Lipid Solvation of the Aqueous Form of the Myelin Proteolipid Apoprotein: Evidence and Characterization of Two Lipid Populations by Fluorescence Polarization, Differential Calorimetry, and Sucrose Gradient Centrifugation†

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ABSTRACT: The interaction between dipalmitoylphosphatidylcholine (DPPC) and the aqueous form of the myelin proteolipid apoprotein (PLA) has been investigated. Lyophilization was found to be an efficient and nonperturbing method for membrane reconstitution. Mixtures of different lipid/protein ratios were analyzed by means of differential calorimetry, fluorescence polarization, and sucrose gradient centrifugation. The presence of two coexisting lipid populations, termed "bulk" and "interacting" lipids, was demonstrated by these three techniques. By differential calorimetry, 23 DPPC molecules per molecule of protein (30 kDa) were shown to be excluded from the lipid phase transition. By fluorescence polarization, we detected above the phase-transition temperature a large perturbation of the lipid acyl chain dynamics induced by the aqueous form of PLA. Increasing the protein content above 35% by weight within the recombinants caused drastic changes in both ΔH values and the fluorescence anisotropy parameter, which could stem from protein aggregation.

Thirty years ago Folch & Lees (1951) first extracted from brain white matter a lipid–protein complex using chloroform–methanol (C–M) mixtures as solvent. They named this complex "proteolipid". Later, proteolipids—which have in common solubility in C–M mixtures—were found to be present in different membranes such as sarcoplasmic reticulum membranes, inner mitochondrial membranes, nerve ending membranes, and bacterial outer membranes (Folch-Pi & Stoffyn, 1972; Laggner & Barratt, 1975; Lees et al., 1979; Schlesinger, 1981). The protein moiety of the Folch–Lees proteolipid (PLA),[1] which accounts for almost half the total myelin proteins, displays the unusual property of being soluble in organic and aqueous solvents. Structural and conformational characteristics of both aqueous and organic forms of the isolated apoprotein have been largely explored (Sherman & Folch-Pi, 1970; Folch-Pi & Stoffyn, 1972; Nicot et al., 1973; Nguyen et al., 1976; Cockle et al., 1978, 1980; De Foresta et al., 1979; Lavialle et al., 1979; Stoffel et al., 1983; Vacher et al., 1984). Its structural characteristics—amino acid composition, primary structure, solubility in chloroform–methanol, and the presence of covalently linked fatty acids—designated it as an integral membrane protein (Tennebaum et al., 1966; Lees et al., 1979, 1983; Stoffyn & Folch-Pi, 1971; Stoffel et al., 1984). Freeze–fracture electron microscopy and fluorescence studies (Cockle et al., 1978; Boggs et al., 1982) supported this hypothesis, suggesting a quite important contribution of PLA to the architectural arrangement of the myelin membrane via hydrophobic lipid–protein interactions.

By use of model membranes composed of well-defined lipids, the consequence of PLA insertion has been studied by physical techniques such as electron spin resonance (Boggs et al., 1976; Silvius et al., 1983; Brophy et al., 1984), freeze–fracture electron microscopy (Papahadjopoulos & Moscarello, 1975; Boggs et al., 1977, 1982; Curatolo et al., 1978; Boggs & Moscarello, 1978), X-ray diffraction (Curatolo et al., 1977; Brady et al., 1979), Raman spectroscopy (Curatolo et al., 1978), nuclear magnetic resonance (Rice et al., 1979), circular dichroism (Cockle et al., 1980), and intrinsic fluorescence (Cockle et al., 1978). All these data describe perturbations induced on the lipid organization by the protein prepared in organic solvents (chloroform–methanol, 2:1 v/v,and 2-chloroethanol).

In the present work we have studied recombinants prepared with the aqueous form of the Folch-Pi apoprotein and dipalmitoylphosphatidylcholine (DPPC) multilayers. Although only a few groups have investigated reconstituted systems prepared with PLA in water (Braun & Radin, 1969; Cockle et al., 1978; Katona et al., 1978; Ting-Beall et al., 1979; Lavialle & Levin, 1980), this protein emerges as an attractive candidate for membrane reconstitution, taking into account recent results on the biosynthesis and transport of this protein to the myelin sheath (Colman et al., 1982; Nussbaum & Roussel, 1983). According to these authors, the proteolipid synthesized on the rough endoplasmic reticulum would be transported to the Golgi apparatus prior to be incorporated into the myelin sheath. Such a route does not exclude the possibility that the protein is transported through the endoplasmic reticulum *lumen*, i.e., through an aqueous environment.

Artificial membranes were prepared with DPPC. The main arguments led to this choice: (1) phosphatidylcholine (PC) represents an important fraction (10%) of the lipid content of the CNS myelin; (2) PC has been reported to be incorporated early into myelin membranes (Szuchet et al., 1983) and therefore is probably closely involved in myelin assembly; (3) the zwitterionic character of DPPC allows an approach of hydrophobic lipid–PLA interactions, which are expected to be of great importance in the myelin membrane due to the integral character of PLA mentioned above. Differential calorimetry and fluorescence techniques were used in this study to yield correlative information on the perturbations induced

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[1] Abbreviations: PLA, myelin proteolipid apoprotein; DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; CNS, central nervous system.

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on the lipid organization by the aqueous form of PLA. The first technique provides a direct measurement of the thermodynamic parameters of the phase transition (phase-transition temperature, phase-transition width, enthalpy change, and cooperativity of the transition) while fluorescence polarization probes lipid-chain organization below and above T_m. Both techniques simultaneously revealed in all recombinants the presence of two coexisting lipid populations, i.e., bulk and perturbed molecules. In order to determine if they were long lived or transient species, we isolated them on sucrose density gradients.

Materials and Methods

Material. Synthetic DPPC was purchased from Serdary (Canada). It was used without further purification after we controlled the absence of lysolecithine.

Protein Analysis. The proteolipid apoprotein (PLA) used throughout this work was prepared as previously described (Nicot et al., 1973). Briefly, the proteolipid was isolated from bovine white matter and delipidated by light petroleum precipitation. The pellet was resuspended with chloroform–methanol, 2:1 v/v, and then dialyzed against acidic and neutral chloroform–methanol, 2:1, mixtures until complete delipidation. At this step of the preparation the phosphorus content of the apoprotein was measured as described by Ames & Dubin (1960). All the preparations contained less than 0.04% by weight of phosphorus.

The chloroform–methanol, 2:1 v/v, extract of the apoprotein was transferred into aqueous solution as described by Sherman & Folch-Pi (1970). Protein concentration was determined spectrophotometrically with a Cary 118 apparatus and an absorption coefficient $A_{280}^{1\%} = 13.5$ (Nicot et al., 1973). The protein content of sucrose gradient fractions was detected by fluorescence intensity measurements on a Jobin-Yvon JY3D. Excitation and emission wavelengths were respectively 280 and 320 nm. Since the quantum yield of tryptophan in such a lipid environment was not determined, these fluorescence measurements were only used as a qualitative test for protein localization.

Lipid Detection. The presence of a small amount of sucrose after dialysis of the gradient elution fractions made unreliable the classical techniques reported for phosphorus content determination. Consequently, lipids were localized by turbidity measurements at 340 nm.

Reconstitution Procedure. DPPC multilamellar structures were prepared by hydration of DPPC powder at 45 °C for 2 h in double-distilled water purged with argon (final water proportion was 90% by weight). Aqueous solutions of PLA at 1.5 mg/mL were added to these preformed lipid multilayers to obtain the required lipid to protein molar ratios. These samples were bubbled with argon to prevent DPPC oxidation and immediately lyophilized. We called these recombinants "initial mixtures". The absence of protein molecules trapped in the water layers located between lipid bilayers was checked according to Bakouche & Gerlier (1980) by sonication of the mixtures at 0 °C. The multilamellar nature of the DPPC–PLA complexes isolated by sucrose gradient centrifugation was assumed from the density of these liposomes compared to that of DPPC–PLA vesicles obtained by injection of chloroform–methanol solutions of DPPC–PLA into water (F. Lavialle, unpublished results).

Hydration Procedure. For fluorescence polarization and sucrose gradient centrifugation experiments, we first added water 50% by weight. After 1 h at 45 °C, the sample concentration was adjusted. This two-step procedure led to homogeneous mixtures. For calorimetric experiments, sample hydration was performed directly in the microcalorimeter sample holders by weighing about 10 mg of initial mixtures and water 52% by weight. These holders were kept in an oven at 45 ± 1 °C for 4 h. Because the water concentration of all samples belongs to the invariants of the eutectoid reactions, $L_\alpha \rightleftharpoons P_{\beta'} + H_2O$ and $P_{\beta'} \rightleftharpoons L_{\beta'} + H_2O$ (Grabielle-madelmont & Perron, 1983), both fluorescence polarization and calorimetric results correspond to the same transitions, even though the water concentration was largely different in the two kinds of experiments.

Steady-State Fluorescence Measurements. They were performed with 1,6-diphenyl-1,3,5-hexatriene (DPH, Aldrich Chemical Co.) as probe. DPH stock solutions were 1 mM in tetrahydrofuran (THF, Merck). The probe was injected in hydrated lipid–protein mixtures and its incorporation considered as complete after incubation for 1 h at 45 °C; DPH:DPPC molar ratio was 1:1000. Measurements were carried out on a T-format SLM 8000 instrument. Excitation was set at 360 nm. Schott K418 filters were used at emission sides. The effect of depolarization due to the light scattering was negligible at the sample concentration used (0.25 mg/mL for DPPC, i.e., an OD at 450 nm < 0.15). The temperature was directly measured in the cuvette by a thermistor immersed in the suspension. The temperature was increased automatically at a rate of 10 °C/h from 25 to 55 °C in a water-circulating apparatus (Hüber HS 40). The anisotropy parameter $\bar{r}_s$ is defined by

$$\bar{r}_s = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)$$

where $I_\parallel$ and $I_\perp$ are respectively the fluorescence intensities parallel and perpendicular to the plane of polarization of the excitation beam.

The steady-state anisotropy $\bar{r}_s$ may be resolved into static and dynamic parts. A structural order parameter S is defined in the equation of Heyn (1979) as $S^2 = r_\infty/r_0$, where r_0 is the limiting anisotropy of the probe ($t \to 0$) and r_∞ the residual anisotropy at long time (relative to the lifetime of the excited state). From Pottel et al. (1983), Van Blitterwijk et al. (1981), and Heyn et al. (1981), it appears that in biomembranes with DPH as probes the order parameter and not "viscosity" can be reliably evaluated from steady-state fluorescence anisotropy. Since the order parameter describes the molecular packing of the lipids, our results will be discussed in terms of lipid packing and/or lipid dynamics. Lifetimes measurements of the excited state of the probe embedded in the recombinants were performed as previously described (Gallay et al., 1981) to control that the observed variations in $\bar{r}_s$ values actually reflected perturbations of the lipid acyl chain ordering and not variations of the probe characteristics.

Calorimetric Experiments. They were performed in a high-sensitivity Arion microcalorimeter of flux type (sensitivity 59.5 V/mW at room temperature and 58.7 V/mW at 40 °C). The apparatus was standardized for temperature and quantitative heat determinations as previously described (Grabielle-Madelmont & Perron, 1983). To obtain a thermodynamic equilibrium, samples were held at room temperature for 20 h. After each calorimetric scan, the water content was redetermined. The calorimetric runs were recorded at 0.1 °C/min. Repeat heating scans (three to five) were performed within less than 10 h. In this time interval, the $P_{\beta'} \rightleftharpoons L_\alpha$ transition temperature was found perfectly stable, and the transition width (ΔT_c) only varied from 2.2 to 2.5 °C (see text).

Transition temperatures (T_c) were taken at the onset of the transition. For ΔH determination, recordings were digitized with a graphic plotter WATANABE (resolution 0.1 mm for

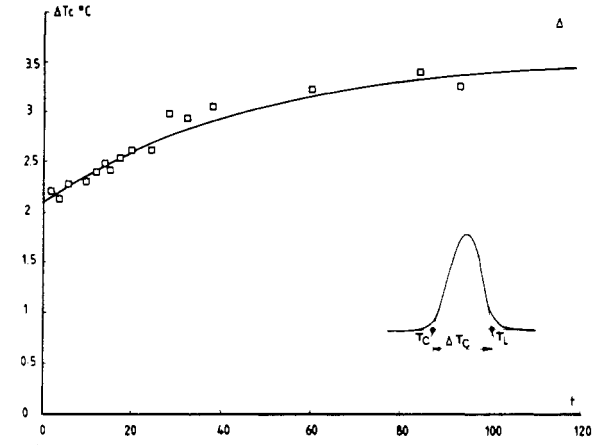

FIGURE 1: Effect of storage time (t) in hours at 45 °C (□) on the ΔT_c (°C) value (expressed as the difference between the onset and completion temperatures) for the 60:1 system. This t value was calculated by taking into account the time between repetitive scans during which the sample was at temperatures just above the main transition, i.e., in the L_α phase. (▲) Effect of storage time (t) at 55 °C. The mean values of ΔH and T_c (onset of the main transition) were respectively equal to 6.62 ± 0.25 kcal/mol of DPPC and 43.9 ± 0.3 °C.

0.01 °C) using a 0.1 °C step. The transition peak areas were calculated with a computer using the Simpson method. The exact fraction of melted lipid (α) was determined by taking into account, at each digitized point, the exponential decrease of the endothermic peak, which mainly arises from the calorimeter constant (Calvet & Camia, 1958). From the slope of the plot $\alpha = f(T)$, the cooperative unit size (U) was estimated as half the transition according to

$$U = [4RT_H^2(d\alpha/dt)_{TH}]/\Delta H_{DPPC}$$

where R is the universal gas constant, T_H the temperature at which $\alpha = {}^1/_2$, $(d\alpha/dt)_{TH}$ the tangential slope at T_H, and ΔH_{DPPC} the enthalpy change per mole of pure DPPC [an average value equal to 8610 cal mol^{-1} was used from Phillips et al. (1969), Hinz & Sturtevant (1972), Mabrey & Sturtevant (1976), Suurkuusk et al. (1976), and Albon & Sturtevant (1978)].

Sucrose Gradient Centrifugation. Discontinuous sucrose gradients were prepared from a stock solution of 50% sucrose (w/w) in double-distilled water. Dilutions were made to give 40, 20, 10, and 5% solutions. Respectively, 5, 12, 12, and 5 mL of these solutions were layered in cellulose nitrate tubes (36-mL capacity). Aliquots of 2–15 mg of the lipid–protein initial mixtures were applied to the top of the gradient to obtain less than 10 mg/band after centrifugation.

Centrifugation was performed on a L5 Beckman centrifuge using a SW 27 swinging-bucket rotor at 25 000 rpm for 15 h at 4 °C. Sucrose layers were collected from the bottom of the tube in 2-mL fractions. The eluted fractions containing the protein (peaks A) were identified as "isolated complexes" (A360, A160, A60). Continuous sucrose gradients were performed as described above, in a stock solution of 50% sucrose (w/w) to give 45–0% solutions. A total of 0.5 mL of each fraction equilibrated at 20 °C was weighed on a Sartorius electronic balance to determine the density.

RESULTS

Initial Mixture Stability. Stability of the initial mixture was first tested by studying the 60:1 (L:P molar ratio) mixture. The effect of the storage time at 45 °C on the enthalpy change and temperature of the main transition was analyzed. These two parameters did not significantly change in time up to 100 h at 45 °C. The main transition width (ΔT_c), calculated as shown on Figure 1, only varied from 2.2 to 2.5 °C after 10 h at 45 °C and reached asymptotically a value of 3.5 °C after 100 h.

Initial Mixture Characterization. Characteristic calorimetric scans and fluorescence polarization temperature profiles of DPPC and different DPPC–aqueous PLA initial mixtures are depicted in Figure 2. Analysis of the experimental data are reported on Figure 3.

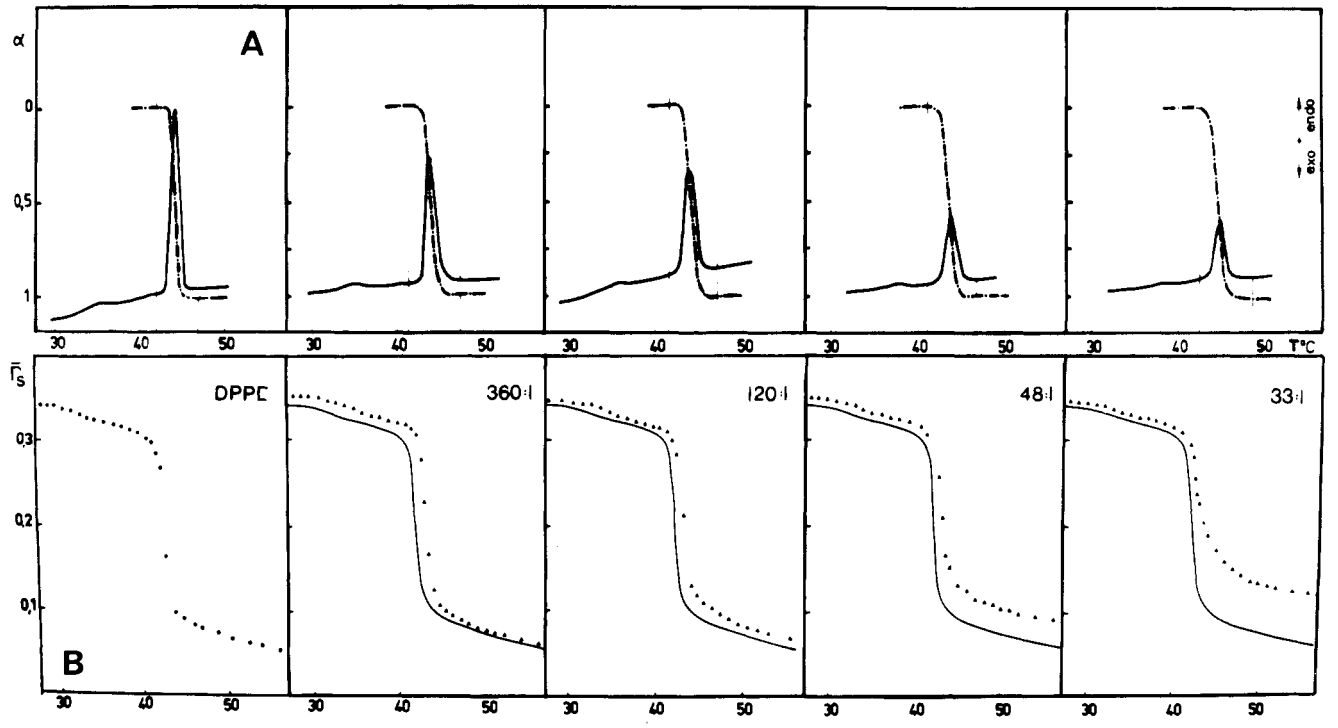

FIGURE 2: (A) (—) Representative calorimetric scans of the $L_{\beta'} \rightleftharpoons P_{\beta'}$ and $P_{\beta'} \rightleftharpoons L_\alpha$ transitions for pure DPPC multilayers and DPPC–PLA mixtures. The heating rate was 0.1 °C/min. (–·–) Melted fraction (α) vs. temperature. (B) (▲) Temperature dependence of the steady-state fluorescence anisotropy ($\bar{r}_s$) of DPH embedded in pure DPPC multilayers and DPPC–PLA mixtures. Solid lines refer to pure DPPC.

Table I: Main Transition Parameters Determined from Calorimetric Measurements[a]

	T_c (°C)	ΔT_c (°C)	ΔH (kcal/mol of DPPC)
DPPC	43.2 ± 0.17	1.79 ± 0.03	7.76 ± 0.12
360:1	43.3 ± 0.3	1.93 ± 0.09	7.30 ± 0.09
185:1	43.3 ± 0.23	2.11 ± 0.1	6.76 ± 0.21
120:1	43.1 ± 0.08	1.97 ± 0.14	6.36 ± 0.05
70:1	42.1 ± 0.15	2.83 ± 0.15	5.64 ± 0.34
60:1	43.9 ± 0.14	2.24 ± 0.07	6.29 ± 0.09
48:1	42.8 ± 0.16	2.32 ± 0.33	6.32 ± 0.29
33:1	43.6 ± 0.15	2.38 ± 0.53	6.80 ± 0.15

[a] T_c, temperature of the onset of the transition (cf. Figure 3A). These values are obtained with samples (DPPC and DPPC–PLA recombinants) first lyophilized and rehydrated with double-distilled water purged with argon. ΔT_c, transition width calculated as in Figure 1. ΔH enthalpy change of the $P_{\beta'} \rightleftarrows L_\alpha$ transition. Each result is the average of three to five measurements performed on the same sample.

(A) Pretransition $L_{\beta'} \rightleftharpoons P_{\beta'}$. By differential calorimetry, the pretransition of DPPC was clearly detected in all the reconstituted systems studied (Figure 2A). Fluorescence anisotropy experiments (Figure 2B) revealed near 36 °C a subtle perturbation in the slope of the curve $\bar{r}_s$ vs. temperature. This perturbation observed for DPPC and all recombinants was attributed to the $L_{\beta'} \rightleftharpoons P_{\beta'}$ transition. In the gel phase (below 30 °C), pure DPPC and all recombinants exhibited closely similar $\bar{r}_s$ values (Figure 2B).

(B) Main Transition $P_{\beta'} \rightleftharpoons L_\alpha$. As shown in Figure 2A, the line shapes of the traces obtained by calorimetry appear for all recombinants similar to that of pure DPPC. The values of the transition temperatures (T_c), transition widths (ΔT_c) and enthalpy change (ΔH) are reported in Table I. In the low protein concentration range (L:P molar ratios equal to 360:1, 185:1, and 120:1) the transition temperature (T_c) taken at the onset of the transition is found to vary less than 0.2 °C and within 1 °C at higher protein content. A slight transition broadening (ΔT_c) is observed for the 70:1, 60:1, 48:1, and 33:1 recombinants. The cooperative unit size (U) of the transition calculated as described under Materials and Methods was equal to 91 for pure DPPC and decreased to 66 after insertion of the first PLA molecules. Such an effect of protein on the phospholipid phase was reported by Correa-Freire (1979) and Chicken et al. (1984). This change was not altered by the protein content of the recombinants: a mean cooperative unit size equal to 62 was calculated for the 360:1 to 33:1 (L:P) recombinants.

As shown in Figure 4A, the enthalpy change ΔH, expressed as kilocalories per mole of DPPC present in the mixture, linearly decreases as the protein content reaches 32% by weight (i.e., P/L = 1.87). Above this PLA concentration, the ΔH value increases. On the basis of (a) the absence of variation of the line shape of the calorimetric curves (Figure 2A) and of the transition temperatures (Table I) and (b) the linear decrease in ΔH as a function of protein content, we assumed that the DPPC molecules present in the recombinants and participating to the transition had an enthalpy change per mole equivalent to that of pure DPPC. Therefore, the linear decrease in ΔH as a function of protein content corresponds to the subtraction of N molecules of DPPC from the $P_{\beta'} \rightleftarrows L_\alpha$ transition; these lipid molecules presumably form strong interactions with PLA. The value of N was calculated by taking into account only the ΔH values obtained for the mixtures with low protein content (P:L < 1:70). The variation of ΔH as a function of the molar ratio PLA:DPPC is given by (Correa-Freire et al., 1979)

$$\Delta H/\Delta H_0 = 1 - N(\text{PLA:DPPC})$$

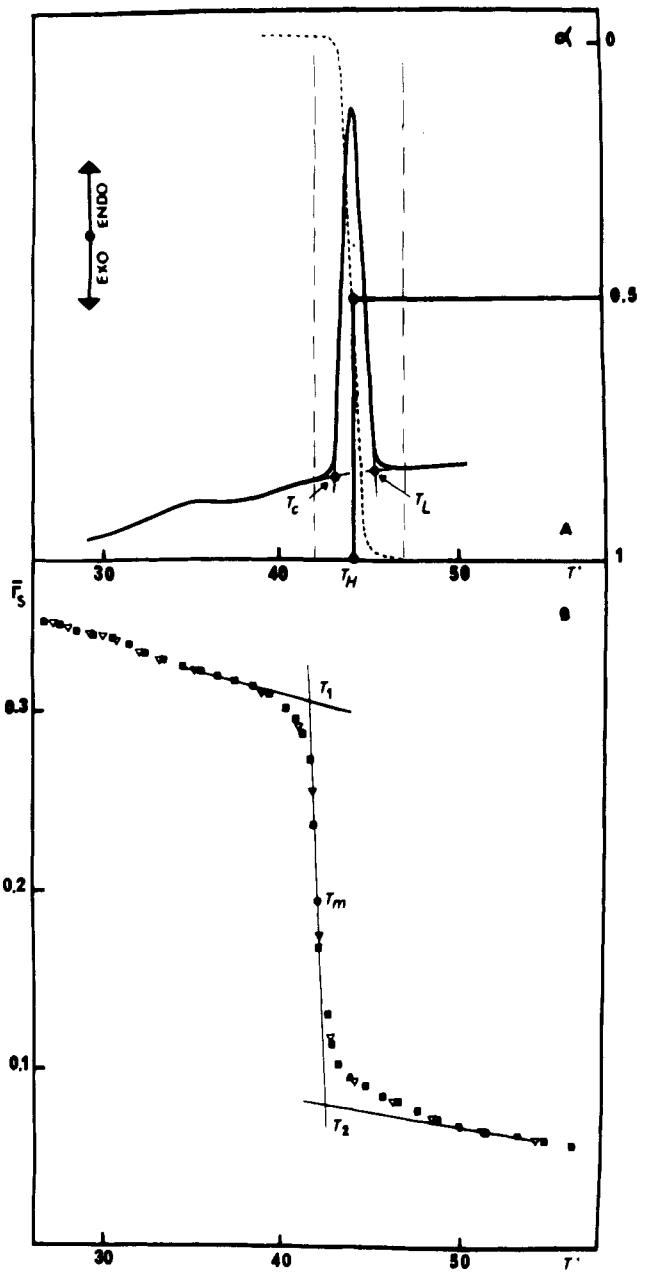

FIGURE 3: Determination of experimental parameters. (A) From calorimetric traces. (– –) Represents the melted fraction (α) for pure DPPC as a function of temperature. Vertical dashed lines indicate the integration limits within which the calculation was done. T_c, temperature of the onset of the transition; $\Delta T_c = T_L - T_c$, transition width; T_H, temperature at which half the melting process is completed ($\alpha = 0.5$); ΔH, peak area. (B) From fluorescence polarization temperature profiles: T_m, temperature at which the transition is half completed, i.e., for $\bar{r}_s = (\bar{r}_{T2} + \bar{r}_{T1})/2$; $\Delta T_m = T_2 - T_1$, transition width; (■) lyophilized DPPC; (▽) nonlyophilized DPPC.

in which ΔH is the enthalpy change obtained with the recombinants and expressed as kilocalories per mole of DPPC present in the mixture and ΔH_0 is the enthalpy change of pure DPPC. We used this equation to calculate the "best fit" linear regression of the experimental straight line. Extrapolation to zero gives the N value. It was found that 23 ± 1 DPPC molecules per PLA monomer (30 kDa) were subtracted for the transition. By means of fluorescence polarization, we observed on pure DPPC and all recombinants that the onset of the main transition (calculated as in Figure 3B) occurs around 42 °C. No significant perturbation of T_1 was detected as we increased the protein content of the DPPC–PLA mixtures. The midtransition temperature (T_m) is measured as

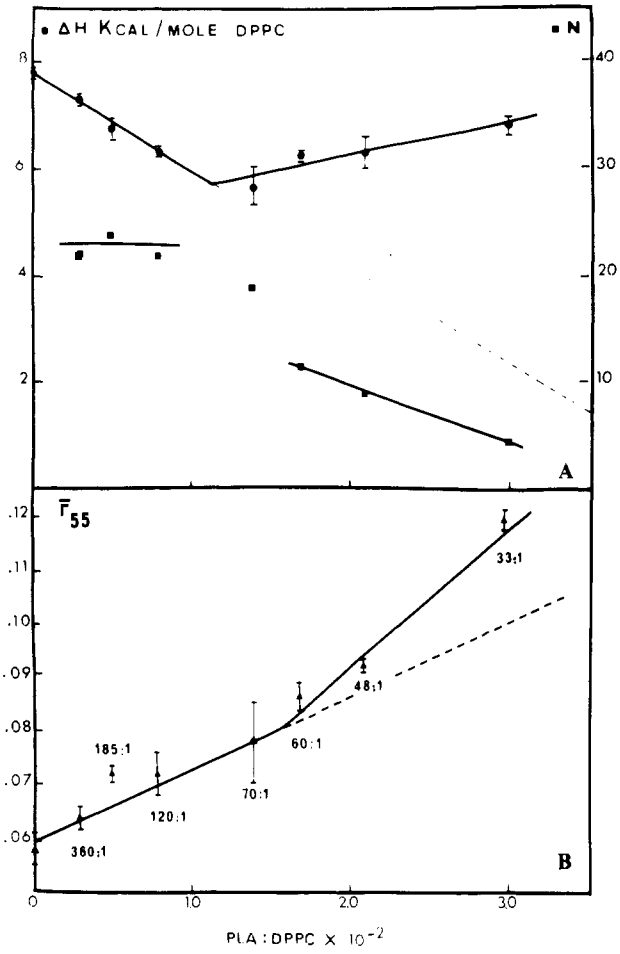

FIGURE 4: Effect of protein content (expressed as PLA:DPPC) of the recombinants. (A) On the enthalpy change ΔH (kcal/mol of DPPC) of the $P_{\beta'} \rightleftharpoons L_{\alpha}$ transition (●) and on the number (N) of DPPC molecules bound per PLA monomer (■). N was calculated from the $\Delta H_0 - \Delta H$ value, ΔH_0 and ΔH being respectively the enthalpy change obtained with pure DPPC and recombinants. (B) On the steady-state fluorescence anisotropy parameter $\bar{r}_s$ measured at 55 °C (▲). Plots were drawn from linear least-squares analysis.

shown in Figure 3B. From Table II it appears that insertion of aqueous PLA slightly increased the T_m value of pure DPPC. The shift is not amplified by increasing the amount of protein within DPPC multilayers. Analysis of Figure 2B reveals that below T_m the slope of the curve r_s vs. temperature is not modified by insertion of PLA in DPPC multilayers. In contrast, above T_m this slope decreases as the protein content in recombinants increases. The plot of $\bar{r}_s$ values measured at 55 °C ($\bar{r}_{55}$) vs. PLA:DPPC exhibits a break around 32% weight PLA (Figure 4B).

By using the equation proposed by Pottel et al. (1983)

$$S^2 = [(4/3)\bar{r}_s/0.384] - 0.28$$

An order parameter S can be calculated from these $\bar{r}_{55}$ values. The results obtained are reported in Table II.

Isolation and Characterization of DPPC–PLA Complexes (A360, A120, and A60). Figure 5a shows the sucrose gradient elution characteristics of pure DPPC multilayers and isolated aqueous PLA at 1.5 mg/mL. From turbidity measurements, pure DPPC multilayers appear to be located at the 20–10% sucrose boundary. Aqueous PLA is detected by means of fluorescence emission at the very top of the gradient. The elution profiles of the initial mixtures (360:1, 120:1, 60:1) are presented in Figure 5b–d. Fluorescence measurements evidenced that PLA is exclusively located in peaks A. Since no free protein was detected in elution profiles of all recombinants

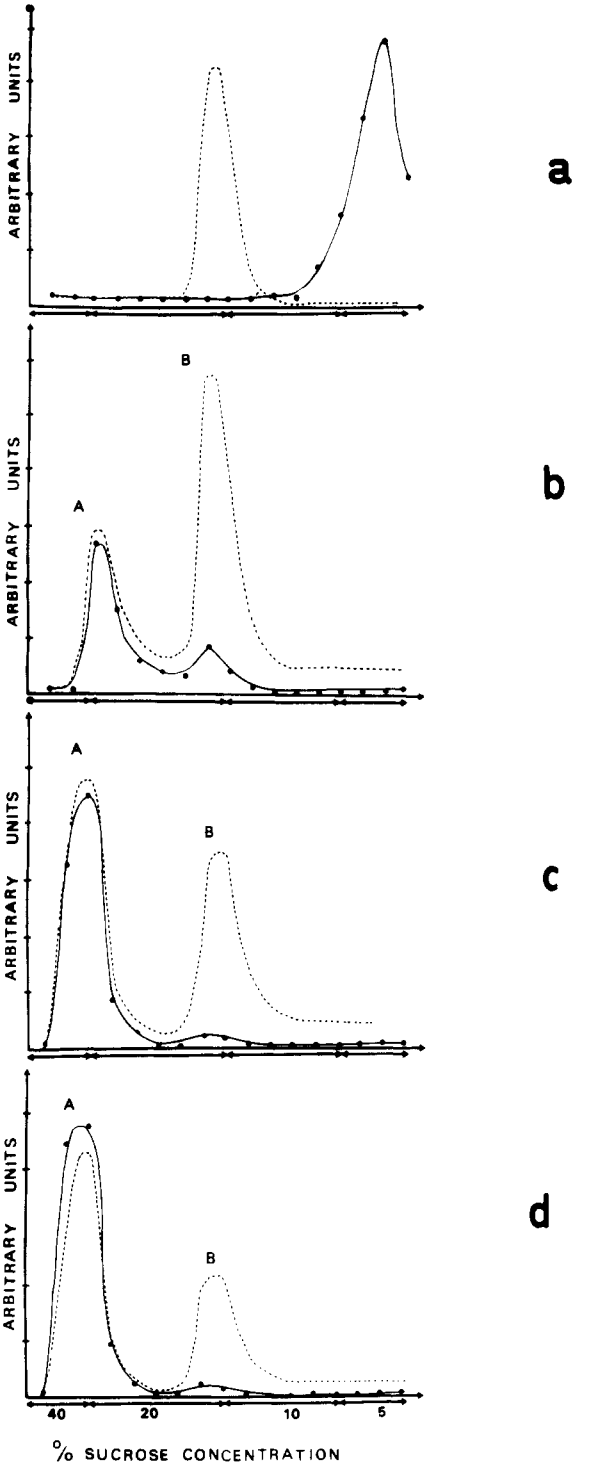

FIGURE 5: Discontinuous sucrose gradient elution profiles: (a) DPPC multilayers and aqueous PLA; (b) lyophilized DPPC–aqueous PLA, 360:1; (c) lyophilized DPPC–aqueous PLA, 120:1; (d) lyophilized DPPC–aqueous PLA, 60:1; (—) tryptophan fluorescence intensity; (– –) lipid turbidity. All recombinants were prepared by mixing aqueous PLA solution and preformed DPPC multilayers. The mixtures were immediately lyophilized. Rehydration was performed as under Materials and Methods. Centrifugations were performed in a SW 27 swinging-bucket rotor at 25 000 rpm for 15 h at 4 °C. The small departure from the base line observed under peaks B can be ascribed to light scattering due to lipid multilayers.

studied, we can conclude that 100% of the protein added to performed DPPC multilayers is incorporated into the artificial membranes. In all initial mixtures studied, the turbidity measurements revealed the presence of two lipid populations located at the 40–20% (peak A) and 20–10% (peak B) sucrose

Table II: Fluorescence Polarization Data[a]

	T_m (°C)	ΔT_m (°C)	$\bar{r}_{55}$	S_{55}
DPPC	41.9	2	0.058	*b*
360:1	43.2	1.6	0.064	*b*
185:1	43.2	2.4	0.072	*b*
120:1	43.2	2.2	0.072	*b*
70:1	43.2	2.2	0.078	*b*
60:1	43.2	2.2	0.086	0.137
48:1	42.8	2.2	0.092	0.199
33:1	43.0	2.7	0.11	0.365
A360	43.3	3.5	0.085	0.123
A120	44.0	5.7	0.103	0.279
A60	43.8	5.9	0.126	0.397

[a] T_m corresponds to the midtransition temperature (cf. Figure 3B). ΔT_m corresponds to the transition width (cf. Figure 3B). $\bar{r}_{55}$ is the anisotropy parameter at 55 °C, and S is the order parameter defined by Pottel (1983) as $S^2 = [(4/3)\bar{r}_{55}/0.384] - 0.28$. [b] $\bar{r}_s$ values lower than 0.08 correspond to a zero value for S (Berlin & Sainz, 1984).

boundaries. By comparison with the results reported in Figure 5a, turbidity and fluorescence measurements, peaks A and B were respectively identified as DPPC–PLA complexes (A360, A120, and A60) and free lipids. After dilution up to 0.25 mg/mL in lipids, the 360:1, 120:1, and 60:1 initial mixtures presented the same elution pattern with a fraction of free lipids, a fraction of lipids in interaction with PLA, and no free protein.

The isolated complexes A360, A120, and A60 (peak A) were further characterized by continuous gradient centrifugation and fluorescence polarization. The presence of a small amount of sucrose remaining in the samples even after extensive dialysis in addition to the sample concentration required for calorimetric measurements precludes the use of this technique for A360, A120, and A60 characterization. Figure 6A illustrates the gradient elution pattern of these three systems. They appear to be homogeneous and to have a density equal to 1.096, 1.116, and 1.129 g/cm^3 for A360, A120, and A60, respectively. At the concentration used for the preparation of the initial mixture, pure PLA solution has an apparent density equal to 1.010 g/cm^3 although DPPC multilayer's density is found to be equal to 1.064 g/cm^3 (Figure 6B). Fluorescence polarization characteristics of A360, A120, and A60 are depicted in Figure 7 and analyzed in Table II. Below the midtransition temperature (T_m), quite similar $\bar{r}_s$ values are obtained for pure DPPC, A360, A120, and A60. A shift in the transition temperature of DPPC is observed for all these "isolated complexes", and a large increase of the transition width can be evidenced. As observed for the DPPC–PLA initial mixtures (360:1, 120:1, 60:1), a drastic perturbation is observed in the liquid-crystal phase transition temperature range. We measured for the isolated complexes A360, A120, and A60 $\bar{r}_{55}$ values respectively equal to 0.085, 0.103, and 0.126 (Table II). These values are significantly higher than those measured for the corresponding initial mixtures (360:1, 120:1, 60:1). This is consistent with the fact that noninteracting lipids have been eliminated by sucrose density gradient. The order parameter S reaches now a value near 0.4 for A60, indicative of constraints on the probe orientation (cf. Discussion). Fluorescence polarization measurements performed on peaks B360, B120, and B60 exhibited the same lipid dynamics as pure DPPC: all temperature profiles perfectly matched.

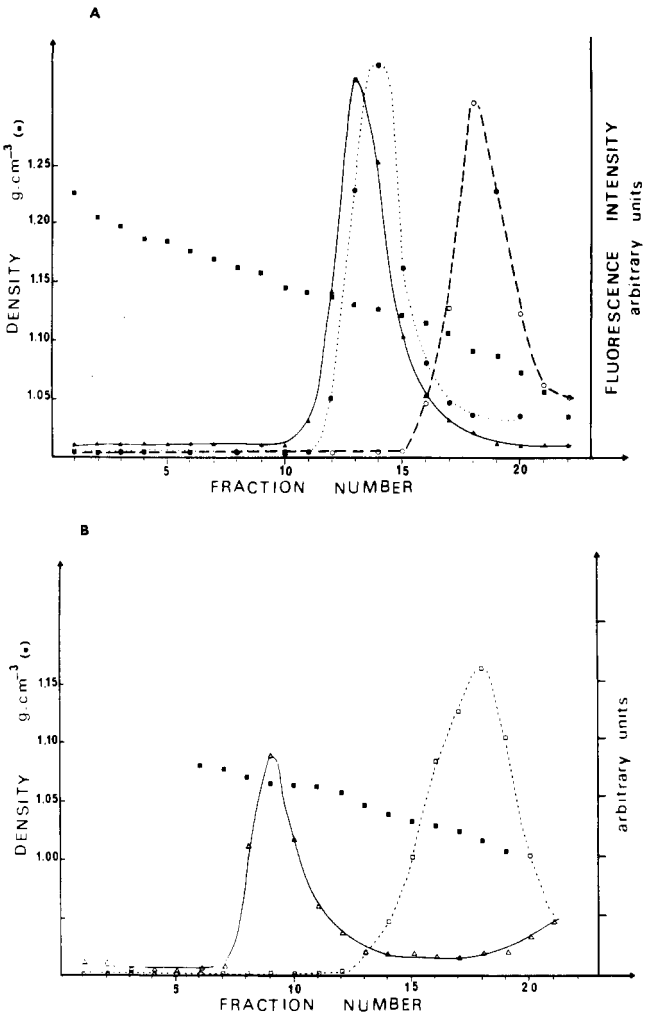

FIGURE 6: Continuous sucrose gradient elution profiles: (A) (○) A360, (●) A120, (▲) A60, and (■) density; (B) (△) lyophilized DPPC multilayers, (□) aqueous PLA, and (■) density.

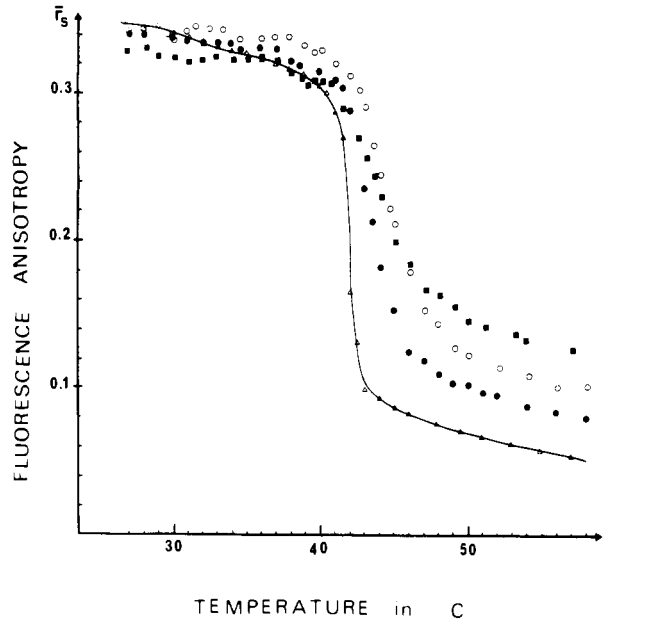

FIGURE 7: Steady-state fluorescence anisotropy parameter $\bar{r}_s$ of DPH in (△) lyophilized DPPC multilayers: (●) A360, (○) A120, and (■) A60.

DISCUSSION

Lyophilization as a Reconstitution Procedure. By lowering the water content of DPPC–aqueous PLA mixtures via lyophilization, we obtained the incorporation of 100% of the aqueous protein into neutral lipid multilayers as evidenced by sucrose gradient experiments. Several groups already published results on phosphadidylcholine (PC)–aqueous PLA mixtures, but Ting-Beall et al. (1979) observed formation of rather unstable recombinants with black lipid membranes,

Braun & Radin (1969) reported that after incubation at room temperature PC interacted with only a fraction of the total protein, and Papahadjopoulos et al. (1975) incorporated 25% of the aqueous solution of the protein in DPPC multilayers after 1 h of incubation at 45 °C. Lyophilization thus appears to be a quite efficient procedure for membrane reconstitution. We demonstrated by differential calorimetry (Figure 2A, Table II) and fluorescence polarization (Figure 3B) that lipid multilayers were unperturbed after lyophilization. We also observed that the protein embedded in artificial membranes by lyophilization and extracted with chloroform–methanol 2:1 (v/v) had the same UV characteristics as the original aqueous protein. Hence, lyophilization appears to be nonperturbing in agreement with the results published by Aquilar et al. (1982) on the Folch–Lees proteolipid and Crowe et al. (1981) on vesicles of sarcoplasmic reticulum. Finally, the observation that dilution from 30 to 0.25 mg/mL in lipids did not modify the lipid–protein assembly (sucrose density data) and the absence of perturbation of T_c and ΔH values after long storage time at 45 °C demonstrate the high stability of our lipid–protein recombinants. This is quite important since it allows reliable comparative studies by physical techniques such as spectrofluorometry and differential calorimetry, which require largely different sample concentrations.

Evidence and Characterization of Two Lipid Populations in DPPC–Aqueous PLA Initial Mixtures. (A) Evidence for Bulk Lipids. By means of fluorescence polarization and differential calorimetry, we detected the $L_{\beta'} \rightleftharpoons P_{\beta'}$ pretransition on recombinants prepared with the aqueous form of PLA. The observation of such a discrete rippled lipid phase is evidence for the presence in these mixtures of a population of unperturbed lipids we identified as bulk lipids.

This statement was supported by the results obtained by calorimetry, which showed only small perturbations, if any, of the thermodynamic parameters of the main transition: the transition temperature at which the $P_{\beta'} \rightleftharpoons L_\alpha$ transition occurs and the temperature range ΔT_c within which the transition is completed remain practically constant as PLA is incorporated in DPPC multilayers. Cooperative unit size calculations indicate that the protein introduces defects in the DPPC phase without phase disruption. By fluorescence polarization, the quite similar ordering detected in the gel phase for pure DPPC and all recombinants also suggests the presence of nonperturbed DPPC bilayers in the initial mixtures studied.

(B) Evidence for Interacting Lipids. From calorimetric measurements, a calculation of the number of lipids excluded from the transition (and therefore in a chain configuration different from those encountered in the $L_{\beta'}$, $P_{\beta'}$, and L_α phases) indicated that 23 ± 1 DPPC molecules were so strongly interacting with the aqueous form of PLA that they were prevented from participating in the transition. These results are in good agreement with those published on recombinants prepared with the organic form of PLA by different groups and estimated from calorimetric data (Boggs et al., 1982) and ESR experiments (Brophy et al., 1984). This suggests closely similar physicochemical properties for both the organic form of PLA and our aqueous preparations. However, the present results differ from those published by Curatolo et al. (1977), who reported that the DPPC molecules bound to the organic form of PLA induced a new transition.

Perturbed lipids were detected also by fluorescence polarization. By this means we demonstrated, above the main transition, a significant change in the lipid acyl chain dynamics induced by the insertion of PLA (Figures 2B and 7). For recombinants with protein content lower than 32%, $\bar{r}_{55}$ is lower than 0.08. According to Berlin and Sainz this suggests that the medium within which the probe rotates can be considered as isotropic. In contrast, for recombinants with protein content higher than 32%, $\bar{r}_{55}$ is >0.08, which reflects rotational constraints imposed on the probe.

Very similar temperature profiles were obtained by Heyn et al. (1981) and Kinosita et al. (1981) on bacteriorhodopsin–DMPC and cytochrome oxidase–DMPC mixtures. From time-resolved fluorescence measurements the latter authors showed that, in the liquid-crystalline phase, cytochrome oxidase manifests itself mainly in the form of reduction in the angular range of the wobbling motion. They suggest that insertion of rigid molecules would reduce the angular range of the wobbling motion although the wobbling diffusion constant would not significantly change as long as the major effect is rigid wall.

The direct demonstration of two lipid populations in the initial mixtures was obtained by sucrose gradient centrifugation. In these experiments, we isolated one DPPC population strongly interacting with the aqueous form of PLA and one population that behaved as pure DPPC (Figure 5). Caroll & Racker (1977) also observed on ficoll gradients that cytochrome oxidase was associated with only a small fraction of the phospholipid population. The observation by fluorescence polarization of a phase transition with A360, A120, and A60, which were expected to be composed of only interacting lipids, may indicate the presence of a new population of lipids undergoing a phase transition.

High Protein Content Recombinants (L:P < 70:1). By increasing the protein content within the recombinants we observed a drastic change in both the ΔH value and fluorescence parameters. Calorimetry revealed a "break" in the dependence of ΔH (expressed as kilocalories per mole of DPPC) on the protein concentration (Figure 4A). Above 32% by weight of PLA (i.e., L:P = 70:1), the ΔH value increased. This indicated that the mean number of DPPC molecules involved in the transition increases, i.e., that fewer DPPC molecules interact per PLA monomer. The hypothesis of a protein aggregation masking the lipid bonding sites would provide a coherent explanation for these results. This process occurs at an [interacting DPPC]:[bulk DPPC] ratio of about 1:2. The nonperturbation in this high protein concentration range of the thermodynamic parameters—T_c and cooperative unit size U—indicates that these lipids possess the thermotropic properties of DPPC bulk phase, in contrast with the results obtained by Chicken & Sharon (1984) on human erythrocyte–concanavalin A receptor–phospholipids recombinants. In this protein concentration range our calorimetric results significantly differ from those published by Boggs et al. (1982) on recombinants prepared with the organic form of PLA. These authors reported that the pretransition was suppressed and the main transition broadened although no drastic change in ΔH values was obtained. Fluorescence polarization data also give evidence of a change in lipid–protein interactions in recombinants with high PLA content (Figure 4B). From the order parameter reported in Table II, this change would occur in mixtures with L:P < 60:1; the $\bar{r}_{55}$ values indicate that constraints imposed by the lipids on the probe motion are now present. These constraints can be explained by the presence of larger patches of aggregated protein in the lipid bilayers. Taking into account the calorimetric data indicating an increase in the number of bulk lipids, the fluorescence data averaged over the global repartition of the probe reflect very strong perturbation of the lipids surrounding these protein patches. Since all recombinants (360:1 to 33:1) were prepared

with protein samples at 1.5 mg/mL, the oligomerization process that might be proposed to explain these results would occur during the lyophilization step: at low L:P molar ratios, as the water molecules are eliminated, the aqueous form of PLA would mimic the hydrophobic interactions provided in the 360:1 to 70:1 recombinants by the surrounding DPPC molecules. This process may involve the fatty acids linked to the protein and would lead to PLA aggregation. Comparable results were reported by Silvius et al. (1983) from ESR experiments on phospholipid–lipophilin systems and by Akhrem et al. (1982) from DSC data on lipid–cytochrome P-450 mixtures.

It is worth noting that we obtained quite coherent sets of results by fluorescence polarization and calorimetry. Detection of midtransition temperature shifts in the presence of PLA only by fluorescence means probably arises from the nature of the events probed by both techniques. It should be remembered that calorimetry permits measurements of thermodynamic changes related to the lipid phase transitions ($L_{\beta'} \rightleftharpoons P_{\beta'}$ and $P_{\beta'} \rightleftharpoons L_{\alpha}$) although fluorescence measurements follow the rotational dynamics of the probe in the gel and crystalline states of the lipid layer. Berlin & Sainz (1984) recently observed that the midtransition temperature of tricylglycerol measured by fluorescence polarization was significantly higher than that obtained by differential scanning calorimetry. A reduced mobility of DPH in these systems was suggested to explain the higher temperature required for complete freedom of the probe.

Conclusions

In the present work we demonstrated that under some specific experimental conditions (low water content) 100% of the aqueous form of PLA was incorporated in lipid bilayers. This indicates—in agreement with previous data (Lavialle et al., 1979)—that the Folch-Pi apoprotein exhibits a conformational adaptability to its surrounding solvent medium. Therefore, it could be suggested that in the cell this protein could migrate from the endoplasmic reticulum lumen to the plasmic membrane under the so-called aqueous form. The existence of stable interacting lipids around PLA demonstrated by differential calorimetry, fluorescence polarization, and sucrose density gradients suggests a structural role of this protein in the myelin membrane.

Registry No. DPPC, 2644-64-6.

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Advantages and Limitations of 1-Palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine as a Fluorescent Membrane Probe†

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ABSTRACT: We have investigated the behavior of 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine (DPHpPC) in synthetic, multilamellar phosphatidylcholine vesicles. This fluorescent phospholipid has photophysical properties similar to its parent fluorophore, diphenylhexatriene (DPH). DPHpPC preferentially partitioned into fluid phase lipid ($K_{f/s}$ = 3.3) and reported a lower phase transition temperature as detected by fluorescence anisotropy than that observed by differential scanning calorimetry. Calorimetric measurements of the bilayer phase transition in samples having different phospholipid to probe ratios demonstrated very slight changes in membrane phase transition temperature (0.1–0.2 °C) and showed no measurable change in transition width. Nonetheless, measurements of probe fluorescence properties suggested that DPHpPC disrupts its local environment in the membrane and may even induce perturbed probe-rich local domains below the phospholipid phase transition. Temperature profiles of steady-state fluorescence anisotropy, limiting anisotropy, differential tangent, and rotational rate were similar to those of DPH below the main lipid phase transition but indicated more restricted rotational motion above the lipid phase transition temperature. As for DPH, the fluorescence decay of DPHpPC could be described by either a single or double exponential both above and below the DPPC phase transition. The choice seemed dependent on the treatment of the sample. The intensity-weighted average lifetime of DPHpPC was roughly 1.5 ns shorter than that of DPH. In summary, the measured properties of DPHpPC and its lipid-like structure make it a powerful probe of membrane structure and dynamics.

For years, fluorescent probes have provided insight into the structure and dynamics of biomembranes. The ideal probe (1) is sensitive to the motions of individual molecules on the nanosecond timescale of fluorescence measurements, (2) has a high extinction coefficient and quantum yield, (3) has broad absorption and emission limits, (4) does little to interfere with the natural packing arrangement of the bilayer, and (5) has a fixed orientation in the bilayer. These characteristics enable the user to (1) obtain high fluorescence intensity with minimal use of probe, (2) to work over a wide range of wavelengths, (3) to avoid measuring artifacts in bilayer behavior induced by bulky probe molecules, and (4) to define the environment surrounding the probe.

Diphenylhexatriene (DPH)[1] has many attributes of an ideal probe and has been successfully used to study lipid bilayer

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[1] Abbreviations: DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; DSPC, 1,2-distearoyl-3-*sn*-phosphatidylcholine; DPHpPC, 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine; DPH, 1,6-diphenyl-*trans*-1,3,5-hexatriene; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene; DPHePC, phosphatidylcholine derived from egg yolk lysophosphatidylcholine with propionyl-DPH esterified to the 2-position of glycerol; POPOP, 2,2′-(*p*-phenylene)bis(5-phenyloxazole); LMV, large, multilamellar vesicle(s).

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