, 6831—6837
20 Eytan, G.D. and Broza, R. (1978) FEBS Lett. 85, 175—178
21 van Zoelen, E.J.J., de Kruijff, B. and van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 508, 97—108
22 de Kruijff, B., van Zoelen, E.J.J. and van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 509, 537—542