Lipid Phase Separation Induced by a Hydrophobic Protein in Phosphatidylserine–Phosphatidylcholine Vesicles†

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ABSTRACT: Differential scanning calorimetry (DSC) was used to detect phase separation induced by a hydrophobic myelin protein, lipophilin, in a mixture of phosphatidylserine (PS) and dipalmitoylphosphatidylcholine (DPPC). Preferential binding of PS to the boundary layer of lipophilin causes a decrease in the PS content of the remaining lamellar phase with a resultant shift in the phase-transition temperature to a higher temperature. The phase diagram for this mixture in the presence and absence of lipophilin is presented. From the phase diagram, it can be estimated that for an equimolar mixture of PS and DPPC, the boundary layer contains only PS, although for higher DPPC contents, some DPPC can also be found in the boundary layer. In the case where partial phase separation is induced in this mixture by Ca^{2+} alone, lipophilin increases the phase separation indicating that it also binds PS preferentially in the presence of Ca^{2+}. Preferential binding of two other acidic lipids, phosphatidic acid and phosphatidylglycerol, to the boundary layer was also found, including a mixture where the acidic lipid was the higher melting component in the mixture.

Biological membranes contain a variety of classes of lipids, each of which may have a special role. Segregation of lipids into clusters or domains may be induced and/or maintained by proteins, divalent cations, and localized changes in pH and may allow them to perform their specialized functions. Phase separation induced by Ca^{2+} has been observed in a mixture of phosphatidylserine and phosphatidylcholine, using spin-labels (Ohnishi and Ito, 1973, 1974; Ito et al., 1975) and by differential scanning calorimetry (Papahadjopoulos et al., 1974). Two studies making use of a spin-labeled lipid as one of the components showed that poly(L-lysine) (Galla and Sackmann, 1975) and cytochrome *c* (Birrell and Griffith, 1976) could induce phase separation. Both of these proteins, which are of the extrinsic type, probably exert their effects through electrostatic interactions with the lipid polar head groups.

Another way in which a protein could induce phase separation is through differential binding of lipid to the boundary layer of an intrinsic membrane protein. In this paper, we wish to present evidence obtained from differential scanning calorimetry (DSC)[1] for phase separation in a mixture of phosphatidylserine (PS) and dipalmitoylphosphatidylcholine (DPPC) induced by a hydrophobic protein, which is isolated from the proteolipid of human myelin (Gagnon et al., 1971) and recently referred to as lipophilin (Boggs et al., 1976; Moscarello, 1976). We recently reported (Boggs et al., 1976) that when this protein is incorporated into lipid vesicles labeled with a fatty acid spin label, the electron spin resonance spectrum possesses two components, one which is due to immobilized lipid and one which is characteristic of a lamellar phase. The immobilized lipid induced by lipophilin is probably in the form of boundary lipid (Jost et al., 1973) surrounding the protein with the lipid fatty acids immobilized by interactions with hydrophobic sites on the protein.

The boundary lipid does not take part in the gel to liquid crystalline phase transition since the immobilized component is still present when the lamellar phase has gone through the phase transition. Furthermore, lipophilin reduces the enthalpy of the phase transition, suggesting that less lipid participates, while the midpoint temperature of the phase transition is not altered (Papahadjopoulos et al., 1975), indicating that the protein has little effect on the remaining lamellar phase.

Although lipophilin interacts with lipids primarily through hydrophobic interactions, electrostatic interactions also seem to be important. Proteolipid from myelin is difficult to delipidate and the lipids which are most firmly bound are acidic lipids (Folch-Pi and Stoffyn, 1972) for which it has been reported to have a high affinity in monolayers (London et al., 1974).

Preferential binding of acidic lipids to the boundary layer of lipophilin will effectively change the composition of the lipid in the remaining lamellar phase. Since the boundary lipid does not take part in the phase transition, a change in composition of the remaining lipid is detectable by DSC as a shift in the phase-transition temperature. The composition of the remaining lamellar lipid can be estimated from the phase diagram for these lipids. Phase separation was also induced by Ca^{2+} to determine whether lipophilin binds preferentially to the Ca^{2+}–PS complex or to DPPC. Vesicles containing phosphatidic acid (PA) or phosphatidylglycerol (PG) with PC were also studied.

Materials and Methods

Preparation of Lipophilin. Myelin was isolated from normal human white matter by the method of Lowden et al. (1966). Lipophilin was extracted and purified by chromatography on Sephadex LH-20 in chloroform/methanol (1:1, v/v) containing 5% of 0.1 M HCl by the method of Gagnon et al. (1971) and stored in the lyophilized form.

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[1] Abbreviations used are: DSC, differential scanning calorimetry; PS, phosphatidylserine; DPPC, dipalmitoylphosphatidylcholine; PA, phosphatidic acid; PG, phosphatidylglycerol; PC, phosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DMPC, dimyristoylphosphatidylcholine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

Lipids. Phosphatidylserine (PS) was isolated from beef brain; phosphatidic acid (PA) and phosphatidylcholine (PC) were isolated from egg yolk, as described earlier (Papahadjopoulos and Miller, 1967). Dipalmitoylphosphatidylglycerol (DPPG) and dimyristoylphosphatidylcholine (DMPC) were synthesized as described earlier (Papahadjopoulos et al., 1973). These lipids were stored in chloroform under nitrogen in sealed ampules at −50 °C. Dipalmitoylphosphatidylcholine (DPPC) was purchased from Serdary Research Laboratory (London, Ontario) and was stored in the dry form under nitrogen. All lipids were chromatographically pure. [^{14}C]Dipalmitoylphosphatidylcholine was purchased from Applied Science Lab. Inc. with a specific activity of 25 mCi/mmol.

2-Chloroethanol was obtained from Coleman and Bell, redistilled periodically, and stored in the cold and in the dark. L-Histidine was purchased from Eastman Organic Chemicals and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) was obtained from Calbiochem.

Preparation of Vesicles. Vesicles were prepared by dialysis from 2-chloroethanol against buffer as described previously (Boggs et al., 1976) or by mixing in chloroform–methanol–water, evaporation, and dispersion of the dried material into buffer at 45 °C (Papahadjopoulos et al., 1975). In order to prepare the samples by dialysis, the chloroform solution of the PS was evaporated and redissolved in 100% 2-chloroethanol. The DPPC was also dissolved in 2-chloroethanol and an aliquot of [^{14}C]DPPC was added to give a specific activity of 2×10^5 dpm/mg. The two lipid solutions were combined in the desired ratio. Lipopilin was dissolved in 90% 2-chloroethanol at a concentration of 5 mg/mL and sonicated briefly with a probe sonicator. An aliquot of the solution of lipid in 2-chloroethanol (to give approximately 5 μmol of total lipid) was used to prepare pure lipid vesicles and an aliquot (2–5 μmol of lipid) was combined with the protein solution to prepare protein-containing vesicles. The 2-chloroethanol solutions were placed in dialysis tubing which had been boiled in an EDTA solution and dialyzed against 2 L of buffer containing NaCl (100 mM), L-histidine (2 mM), Hepes (2 mM), and EDTA (1 mM) or $CaCl_2$ (10 mM) adjusted to pH 7.4. Dialysis was carried out in a temperature-controlled bath at 45 °C for approximately 18 h with two changes of buffer.

The resulting suspensions were centrifuged at 40 000 rpm for 1 h at 4 °C in a SW 50.1 rotor. Initially, the pellet was purified by centrifugation on a discontinuous sucrose gradient to remove uncombined lipid and protein; however, the amounts of uncombined lipid and protein were negligible so this step was later dispensed with. Aliquots (50–100 μL) of the pellet suspended in buffer and the initial solution of lipids in 2-chloroethanol were evaporated in scintillation vials and counted for ^{14}C on a Nuclear-Chicago Mark I liquid scintillation system to obtain the DPPC content and similar aliquots were analyzed for total phospholipid content by measuring phosphate according to a modified Bartlett procedure (1959). Aliquots of the supernatant from the pellets were also analyzed for ^{14}C and total phosphate. PS content was obtained by subtracting DPPC content from total phospholipid. The two lipids were assumed to have the same mol wt, 760; thus, weight ratios are equal to molar ratios. Protein content was measured by amino acid analysis on a Technicon TSM amino acid analyzer. Serine and histidine content were corrected according to the known percentage of these two amino acids in lipophilin (Gagnon et al., 1971).

Differential Scanning Calorimetry. The temperature and heat content of the thermotropic transition of the phospholipids in vesicle preparations with and without protein was determined with a differential scanning calorimeter (Perkin-Elmer DSC-2) using a scanning rate of 10 °C/min. Vesicle pellets were taken up in micropipets (10–15 μL) and transferred to the sample pans. The reference pan contained buffer. The area of the peaks was measured, using an electronic planimeter from Numonics Corp. The transition temperature, T_m, is defined as the peak of the endothermic transition. The lower and upper temperatures, T_l and T_h, plotted in the phase diagram were obtained by extrapolating the linear part of the main component of the peak to the baseline from the heating curve.

TABLE I: Phosphatidylserine–Phosphatidylcholine Ratio of Vesicles with and without Lipophilin.

	PS:DPPC ratio before dialysis[a]	PS:DPPC ratio after dialysis[b]	
		Lipid vesicles	Lipid–protein vesicles
Set 1	53:47	53:47	54:46 ± 5.0%
Set 2	62:38	61:39 ± 1.7%	65:35 ± 0.8%

[a] Based on analysis of solution of lipids in 2-chloroethanol. [b] Values for each set are average of two samples dialyzed at the same time. Standard deviation of the two samples from the average is indicated. Sets 1 and 2 were prepared and dialyzed at different times.

Results

Lipophilin was incorporated into lipid vesicles using the dialysis procedure from 2-chloroethanol since this method results in nearly complete incorporation of protein. The lipid–protein vesicles are homogeneous as judged by both freeze fracture electron microscopy which shows the presence of intramembranous particles and centrifugation on a sucrose gradient which results in a single sharp band at a density corresponding to the amount of protein incorporated (Boggs et al., 1976). The amount of protein incorporated was similar in vesicles of all compositions of PS/DPPC and is slightly less than the initial amount, e.g., with 40% protein by weight in the initial solution, 34–37% protein was recovered in the vesicle pellet.

In the absence of Ca^{2+}, not all the lipid and protein sedimented; approximately 33% of the original starting material was left in the supernatant for the pure lipid sample and 5% for the lipid–protein sample. Some of the lipid and protein may also have been lost during the dialysis procedure. However, as shown in Table I, the ratio of PS to DPPC as determined from [^{14}C]DPPC and total phospholipid determined from total phosphate in the recovered lipid and lipid–protein pellets was similar to the ratio in the initial 2-chloroethanol solution of the lipids. The PS to DPPC ratio in vesicles containing protein was a little greater than in vesicles of lipid only.

The effect of lipophilin (34% by weight) on the phase transition temperature of PS/DPPC (30:70) is shown in Figure 1. The protein has no effect on the temperature of the endothermic transition of PS and, as reported earlier (Papahadjopoulos et al., 1975), has no effect on the midpoint of the transition in DPPC although it broadens it slightly (Table II). No transition was observed in the range 0–50 °C for lipophilin incorporated in egg phosphatidylcholine vesicles, showing that lipophilin itself does not absorb any significant amount of heat in this temperature range. In the 30:70 mixture (Figure 1B) a broad peak is observed with the midpoint at 28 °C, indicating that the lipids are incorporated into the same bilayer since otherwise two transitions corresponding to PS and DPPC (Figures 1A and 1D) would be observed. When lipophilin is

TABLE II: Phase Transition Temperatures (°C) of PS-DPPC Vesicles in the Presence and Absence of Lipophilin.

	Lipid			Lipid and protein		
PS:PC (w/w)	T_m[a]	T_l	T_h	T_m[a]	T_l	T_h
100:0	8.1	−1.0 ± 0.5	15 ± 1	7.8	−0.7 ± 0.5	14.3 ± 1
59:41	21.2	3.0 ± 1	32 ± 1	25.5	7.5 ± 1	35.0 ± 1
38:62	27.0	5.0 ± 1	38 ● 1	32.0	11.0 ± 1	40.7 ± 0.5
30:70	28.0	9.5 ± 1	40.5 ± 0.5	35.2	22.3 ± 1	41.2 ± 0.5
15:85	37.3	26.0 ± 1	44 ± 0.5	39.5	33.5 ± 1	43.0 ± 0.5
0:100	43.0	42.0 ± 0.2	44.5 ± 0.2	43.3	42.0 ± 0.2	44.8 ± 0.2

[a] Estimated error ± 0.2 °C.

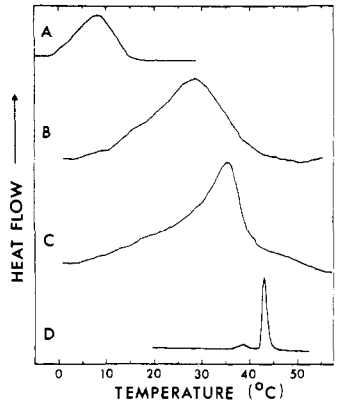

FIGURE 1: Differential scanning calorimetry thermograms for vesicles of (A) PS; (B) PS/DPPC, 30:70; (C) PS/DPPC, 30:70, + 34% lipophilin; and (D) DPPC, all in the presence of EDTA; heating rate, 10 °C/min. Different amounts of sample are present and different instrument sensitivities were used so the heat absorbed cannot be compared.

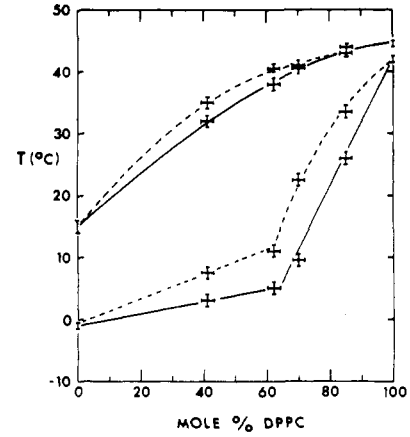

FIGURE 2: Phase diagram for mixtures of PS and DPPC without protein (—) and with 34% lipophilin (- - -), plotted as the width of the transition against mole percent DPPC. T_l and T_h are the temperatures corresponding to onset and completion of melting of the primary component. The error bars indicate the estimated errors in the composition and in measuring the temperatures by extrapolation. The behavior between 0 and 40 mol % DPPC and between 85 and 100 mol % DPPC was not determined and was extrapolated.

incorporated into the vesicles, it shifts the temperature, T_m, of the transition of the mixture from 28 to 35.2 °C, an increase of 7.2 °C, and results in a sharpening of the peak (Figure 1C). The temperature of the transition in the presence of lipophilin is characteristic of a lipid mixture of a lower PS/PC ratio. From Table II, it can be seen that lipophilin produces an upward shift in the transition temperature (T_m) as well as the temperatures corresponding to onset (T_l) and completion (T_h) of melting of the main component present for all mixtures of PS and DPPC. The transition temperatures and shifts produced by lipophilin were reproducible through at least three or four heating and cooling cycles, indicating reversibility of the transition. Similar shifts in temperature were obtained from the cooling curves.

The phase diagrams for PS/DPPC mixtures prepared by dialysis with and without 34% lipophilin obtained from T_l and T_h are shown in Figure 2. The solidus curve of the phase diagram for this mixture of lipids shows considerable deviation from linearity as was also reported for PC/phosphatidylethanolamine mixtures (Shimshick and McConnell, 1973; Chapman et al., 1974) indicating that homogeneous mixing of the lipids does not occur at high PS concentrations and there may be clusters of PS/DPPC of varying composition. However, complete phase separation does not occur or two peaks would be seen in the thermogram corresponding to the individual components as observed for mixtures of dioleoylphosphatidylcholine with saturated phosphatidylcholines (Phillips et al., 1970). Incorporation of lipophilin shifts the phase diagram upward, indicating that the lipid which takes part in the phase transition has a lower PS/DPPC ratio (greater mole percent DPPC) than that present in the entire membrane, according to the analysis of the PS/DPPC ratio recovered in the pellet.

The lipid-proten vesicles had a PS/DPPC ratio similar to or slightly greater than that in lipid vesicles (Table I) which would tend to lower the transition temperature rather than raise it so that the observed increase in transition temperature is clearly not due to decreased incorporation of PS into the vesicles in the presence of protein. Furthermore, samples which were prepared by dispersion of dried material into buffer had the advantage that all of the material could be put into the sample pans with no possibility of differential loss of one lipid. Lipophilin had a similar effect on the phase diagram of these samples.

These results can be rationalized by assuming that lipophilin binds PS preferentially to its boundary layer, thus removing it from participation in the phase transition and decreasing its concentration in the remaining lipid. Alternatively, if several components with varying PS/DPPC ratio are contributing to the endothermic peak, lipophilin binds preferentially to the component with the higher PS content and prevents it from absorbing heat so that only the higher melting components undergo the phase transition.

Lipophilin incorporated into vesicles containing equimolar amounts of egg phosphatidic acid (PA) and DPPC shifted the temperature upward by 1.5 °C. Furthermore, when incorporated into vesicles in which the acidic lipid was the higher

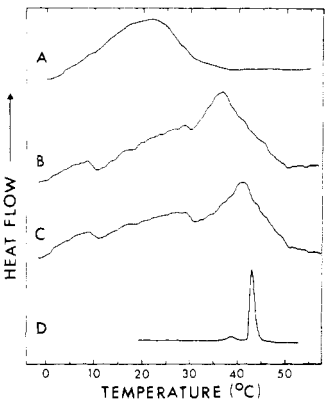

FIGURE 3: Differential scanning calorimetry thermograms for vesicles of (A) PS:DPPC, 59:41, in the presence of EDTA; (B) PS:DPPC, 59:41, in the presence of 10 mM Ca^{2+}; (C) PS:DPPC, 59:41, plus 23% lipophilin in the presence of 10 mM Ca^{2+}; and (D) DPPC; heating rate, 10 °C/min. The heat absorbed cannot be compared for different samples.

melting component, equimolar egg phosphatidylcholine (PC) or dimyristoylphosphatidylcholine (DMPC) with dipalmitoylphosphatidylglycerol (DPPG), lipophilin shifted the temperature downward by 1–2 °C. Thus, lipophilin also binds more PA or PG than PC to its boundary layer although the phase separation with these lipids is less than that observed with PS. The results with the egg PC–DPPG and DMPC–DPPG vesicles also demonstrate that lipophilin is not simply binding to the lipid which melts first nor preferentially to lipids with unsaturated fatty acids, but rather to lipids with acidic polar head groups.

Figure 3B shows the effect of Ca^{2+} (10 mM) on the phase transition of PS/DPPC (59:41) prepared by dialysis at 45 °C. Partial phase separation is obtained resulting in a shift of the main transition from 21 to 36 °C with a smaller broad peak at 11–30 °C and another small peak at 8 °C. These peaks may represent clusters of PS/DPPC–Ca^{2+} and/or PS–Ca^{2+}. Low concentrations of Ca^{2+} (0.5 mM) shift the transition temperature of PS to 18 °C while at the higher concentration of Ca^{2+} (10 mM) no transition would be seen in the temperature range used here (Jacobson and Papahadjopoulos, 1975). However, the presence of DPPC may alter this behavior so that the composition of the lipid domains contributing to the peaks seen in Figure 3B cannot be determined. Greater phase separation is obtained when dialysis is carried out at 4 °C (compared to 45 °C) resulting in only one moderately narrow peak at 41 °C.

Lipophilin can also be incorporated into PS/DPPC membranes following dialysis in the presence of Ca^{2+} (10 mM). With an initial concentration of 20% protein, 23% was recovered in the vesicles regardless of temperature of dialysis. Less than 2% of the original material remained in the supernatant after centrifugation—therefore, the PS/DPPC ratio must be similar in the vesicles in the presence and absence of protein.

The effect of 23% lipophilin and Ca^{2+} on the phase transition of PS/DPPC (59:41) dialyzed at 45 °C is shown in Figure 3C. Lipophilin causes a shift in the main peak of heat absorption from 36 to 41 °C indicating that in the presence of Ca^{2+} also, it decreases the PS/DPPC ratio of the bulk lipid which undergoes the phase transition. It is possible that lipophilin may bind to the lipid domains of high PS/DPPC ratio giving rise to the two lower melting peaks shown in Figure 3C. The resultant decrease in enthalpy would be very difficult to detect quantitatively. However, it appears very likely that lipophilin may also bind some PS from the domain normally giving rise to the peak at 36 °C (Figure 3B), thus decreasing the PS concentration of this phase and shifting the transition temperature upward.

The PS–Ca^{2+} complex probably has a rigid crystalline structure (Jacobson and Papahadjopoulos, 1975) so it was of interest to determine if lipophilin could bind to PS in the presence of Ca^{2+}. PS and lipophilin at an initial concentration of 30% protein by weight were dialyzed against buffer containing 10 mM Ca^{2+} at 45 °C. The material precipitated in the dialysis bag and upon centrifugation less than 1% of the original material was found in the supernatant. The pellet gave a single sharp band at the 40% sucrose level after centrifugation on a sucrose gradient. The pellet contained 21% lipophilin which is only a little less than that incorporated into mixtures of PS–DPPC with or without Ca^{2+}. Therefore, it is evident that lipophilin can bind to a PS–Ca^{2+} domain in vesicles formed of PS/DPPC, resulting in increased phase separation.

Discussion

We have previously shown that when lipophilin is incorporated into a lipid model membrane, two different lipid phases are found—one which consists of an immobilized layer surrounding the protein and another which is a lamellar phase similar to that in the absence of protein (Boggs et al., 1976). The present results show that if a mixture of two different lipid classes is present, lipophilin also induces lipid class separation by preferentially binding one of the lipids to its boundary layer. Preferential binding of PS to the boundary layer results in a change in composition of the remaining lamellar phase to one with a lower PS/DPPC ratio so that the mixture melts at a higher temperature. When partial phase separation has been induced by Ca^{2+}, lipophilin increases the phase separation by binding PS to its boundary layer.

An estimate of the decrease in PS/DPPC ratio in the bulk lipid undergoing the phase transition can be obtained from the phase diagram. Thus, in lipid vesicles of an initial composition of PS/DPPC 50:50 and using the lower set of curves, lipophilin shifts T_l from 4 to 9 °C. By extrapolating to the lipid curve to find the lipid composition which has a T_l of 9 °C it can be estimated that the composition of the bulk lipid which melts in the presence of lipophilin is 32:68 (68 mol % DPPC). Using the upper set of curves, the composition of the bulk phase is estimated at 39:61. Thus, lipophilin decreases the PS content of the bulk lipid undergoing a phase transition from 50 mol % to 39–32 mol %. A similar result is obtained if only the T_m values in the presence and absence of lipophilin are used.

It was earlier estimated from the decrease in enthalpy produced by lipophilin in DPPC and DPPG that the boundary layer contains approximately 15 lipid molecules per molecule of lipophilin assuming a mol wt of 25 000 (Papahadjopoulos et al., 1975). The number of molecules in the boundary layer should be similar for PS although this would be difficult to determine accurately from differences in enthalpy for such a broad peak. Thus, when the vesicles contain 34% lipophilin by weight, this corresponds to 63 molecules of lipid per molecule of protein, 15 of which are boundary lipid and 48 of which are lamellar phase. The ratio of PS to DPPC in the boundary layer of one molecule of lipophilin, PS_b/PC_b, can be obtained from

$$\frac{PS_b}{PC_b} = \frac{PS_i - PS_f}{PC_i - PC_f}$$

where the initial amount of lipid per molecule of lipophilin PS_i

+ PC_i = 63 and the final amount of lipid in the lamellar phase which melts $PS_f + PC_f$ = 48. The actual values of PS_i, PC_i, PS_f, and PC_f can be obtained from the initial ratio of the mixture and the final ratio from the phase diagram.

Thus, with an equimolar mixture of PS and DPPC, PS_i and PC_i are each 31.5 and using an average of the ratios obtained from the upper and lower curves of the phase diagram for the composition of the bulk lipid in the presence of lipophilin, 34:66, PS_f = 16.3 and PC_f = 31.7. Thus, $PS_b/PC_b \simeq 15/0$ and the boundary layer consists primarily if not entirely of PS. PC is incorporated into the boundary layer if only PC is present so that at lower PS/DPPC ratios, the boundary layer does contain some DPPC but the PS/DPPC ratio of the boundary layer is still higher than the starting ratio of the mixture due to preferential binding of PS.

The results indicate that lipophilin clearly has a preference for acidic lipids, PA and PG as well as PS, and does not merely bind to the lipid which melts first. It also causes phase separation when the acidic lipid is the higher melting component (e.g., PC/DPPG and DMPC/DPPG). A similar shift in temperature is also seen for the exothermic transition upon cooling, indicating that the lipid organization induced by lipophilin is maintained upon melting. Furthermore, the binding of lipophilin to DPPC in the gel state (Boggs et al., 1976) and to the rigid Ca^{2+} complex of PS is as great as to lipid in the liquid crystalline state. The intramembranous particles of lipophilin in DPPC seen by freeze fracture electron microscopy are still randomly distributed when the quenching is performed below the phase transition temperature (Papahadjopoulos et al., 1975) rather than aggregated as was observed for ATPase from sarcoplasmic reticulum (Kleemann and McConnell, 1976).

The preferential binding of PS to the boundary layer of lipophilin confirms earlier findings that myelin proteolipid binds more strongly to acidic lipids (London et al., 1974; Braun and Radin, 1969). However, the present results show that in addition, when lipophilin is confronted with a choice of an acidic lipid and a neutral lipid mixed randomly in the same bilayer, it also binds preferentially to the acidic lipid and removes it from the bulk lipid. Both hydrophobic and electrostatic interactions are required for this type of phase separation in which one class of lipid is bound preferentially to the boundary layer of a protein.

The purpose of this phase separation in myelin is not known although it could certainly fulfill a structural role and may contribute to the asymmetry of myelin seen by x-ray diffraction (Caspar and Kirschner, 1971). Preferential binding of certain lipid classes to the boundary layer of an intrinsic protein is one mechanism by which lipid phase separation and asymmetric distribution can be induced and/or maintained in a membrane.

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