A simple assay to study protein-mediated lipid exchange by fluorescence polarization

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We investigated the protein-mediated phospholipid transfer between small vesicles by fluorescence polarization measurements with diphenylhexatriene as optical probe. Thermotropic-phase-transition curves were taken after mixing two vesicle preparations of lipids exhibiting different gel-to-liquid phase transitions. From the heights of each phase-transition step we were able to follow the lipid transfer process without separating the two vesicle preparations.

Extensive studies of the distribution and trans-bilayer movement of phospholipids have been performed in biological and artificial membranes (Rothmann & Lenard, 1977; Bergelson & Barsukov, 1977) by using chemical labelling (Bretscher, 1972) and phospholipase hydrolysis (Verkley *et al.*, 1973). For several years it has been known that the transfer of phospholipids between natural membranes occurs via soluble proteins (Wirtz & Zilversmit, 1969). So-called phospholipid-transfer proteins have been isolated from liver and other tissues (Wirtz, 1974; Kamp & Wirtz, 1974; Zilversmit & Johnson, 1975). These cytosolic proteins bind specific classes of phospholipids and catalyse the transfer of phospholipids between membrane interfaces (Wirtz *et al.*, 1976; Zilversmit & Hughes, 1976, 1977; Crain & Zilversmit, 1980; Kasper & Helmkamp, 1981).

In the present study we have investigated the lipid-transfer process in phospholipid bilayer vesicles by using fluorescence polarization measurements. It was shown previously (Hartmann *et al.*, 1978; Sixl & Galla, 1979) that it is possible to quantify the amount of lipid in a given state from the thermotropic gel-to-liquid phase-transition curve. We can now follow directly the exchange process between phospholipid vesicles that exhibit a phase transition without separating the two vesicle populations.

Materials and methods

Phospholipid-transfer protein

The transfer protein was isolated from fresh beef liver by a slight modification of the method described by Kamp & Wirtz (1974). Chromatography on DEAE-cellulose was performed with 5 mM-sodium phosphate/10 mM- β -mercaptoethanol, pH 8.0, as eluent. We subjected this crude protein to gel

filtration on Ultrogel AcA 44 from LKB Produkter AB (Bromma, Sweden) in 50 mM-NH₄HCO₃ (pH 8.5). The phospholipid-transfer protein was present in the second peak. Protein concentration was determined by the method of Lowry *et al.* (1951). The transfer protein was stored at –20°C in 50% glycerol.

Lipids and vesicle preparation

Dipalmitoyl- and distearoylglycerophosphocholine were obtained from Fluka (Neu-Ulm, Germany) and used without further purification. Lipids and 1% diphenylhexatriene (Fluka) were dissolved in chloroform and evaporated under N₂ in a flask to yield a film on the flask wall. Buffer (0.1 M-sodium phosphate, pH 7) was added to the dried film to make up a lipid concentration of 0.5 mg/ml. Sonication was performed with a Branson Ultrasonifier for 12 min at 30 W and at a temperature 10°C above the corresponding lipid phase-transition temperature. The transfer protein was added after mixing of the desired preparations.

Fluorescence polarization measurements

Fluorescence polarization $P = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$, where $I_{\parallel}$ is the fluorescence intensity measured parallel and $I_{\perp}$ is the fluorescence intensity measured perpendicular to the polarized excitation. The fluorescence polarization was followed automatically with temperature with the use of a Schoeffel RRS 1000 fluorescence spectrometer equipped with two sets of monochromator/photomultiplier. This arrangement allowed the display of the degree of polarization calculated by an analog computer continuously with temperature. The heating rate was 3°C/min for the small sample volume of 0.2 ml in a microcuvette. This corresponds to an assay time of

about 10 min. Temperature changes were achieved by using a Peltier-element, and temperature was measured by a thermocouple to an accuracy of $\pm 0.2^\circ\text{C}$.

Results

Thermotropic-phase-transition curves of phosphatidylcholine vesicles were obtained by measuring the fluorescence polarization as a function of temperature. Fig. 1 shows the curves taken

Fig. 1. Determination of phase transition by fluorescence polarization

Phase-transition curves of pure and mixed phosphatidylcholine vesicles obtained from fluorescence polarization measurements with diphenylhexatriene as optical probe are shown. (a) Dipalmitoylglycerophosphocholine (DPPC) vesicles; (b) distearoylglycerophosphocholine (DSPC) vesicles; (c), (d) and (e), mixtures of separately formed DPPC and DSPC vesicles containing respectively 24, 50 or 75% DPPC vesicles; (f) co-sonicated vesicles containing DPPC and DSPC in an equimolar ratio; (g) mixture of equal amounts of DPPC, DSPC and mixed-DPPC/DSPC vesicles. The phase transitions of the pure DPPC or DSPC vesicles are clearly visible. The slope of the medium temperature range between 42 and 50°C is typical for lipid bilayer containing both types of lipids (mixed DPPC/DSPC vesicles as shown in curve f). All phase-transition curves were taken immediately after mixing of the different vesicle preparations. Note that the height of each transition step is a measure of the amount of lipid that melts at the corresponding phase-transition temperature.

separately from dipalmitoyl- (a) and distearoylglycerophosphocholine vesicles (b). If the two vesicles preparations were mixed and measured immediately we observed a two-step phase-transition curve with an equal step height for a 1:1 mixture (e.g., Fig. 1d). A change in the concentration ratio of the two vesicle preparations can be easily followed by an equivalent change in the step-height ratio obtained from the phase-transition curves as shown in Figs. 1(c) and 1(e). Each vesicle preparation exhibits its characteristic phase-transition temperature. In the intermediate range between 42 and 50°C the degree of polarization remains almost constant. Complete mixing of the two lipids obtained by co-sonication leads to one broad phase transition between 35 and 55°C (Fig. 1f). If a lipid transfer between two different vesicles takes place, we have to expect a mixed outer layer containing both lipids, as well as remaining pure fractions of each lipid component. Such a superposition is demonstrated in Fig. 1(g). The phase-transition curve of a mixture containing equal amounts of vesicles of pure dipalmitoylglycerophosphocholine, pure distearoylglycerophosphocholine and a cosonicated mixture of the two phospholipids is shown. We clearly observe the phase transition of the pure lipids and in an intermediate range between 42 and 55°C of the mixed-lipid layer. This measurement simulates a situation where one-third of the lipids in each vesicle preparation is exchanged. The step height of the mixed-lipid-layer transition with respect to the total height of the phase-transition curve can be taken as a measure of the relative amount of lipid in the mixed-lipid layer.

We used the fluorescence polarization measurements to determine the activity of exchange protein in the fractions collected after gel filtration during the purification described in the Materials and methods section.

Fig. 2(a) shows the separation of the transfer protein band after gel filtration monitored by the protein absorbance at $\lambda = 280\text{nm}$. The fractions were tested for exchange activity with distearoyl- and dipalmitoyl-glycerophosphocholine vesicles. Typical fluorescence polarization phase-transition curves are shown in Fig. 2(b) after addition of $50\mu\text{l}$ of the corresponding protein fraction and incubation at 42°C for 1 h. Fraction nos. 80–94 showed no exchange activity. The corresponding absorbance shown in Fig. 2(a) is due to impurities separated during gel filtration. An increased protein transfer activity was observed between fraction nos. 95 and 105, with maximum transfer activity (Fig. 2c) coinciding with the maximum absorbance measurements (Fig. 2a). These fractions were pooled and used to assay the lipid-transfer process. The step-height ratio H_m/H_t (see Fig. 2b) gives the relative amount of transferred lipids. This is demonstrated in

Fig. 2. Application of the fluorescence polarization assay

(a) Elution profile for exchange protein after Ultrogel filtration. Fraction volume was 10 ml; absorbance was measured at 280 nm. (b) Phase-transition curves obtained from mixtures of DPPC and DSPC vesicles after addition of 50 μ l of the fractions obtained by gel filtration. Fluorescence polarization measurements were used to determine the exchange activity. The step-height ratio H_m/H_i is a measure of the amount of lipid in the mixed layer, which in turn is a measure of the fraction of transferred lipid. (c) The protein exchange activity is given as the fraction of transferred lipid for different fractions obtained by Ultrogel filtration. The activity profile agrees exactly with the elution profile obtained by absorbance measurements.

Fig. 3. Dependence of lipid exchange on time and concentration of exchange protein

The relative amount of transferred lipid obtained from phase-transition curves is given (a) as function of incubation time (the protein concentration was

Fig. 3(a) as function of incubation time. The incubation temperature was 42°C, which is slightly above the phase-transition temperature of dipalmitoylglycerophosphocholine but below the phase-transition temperature of the distearoylglycerophosphocholine vesicles. The protein concentration was 13 μ g/ml of lipid dispersion. A relatively rapid transfer process, completed after about 30 min, is followed by a very slow component. Fig. 3(b) shows the protein concentration dependence of the transfer process at $T = 42^\circ\text{C}$ after an incubation time of 1 h. Saturation is reached at a protein concentration of 13 μ g/ml added to 1 ml of lipid dispersion containing 0.5 mg of lipid/ml.

Discussion

Fluorescence polarization measurements were used to determine the transfer activity of phosphatidylcholine transfer protein from phase-transition curves of phospholipid vesicles. The advantage of this method is that there is no necessity to

13 μ g/ml of total lipid dispersion; the incubation temperature was 42°C and (b) as a function of protein concentration. The incubation time was 60 min at 42°C.

separate the two different types of vesicles, which otherwise has to be done, for example, by an antigen-antibody reaction to separate antigen-sensitized liposomes (Ehnholm & Zilversmit, 1972) or by anion-exchange chromatography to separate liposomes containing negatively charged probes (Hellings *et al.*, 1974). Fluorescence polarization measurements were shown to be an easier way to determine the transfer activity, for example, during the purification of the protein. This method may be applied to study the mechanism of the transfer process. The limitation of our method for kinetic studies is the time required for a temperature scan in the phase-transition region. Thus kinetic processes faster than 7 min cannot be investigated by measuring the total transition curves. However, this disadvantage may be overcome by measuring the degree of polarization at a given temperature, where changes occur in the degree of polarization after addition of transfer protein. In our case, this is at the end of the dipalmitoylglycerophosphocholine phase transition (42°C) or at the onset of the distearoylglycerophosphocholine phase transition (50°C).

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