Interaction of Myelin Basic Protein with Dipalmitoylphosphatidylglycerol: Dependence on the Lipid Phase and Investigation of a Metastable State†

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ABSTRACT: The basic protein of myelin binds electrostatically to acidic lipids but has several hydrophobic segments which are believed to intercalate into the lipid bilayer. Differential scanning calorimetry (DSC) and fatty acid spin-labels were used to investigate the dependence of this intercalation on the phase state of dipalmitoylphosphatidylglycerol. After interaction with the lipid in the liquid-crystalline phase, basic protein decreased the phase transition temperature but had a much greater effect on cooling scans than on heating scans, if heating was performed from a low temperature. If the sample had been supercooled, an exothermic transition also occurred in the heating scans, suggesting that the phase formed on supercooling is metastable. Incubation at the temperature of the exothermic transition for a short time resulted in conversion of the sample to a state which melted with a temperature and enthalpy only slightly less than those of the pure lipid. This could also be achieved by prolonged storage of the sample at a low temperature. Below the phase transition, the protein had a pronounced immobilizing effect on a spin-labeled fatty acid with the nitroxide moiety located near the terminal methyl. Supercooling, prolonged cooling, and incubation at the temperature of the exothermic transition for a few minutes all increased the degree of this immobilization, which was greater than that produced by polylysine or divalent cations. The DSC results can be explained by a mechanism in which the hydrophobic segments of the protein are squeezed out of the bilayer as the lipid fatty acid chains refreeze during the exothermic process. The protein would then interact primarily electrostatically in the state which melts with a T_c and ΔH similar to those of the pure lipid. However, this mechanism does not explain the high degree of immobilization of the fatty acid spin-labeled near the terminal methyl. It is suggested that this immobilization may be due to interdigitation of the acyl chains in the lipid–protein complex. This would compensate for the lipid expansion due to intercalation of the protein part way into the bilayer and stabilize the complex, particularly below the phase transition temperature.

The interaction of myelin basic protein with phosphatidylglycerol has been found to depend on the phase state of the lipid and on the sample history. This protein contains hydrophobic segments which are believed to intercalate into the hydrocarbon region of the lipid bilayer (Gould & London, 1972; London & Vossenberg, 1973; London et al., 1973; Demel et al., 1973; Papahadjopoulos et al., 1975). This was subsequently found to be true primarily for lipids which do not interact intermolecularly by hydrogen bonding, such as phosphatidylglycerol, or whose hydrogen-bonding groups are involved in electrostatic binding to positively charged residues on the protein, such as phosphatidic acid (Boggs & Moscarello, 1978a; Boggs et al., 1980, 1981; Stollery et al., 1980).

In the case of phosphatidic acid (dimristoyl- and dipalmitoyl-), the effect of this intercalation on the lipd organization is predictable. It results in a decrease in the enthalpy and temperature of the phase transition and a decrease in the amplitude of motion of fatty acid spin-labels close to the polar head-group region but not in the interior of the bilayer (Boggs & Moscarello, 1978a; Boggs et al., 1980, 1981). In contrast, proteins such as polylysine, which only interact electrostatically with lipids, increase the temperature and enthalpy of the lipid phase transition and decrease the amplitude of motion of fatty acid spin-labels both near the polar head-group region and in the interior of the bilayer (Papahadjopoulos et al., 1975; Galla & Sackmann, 1975; Boggs et al., 1981).

However, in phosphatidylglycerol, the interaction is much more complicated and depends on the phase state of the lipid. Results reported earlier indicated that it was necessary for the protein to interact with liquid-crystalline phase lipid in order to subsequently decrease the temperature of the gel to liquid-crystalline phase transition, suggesting that intercalation into the gel phase did not occur to a significant extent (Papahadjopoulos et al., 1975). After interaction with the liquid-crystalline phase, the protein had as great an effect on the liquid-crystalline to gel phase transition of phosphatidylglycerol (determined from cooling scans) as of phosphatidic acid (from heating or cooling scans). However, on reheating, the effect on the gel to liquid-crystalline transition was greatly decreased, and an exothermic transition was seen under some conditions, indicating the formation of a metastable state on refreezing of the sample (Boggs & Moscarello, 1978a; Boggs et al., 1980, 1981).

Furthermore, basic protein was found to decrease the amplitude of motion of a fatty acid spin-label in the interior of the bilayer in the liquid-crystalline state of phosphatidylglycerol much more than would be expected for a protein which intercalates only partway into the hydrocarbon region. Indeed, its effect was comparable with that of polylysine in contrast to results with phosphatidic acid (Boggs et al., 1981). In the gel state of dipalmitoylphosphatidylglycerol (DPPG),[1] basic protein was found to have an even greater effect on fatty acid motion in the interior of the bilayer than in the liquid-crystalline phase. It had a pronounced immobilizing effect on the fatty acid spin-label while polylysine and divalent cations had a much smaller effect (Boggs et al., 1981). The phase behavior

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[1] Abbreviations used: ESR, electron spin resonance; DSC, differential scanning calorimetry; DPPG, dipalmitoylphosphatidylglycerol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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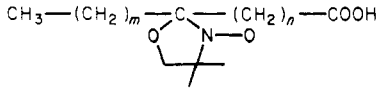
of the complex of basic protein and dipalmitoylphosphatidylglycerol is investigated further in the present study by DSC and ESR to understand the phases which occur above and below the phase transition after various treatments.

Materials and Methods

The fatty acid spin-labels 5-S-SL [I(12,3)], 12-S-SL [I-(5,10)], and 16-S-SL [I(1,14)] were purchased from Syva (Palo Alto, CA).



I(m,n)

Dipalmitoylphosphatidylglycerol was purchased from Supelco (Bellefonte, PA) and was chromatographically pure. It was stored in the dry form at −20 °C. *N*-2-(Hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (Hepes) was obtained from Calibochem.

Basic protein was extracted from isolated human myelin by the method of Lowden et al. (1966) and was stored in the lyophilized form.

Preparation of Lipid–Protein Vesicles. Vesicles were prepared by dispersing the dry lipid (2 mg/0.5 mL) by vortex shaking in aqueous Hepes buffer (10 mM) containing 10 mM NaCl and 1 mM EDTA at pH 7.0 at 45 °C. The protein solution (5 mg/1 mL of buffer) was then added to give the desired concentration, and the sample was dispersed again at 45 °C for 10 min. For ESR, the spin-label and lipid were dissolved together in chloroform–methanol (2:1) at a molar ratio of lipid to spin-label of 200:1. The solvent was evaporated under nitrogen, and the vesicles were prepared as described above. the resulting suspension was sedimented by centrifugation at 13000g at room temperature for 5 min in an Eppendorf microcentrifuge, and the pellets were used for calorimetry or ESR spectroscopy. The lipid/protein ratio of the samples was determined by phosphorus analysis (Bartlett, 1959) and amino acid analysis on a Durrum D-500 amino acid analyzer, after hydrolysis with 5.7 N HCl for 19 h at 110 °C.

Cycling of the samples through a low temperature was necessary for the observed changes in the DSC scan and ESR spectra. The lowest temperature used in the ESR experiments was 9 °C, and this was sufficiently low. In the DSC measurements, the samples were cooled to −3 °C (270 K) (the water did not freeze because of the salts present), but it was not necessary to use such a low temperature. For incubation of the samples at a low temperature overnight, they were removed to a refrigerator at 4 °C.

Differential Scanning Calorimetry. Samples were run on a Perkin-Elmer DSC-2 at heating or cooling rates of 2.5–10 °C/min. The midpoint of each peak was defined as the phase transition temperature (T_c). Enthalpy determinations were made as described earlier (Boggs & Moscarello, 1978b).

Electron Spin Resonance Measurements. The motional parameter, τ_0, or order parameter, S, of the fatty acid spin-labels was measured as described earlier (Boggs & Moscarello, 1978a) from the expressions

$$\tau_0 = (6.5 \times 10^{-10}) W_0[(h_0/h_{-1})^{1/2} - 1]$$

$$S = \frac{T_\parallel - T_\perp}{T_{zz} - T_{xx}}$$

where W_0 is the width of the center line, h_0 and h_{-1} are the heights of the center and high-field lines, respectively, and $T_\parallel$ and $T_\perp$ are the experimentally determined outer and inner hyperfine splittings. In some cases, $T_\parallel$ is also used as a measure of the amplitude of motion of the molecular long axis about the average orientation of the fatty acid chains in the lipid bilayer. Spectra were measured on a Varian E-104 spectrometer with a Varian temperature control accessory. The microwave power used was 10 mW.

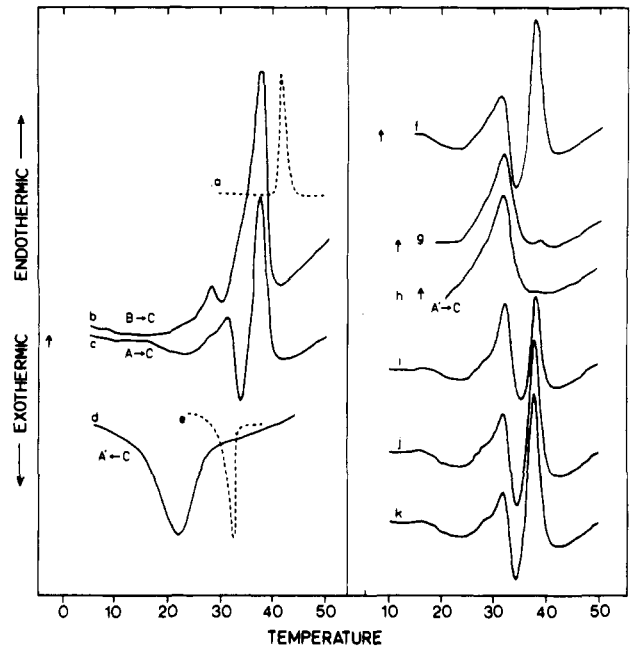


FIGURE 1: Differential scanning calorimetric thermograms of dipalmitoylphosphatidylglycerol alone [dashed lines (a) and (e)] and complexed with 52.4 wt % basic protein (solid lines). (d) and (e) are cooling scans while all others are heating scans. (b) First heating scan of sample stored at 4 °C overnight, heated from −3 °C; (c) second heating scan, heated from −3 °C; (f) reheating scan, heated from 7 °C; (g) reheating scan, heated from 12 °C; (h) reheating scan, heated from 17 °C; (i) reheating scan after storage for negligible time at −3 °C; (j) reheating scan after storage for 5 min at −3 °C; (k) reheating scan after storage for 2 h at −3 °C. Heating and cooling rates are 10 °C/min. The arrows (↑) indicate the temperature to which the sample was cooled and from which it was heated. Phase states above and below transitions are indicated as described in the text.

Results

The effect of basic protein on the phase transition of DPPG is greater on the second and repeated heating scans than on the first heating scan after preparation of the vesicles or after storage at 4 °C overnight. It has an even greater effect on cooling scans than on heating scans. DSC scans for a sample containing 52.4 wt % basic protein after varying treatments are shown in Figure 1. On the first heating scan (Figure 1b), after the sample was stored at 4 °C overnight the main transition is only 3 °C below the T_c of the pure lipid (Figure 1a), and a much smaller peak at 27.9 °C is present. The first heating scan after preparation of the vesicles at 45 °C and storage at room temperature for 1–2 h is similar to that in Figure 1b except that the lower melting peak at 27.9 °C is absent. Storage at 4 °C overnight does not always abolish this peak. The enthalpy of the first heating scan of freshly prepared samples or those stored at 4 °C overnight is only slightly less than that of the pure lipid (Table I). When the sample is immediately reheated from −3 °C, the main transition decreases in intensity (Figure 1c). A lower melting endothermic peak at 31.3 °C and an exothermic transition at 34 °C are present. Repeated heating scans are similar to Figure 1c. The combined enthalpy of both endothermic peaks is about half that of the pure lipid. On cooling (Figure 1d), a single broad transition occurs 10.5 °C below the T_c of the pure lipid (Figure

Table 1: Enthalpy of DPPG Alone and Complexed with Basic Protein after Varying Treatments

	ΔH^a (kcal/mol)
DPPG (distilled water)	8.6 ± 0.8
DPPG (buffer)[b]	8.3 ± 0.7
DPPG-basic protein	
first heating scan after preparation	7.4 ± 0.4
second heating scan from −3 °C	4.4 ± 0.6[c]
heating scan from 17 °C	4.0 ± 0.3
cooling scan	5.5 ± 0.3
heating scan from −3 °C after incubation at 32 °C for 15 min	7.7 ± 0.5
first heating scan after storage at 4 °C overnight	7.9 ± 0.8

[a] Values for at least four different samples are averaged. [b] 10 mM NaCl, 10 mM Hepes, and 0.1 mM EDTA. pH 7.0. [c] Both endothermic peaks.

1e) with an enthalpy of 5.5 kcal/mol compared to 8.4 kcal/mol for the pure lipid. (The T_c of all samples on cooling scans is 8.5 °C below that on heating at the heating and cooling rates used, 10 °C/min, due to instrumental hysteresis.)

The ratio of the two endothermic peaks in Figure 1c depends on the starting temperature of the scan, as shown in Figure 1f,g, and on how long the sample is held at the lower temperature, as shown in Figure 1i–k. Heating from 7 °C results in a decrease in amplitude of the higher temperature endothermic transition and an increase in amplitude of the lower temperature one (Figure 1f). Heating from 12 °C results in almost complete loss of both the upper temperature endothermic peak and the exothermic peak (Figure 1g). Heating from 17 °C (Figure 1h) results in only a single broad endothermic peak at a temperature 9.2 °C below that of the pure lipid and with an enthalpy of 4.0 kcal/mol (Table I), similar to the results seen on the cooling scan (Figure 1d). If the sample is stored at −3 °C for increasing lengths of time up to 2 h (Figure 1i–k), the intensity of the higher temperature peak increases and that of the lower temperature peak decreases, but the exothermic transition is retained and the amplitude never reaches that seen on leaving the sample at 4 °C overnight (Figure 1b).

The results suggest that the gel state of the lipid–protein complex after storage at 4 °C for a prolonged time is different from the gel state present immediately after cycling through the phase transition. This difference is probably due to the increased hydrophobic interaction of the protein with the lipid bilayer which occurs above the phase transition temperature. The slightly perturbed gel state which undergoes a transition similar to that in Figure 1b at a temperature only a few degrees below that of the pure lipid and with an enthalpy nearly as great as that of the pure lipid will be defined as state B. The gel state which undergoes a transition similar to that in Figure 1c with a lower temperature component and an exothermic transition will be defined as state A, and the liquid-crystalline state which occurs in all cases will be defined as state C. The exothermic transition observed on reheating (Figure 1c) indicates that state A, in which the protein continues to interact hydrophobically with the lipid, is metastable and goes into a different state at ~34 °C, which then melts only 3 °C below the melting temperature of the pure lipid. Supercooling to below 7–12 °C is necessary to reach state A. If the sample is only cooled to 17 °C, it goes into a phase which melts 10 °C below the melting temperature of the pure lipid and with a lower enthalpy (Figure 1h). This greatly perturbed gel phase will be defined as state A′. Supercooling converts state A′ to A. When stored at a low temperature for a prolonged time (4 °C overnight), the hydrophobic interaction is partially

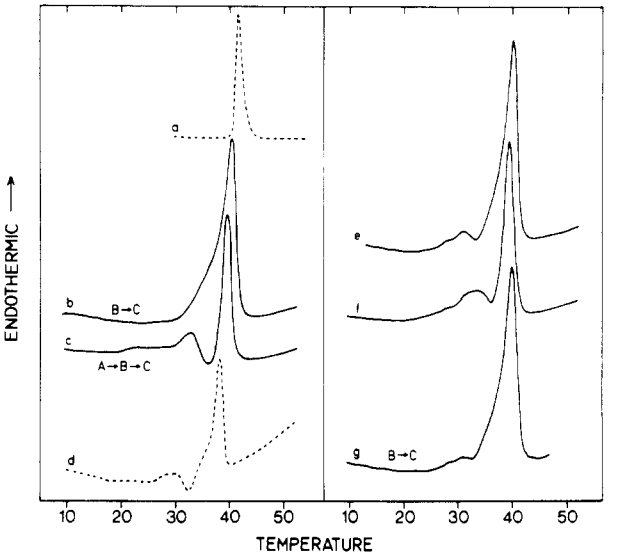


FIGURE 2: Differential scanning calorimetric thermograms of dipalmitoylphosphatidylglycerol (a) alone and (b–g) complexed with 45 wt % basic protein. The heating rate is 10 °C/min except for (d) which is 2.5 °C/min. (b) First heating scan after storage at 4 °C overnight; (c) second heating scan; (d) reheating scan at 2.5 °C/min; (e) heating scan after cycling from 50 → −3 → 31.5 °C and incubation at 31.5 °C for 15 min; (f) heating scan after cycling from 50 → −3 → 31.5 °C and incubation at 31.5 °C for 1 min; (g) heating scan after cycling from 50 → −3 → 30 °C and incubation at 30 °C for 15 min. All scans are from −3 °C. Phase states above and below transitions are indicated as described in the text.

reversed, and state A is converted to state B.

Results in Figure 2 for a different sample containing 45% protein show that conversion to state B also occurs at the temperature of the exothermic transition. As observed for the sample represented in Figure 1, the second heating scan (Figure 2c) is different from the first (Figure 2b) with reduced amplitude of the main transition and the presence of an exothermic transition, which is less distinct than that for the sample in Figure 1. The sample was reheated at a slower rate (2.5 °C/min) to obtain the temperature of the exothermic transition more accurately (Figure 2d). The sample was then heated from −3 °C to this temperature (31.5 °C) and held there for varying periods of time, cooled, and rescanned from −3 °C. The effect of incubation at 31.5 °C for 15 min is shown in Figure 2e. The DSC scan now more closely resembles the first heating scan in Figure 2b. The amplitude of the main transition is increased and shifted to a slightly higher temperature, and the enthalpy is 7.7 kcal/mol, nearly as great as that in the pure lipid (Table I). The exothermic transition is gone although there is still a small lower melting peak. Incubation at 33.5 °C for 15 min was equally effective. If the sample was incubated at 31.5 °C for only 5 min, the DSC scan had a similar appearance, but the amplitude of the main transition was slightly less (not shown). Incubation at 31.5 °C for only 1 min was not as effective (Figure 2f); the height of the main transition is less, and the amplitude of the lower temperature component is greater. Incubation at 30 °C for 15 min (Figure 2g) reduced the amplitude of this lower temperature transition to the greatest extent, but the amplitude of the main transition was not quite as great as that in Figure 2e. These results indicate that near the temperature of the exothermic transition, 30–34 °C, state A is converted to a state which melts with a slightly reduced enthalpy and only a few degrees below the T_c of the pure lipid, suggesting that state A has been converted to state B. The conversion to state B thus occurs much faster at this temperature than at −3 °C.

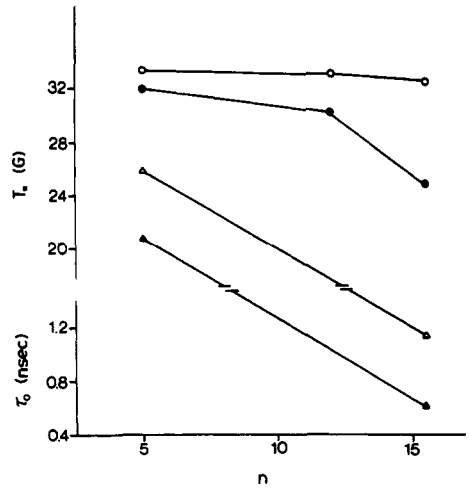


FIGURE 3: Spectral parameters $T_\parallel$ and τ_0 of fatty acid spin-labels plotted against n, the number of the carbon to which the nitroxide moiety is bound, counting from the carboxyl end. Dipalmitoylphosphatidylglycerol complexed with 55.3% basic protein at 9 °C (state B) (○) and at 59 °C (△) and dipalmitoylphosphatidylglycerol alone at 9 °C (●) and at 59 °C (▲).

Basic protein has a pronounced immobilizing effect on fatty acid spin-labels in state B. This is depicted in Figure 3 where the spectral parameters are plotted against n, the number of the carbon which is spin labeled, counting from the carboxyl end, for both the liquid-crystalline phase (at 59 °C) and the gel phase (at 9 °C). The $T_\parallel$ value of 16-S-SL in state B (the lipid–protein complex at 9 °C) is similar to those of 5-S-SL and 12-S-SL although in the gel state of the pure lipid $T_\parallel$ of 16-S-SL is much less than that of 5-S-SL. In the liquid-crystalline phase, the protein decreases the amplitude of motion of both 5-S-SL and 16-S-SL but does not significantly alter the slope of the plot of $T_\parallel$ and τ_0 against n.

Since the effect of the protein on 16-S-SL at temperatures below T_c is most dramatic, its effect on 16-S-SL was measured throughout the phase transition for heating, cooling, and reheating scans. The spectral parameters for a sample containing 55.3% basic protein on the first heating scan (curve 1), cooling scan (curve 2), second heating scan (curve 3), and the heating scan after incubation at 31 °C for 10 min (curve 4) are shown in Figure 4. $T_\parallel$ is plotted at lower temperatures, and τ_0 is plotted at higher temperatures where the probe has more isotropic motion. This plot is intended to show the effect of the protein on fatty acid motion above and below T_c and not the phase transition temperatures. The immobilizing effect on 16-S-SL below T_c can be seen on all heating scans (curves 1, 3, and 4) but is less pronounced on the second heating scan (curve 3) than on the first (curve 1).

The motion of 16-S-SL in state A′ can be determined from the spectra obtained on cooling. A plot of the height of the center peak of the spectrum against temperature (not shown)[2] shows the phase transition temperatures better than Figure 4 and indicates that the cooling transition is over by 19–22 °C. Thus, the sample is in state A′ at this temperature. The DSC scan of the sample heated from 12 or 17 C (Figure 1g,h) also indicates that the sample is in state A′ up to 22 °C. The spectra on cooling are multicomponent in this temperature range, so this region of the cooling curve is indicated by a dashed line in Figure 4 (curve 2). However, comparison of the spectra of 16-S-SL at 19 °C on the first heating scan (state B) and the cooling scan (state A′) (Figure 5A, solid line and dashed line, respectively) shows that the spin-label is much more mobile in state A′ than in state B. Only a small percentage of immobilized component is present in the spectrum on cooling.

As the sample is cooled to lower temperatures, giving state A, two values of $T_\parallel$ can be measured at 14 °C as indicated in Figure 4 (curve 2), but both are less than that found on the first heating scan at 14 °C. A single component powder-type spectrum is not obtained until 9 °C, and the value of $T_\parallel$ is less than that at 9 °C on the first heating scan. These results are consistent with DSC results which indicate that cooling to

[2] Figures depicting the data described in the text are available upon request from J.M.B.

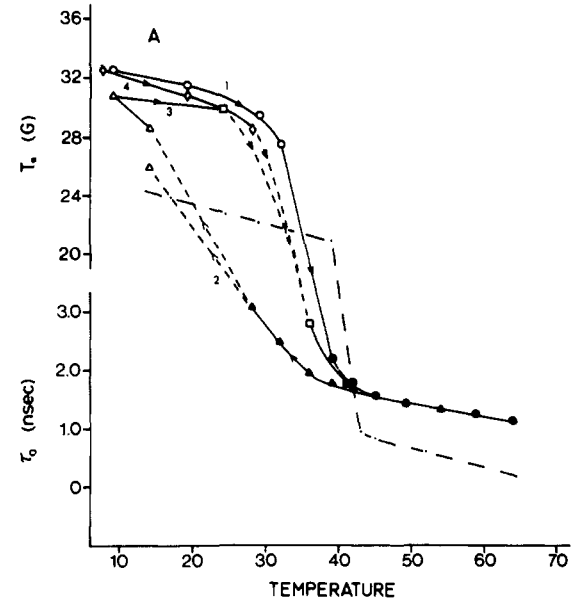


FIGURE 4: Dependence of spectral parameters of 16-S-SL in dipalmitoylphosphatidylglycerol complexed with 55.3% basic protein on temperature. (A) $T_\parallel$ and τ_0 are plotted for the first heating scan (curve 1, ○), cooling scan (curve 2, △); reheating scan (curve 3, □), and heating scan after cycling from 59 → 9 → 31 °C and incubation at 31 °C for 10 min (cuve 4, ◇). $T_\parallel$ is plotted at low temperatures (open symbols), and τ_0 is plotted at high temperatures (closed symbols). The transition of pure lipid is also shown (·····). The region from 14 to 28 °C on curve 2 is indicated by a dashed line since spectra were multicomponent, and neither $T_\parallel$ nor τ_0 could be measured.

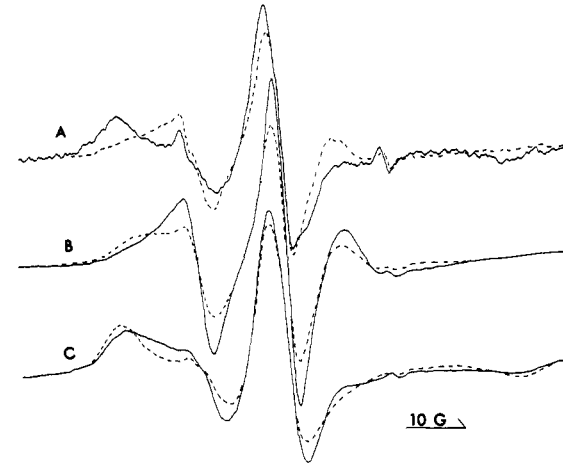


FIGURE 5: ESR spectra of 16-S-SL in dipalmitoylphosphatidylglycerol complexed with 55.3 wt % basic protein: (A) at 19 °C on first heating from 9 °C (—) and on cooling from 59 °C (---) (the gain setting was 1.6 times higher for the spectrum shown by the solid line); (B) at 31 °C after cycling from 59 → 9 → 31 °C and measured immediately after equilibration (—) and after 45 min at 31 °C (---) (same gain setting was used for both spectra); (C) at 9 °C after immediately cooling from 59 °C (—) and at 9 °C after cycling from 59 → 9 → 31 °C and incubation at 31 °C for 45 min (---) (same gain setting was used for both spectra).

between 7 and 12 °C is necessary to convert state A′ to A. It also indicates that 16-S-SL is more immobilized in state A than in A′ but not as immobilized as in state B. The spectrum of 16-S-SL in state A at 19 °C on reheating (not shown)[2] contained more immobilized component than that in Figure 5A (dashed line) (state A′) but less than that in Figure 5A (solid line) (state B). The lower temperature transition observed on reheating by DSC (Figure 1c) as state A starts to melt before it goes into state B cannot be detected from the plots of $T_{\parallel}$ (Figure 4, curve 3) or spin-label peak height against temperature. The reason for this is the very slow heating rate used for the ESR measurements as will become apparent below.

Changes in fatty acid motion during the exothermic transition observed by DSC were monitored by measuring spectra at frequent intervals at 31 °C after taking the sample through the cycle 59 →9 →31 °C. The spectra of 16-S-SL upon measurement immediately after equilibration at 31 °C and after 45 min at 31 °C are shown in Figure 5B (solid line and dashed line, respectively). The spin-label is initially more mobile (Figure 5B, solid line) than in pure DPPG at 31 °C (not shown)[2] but becomes more immobilized than in pure DPPG with time. As shown in Figure 5B (dashed line), a more immobilized component appears, and the peak height decreases. A further degree of immobilization of 5-S-SL and 12-S-SL (increase in $T_{\parallel}$ and decrease in peak height) also occurs, but the change is not as dramatic as for 16-S-SL since 5-S-SL and 12-S-SL are already relatively immobilized in pure DPPG at this temperature (data not shown).[2]

Spectra of 16-S-SL obtained at 9 °C after (i) cooling directly from 59 °C (resulting in state A) and (ii) taking the sample through the cycle 59 → 9 → 31 °C and incubation at 31 °C for 45 min (resulting in state B) are compared in Figure 5C (solid line and dashed line, respectively). Conversion to state B results in somewhat lower peak height, an increase in $T_{\parallel}$, and a line shape which more closely resembles a powder spectrum (Figure 5C, dashed line). This spectrum in Figure 5C resembles the spectrum obtained at 9 °C on the first heating scan or after storage at 4 °C for a prolonged time (not shown). Similar but smaller changes were also seen in the spectra of 5-S-SL (not shown).[2] Incubation at 31 °C also caused the spectrum of 16-S-SL on subsequent reheating from 9 to 19 °C (not shown)[2] to resemble the spectrum obtained at 19 °C on the first heating scan (Figure 5A, solid line). After this treatment, 16-S-SL was more immobilized at 19 °C than on the second heating scan (compare curves 3 and 4 at 19 °C in Figure 4).

These results agree with the DSC results indicating that the transition to state B occurs more rapidly at 31 °C than at temperatures below 9 °C. The cycle 59 → 9 → 31 °C is necessary for these spectral changes to occur rapidly as it is for the conversion of the DSC scan from that shown in Figure 2c to that shown in Figure 2e. Thus, conversion from state A′ to A is necessary in order to obtain rapid conversion to state B at 31–34 °C.

The rate of the transition to state B at 29–33 °C was monitored from the decrease in peak height by setting the magnetic field value at the of the center peak and scanning with time instead of magnetic field (Figure 6). The peak height was measured at 9 °C for a few minutes, and then the temperature was increased to the desired value while manually maintaining constant detector current. The increase in peak height, upon raising the temperature, due to the greater fatty acid motion in the lipid at the higher temperature can be seen, followed by a decrease in peak height as the lipid goes into

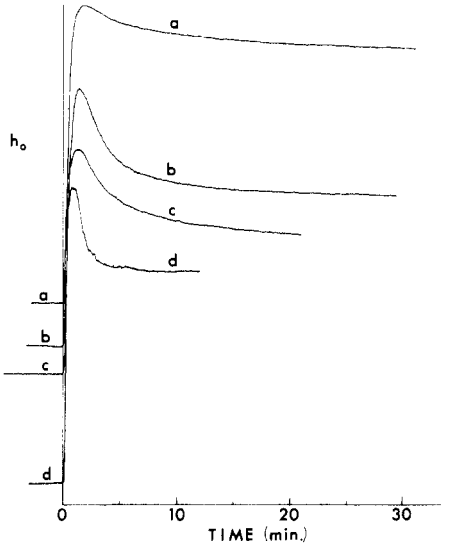


FIGURE 6: Change in center peak height (h_0) of 5-S-SL (a) and 16-S-SL (b–d) with time in dipalmitoylphosphatidylglycerol complexed with 55.3 wt % basic protein after cycling from 59 → 9 °C and raising the temperature from 9 to 31 °C (a and b), 29 °C (c), and 33 °C (d) at 0 min. The value of h_0 at 9 °C for each curve is indicated to the left of the y axis. The detector current was manually maintained constant as the temperature equilibrated.

the more immobilized state B. The loss in peak height upon going into state B at 31 °C was as much as 40% of the gain in height upon increasing the temperature from 9 to 31 °C (curve b, Figure 6). The magnitude of the change for 5-S-SL (curve a, Figure 6) was less than that for 16-S-SL. The rate was fastest at 32–33 °C (curve d, Figure 6) and decreased in the order 33 = 32 > 31 > 29 °C. The transition was nearly complete in 3 min at 32 and 33 °C while it was nearly complete in 10 min at 29 °C. This rapid rate means that the transition to state B will occur completely while scanning the phase transition by measuring ESR spectra at different temperatures, and thus the only transition which can be observed by ESR is the high temperature transition (curves 1, 3, and 4 of Figure 4). The scan rate by DSC (10 °C/min) is fast enough that the transition does not go to completion unless the sample is held at 30–33 °C.

Discussion

The DSC results suggest that the complex of basic protein with DPPG can occur in several phase states which undergo transitions between themselves under different conditions as given below:

$$A \xrightarrow{\text{heat}} B \xrightarrow{\text{heat}} C$$

$$A \xleftarrow[\text{supercool}]{} A' \underset{\text{cool}}{\overset{\text{heat}}{\rightleftharpoons}} C$$

A can be converted to B by incubation at a temperature below 10 °C for a prolonged time (2–18 h) or by incubation at 30–34 °C for a short time (<15 min). The thermotropic transition between B and C is not reversible. Instead, state C goes into state A′ on cooling. This transition is reversible provided the sample has not been supercooled to a temperature below 12 °C. Supercooling of the sample converts state A′ to a metastable state A.

These transitions from one state to another probably involve all or almost all of the lipid in the sample (unless some of the lipid does not undergo any detectable transition). When the sample is in state A′ or B, almost all of the lipid present must be in these states since only a single peak is usually observed

in the DSC scan as in Figure 2b,g (state B) and Figure 1d,h (state A'). This is also true of A since conversion to state A is necessary for conversion to state B at 30–34 °C, and all or almost all of the sample can be converted to state B. In some samples, there is a small lower temperature peak as in Figure 1b even when most of the sample is in state B. This peak is at a lower temperature than the transition temperature of A' → C and is probably present in all scans in Figure 1. Its cause is not understood, but it represents a small percentage of the total enthalpy.

The large decrease in T_c and ΔH of the transitions A' → C and C → A' and the greater mobility of 16-S-SL in state A' relative to the pure lipid suggest that states A' and C are greatly perturbed, probably due to intercalation of the protein into the bilayer as concluded earlier. The T_c and ΔH values of the transition B → C are not decreased as much while 16-S-SL is highly immobilized in state B, indicating that state B is less perturbed than states A' or C. The lesser effect on T_c and ΔH could be explained by a lower degree of intercalation of the protein due to partial freezing out of the hydrophobic segments in state B. Differences in the extent of intercalation may be due to differences in the depth to which the hydrophobic segments penetrate or to the number of segments which penetrate or both.

The DSC results further suggest that the protein is probably intercalated into state A to a similer extent as in states A' and C since the metastable state A starts to melt at a temperature 10 °C below the T_c of pure DPPG. The motion of 16-S-SL is more restricted in state A than in state A' but not as much as in state B. Therefore, supercooling probably decreases the number of gauche rotations in the acyl chains and increases the packing density of the complex. The fatty acid chains may not be able to accommodate the protein as well while the protein may itself be constricted into an unstable configuration. When the sample is reheated and starts to melt at 30–34 °C, molecular reorganization into a more stable structure can occur. The protein may be forced out of the bilayer, the lipid chains refreeze and heat is released, and 16-S-SL becomes more immobilized. At a heating rate of 10 °C/min, this process does not have time to go to completion, but holding the sample at 30–34 °C for a few minutes or use of a slower heating rate allows the sample to go completely into state B in which 16-S-SL is highly immobilized and which then melts with a T_c and ΔH only slightly less than the pure lipid.

Although this explanation of intercalation of the protein above the T_c and freezing out of the hydrophobic segments under conditions which convert the samples to state B accounts for the DSC results, it does not explain the high degree of immobilization of 16-S-SL in states B and A nor the decrease in τ_0 of 16-S-SL produced by basic protein in state C. It is unlikely that basic protein restricts the fatty acid motion in the manner of intrinsic proteins (Jost et al., 1973). It is probably not hydrophobic enough to span the bilayer or even one monolayer. Freeze–fracture electron microscopy of vesicles containing basic protein does not show intramembranous particles (J. M. Boggs, I. R. Clement, and M. A. Moscarello, unpublished experiments). Basic protein does not result in a powder-type spectrum above the phase transition temperature as intrinsic membrane proteins do, but only below the phase transition temperature.

Freezing out of the protein in state B so that it interacts mainly electrostatically would be expected to result in a decrease in the amplitude of motion of 16-S-SL relative to that of the pure lipid or state A and A' but not to the extent observed. Polylysine and divalent cations (Ca^{2+} and Mg^{2+})

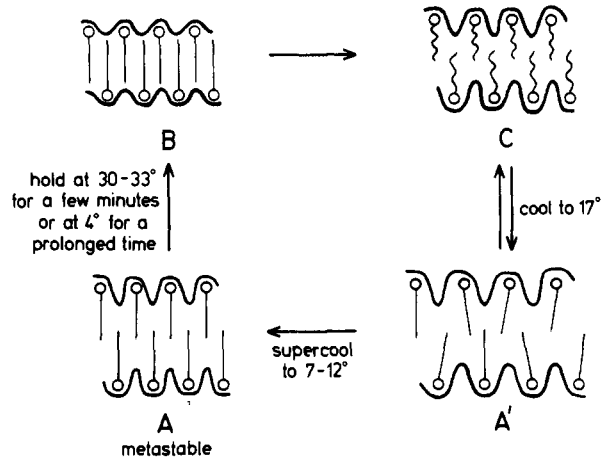


FIGURE 7: Diagrammatic representation of the phase states of the dipalmitoylphosphatidylglycerol–basic protein complex obtained on heating, cooling to 17 °C, supercooling to 7–12 °C, and incubation at 30–34 °C for a few minutes or storage at 4 °C overnight. A, A', and B are the states found below the phase transition temperature, and C is the state found above the phase transition temperature. The detailed molecular structures of states A' and A are not understood as well as those of states B and C, but it is known that the degree of immobilization of the fatty acid terminal end increases in the order C << A' < A < B. Interdigitation as shown is a suggested mechanism to explain the high degree of immobilization of 16-S-SL in state B.

do not restrict the motion of 16-S-SL in the gel phase of DPPG to the same extent as basic protein (Boggs et al., 1981). A concentration of 0.1 M $CaCl_2$ increased $T_{\parallel}$ to 27.3 G at 9 °C in contrast to the value of 32.5 G produced by basic protein (J. M. Boggs, D. Stamp, and M. A. Moscarello, unpublished experiments). Nevertheless, it must be considered possible that the positively charged residues of the basic protein are arranged in such a way that when the protein interacts mainly electrostatically (state B) it tightens up the lipid packing so that the motion of all the fatty acid spin-labels including 16-S-SL is restricted to the extent found.

An alternative explanation given below can account for both the DSC and ESR data. The hydrophobic segments of the protein must not be frozen out of phosphatidic acid (dimyristoyl- and dipalmitoyl-) below the T_c since the protein decreases the T_c to a similar extent on heating as on cooling (Boggs et al., 1981). Perhaps they are not frozen out of DPPG either, but instead the fatty acid chains become interditigated in state B as shown in Figure 7. This should cause the motion of 16-S-SL in the gel phase to be restricted to the same extent as 5-S-SL and is a plausible consequence of intercalation of the hydrophobic segments of the protein only partway into the bilayer since it would allow maximum van der Waals interactions between the fatty acid chains in state B while allowing the polar head groups to be separated enough to accommodate the hydrophobic segments of the protein. This phenomenon would not be expected to happen in the complex of basic protein with phosphatidic acid due to the intermolecular hydrogen-bonding properties of this lipid when it is not bound electrostatically to positively charged residues of the proteins. If interdigitation occurs, it would be necessary for all the lipid in the bilayer to participate. Any phosphatidic acid which is not bound directly to the protein would be involved in intermolecular hydrogen bonding with adjacent lipids. This would counteract the stabilization to be gained by interdigitation.

In state C, the degree of intercalation of the protein may increase while the fluid fatty acid chains would probably interdigitate less. Even a small degree of interdigitation would explain the decrease in τ_0 of 16-S-SL produced by basic protein above the T_c. Interdigitation in states A and A' should be

comparable to that in state C. 16-S-SL is more immobilized in state A than the pure lipid but not as much as in state B. During the exothermic transition, 16-S-SL becomes even more immobilized, suggesting that the complex may refreeze during this transition by further interdigitation of the acyl chains.

Interdigitation should decrease the bilayer thickness relative to pure DPPG, particularly in the gel state, which would be detectable by X-ray diffraction. Such a study is planned. An X-ray diffraction study on the complex of basic protein with the liquid-crystalline phase of phosphatidylglycerol (prepared from egg phosphatidylcholine) showed that basic protein increased the electron density peak to peak distance between the polar head-group regions of the bilayer by only 5 Å (G. W. Brady, N. S. Murthy, D. B. Fein, D. D. Wood, and M. A. Moscarello, unpublished experiments). This would be consistent with a contribution to an increase in bilayer thickness due to portions of the protein at the polar head-group-aqueous interface combined with a contribution to a decrease in bilayer thickness due to partial interdigitation. Consistent with this interpretation, the protein also caused an increase in the electron density in the center of the bilayer compared to that of the pure lipid.

Interdigitation of the fatty acid chains in the gel state of pure DPPG has been found by X-ray diffraction to occur in the absence of electrolytes due to repulsion of the negatively charged head groups (Ranck et al., 1977). However, we have not been able to detect any significant difference in DPPG in the presence and absence of salt by DSC or ESR. It melts with a similar enthalpy (Table I) and at a temperature only 1 °C higher in distilled water than in the presence of salt (10 mM NaCl). Fatty acid spin-label motion is also similar in DPPG in distilled water as in the presence of salt; i.e., 16-S-SL is not immobilized in the gel phase of DPPG in distilled water as it is in the presence of basic protein. Thus, if the interg-itiation occurs in the pure lipid as reported, it does not have the same effect on fatty acid motion as in the presence of the protein. However, greater separation of the lipid molecules may occur in the absence of salt or protein, resulting in less restriction of fatty acid motion, than in the presence of basic protein which is also bound electrostatically to the polar head groups. Alternatively, some of the restriction of motion of 16-S-SL in the interdigitated state may be due to the steric hindrance provided by the protein hydrophobic segments as observed for 5-S-SL even in the liquid-crystalline phase (Boggs & Moscarello, 1978a). Some species of sphingomyelin and cerebroside containing unequal fatty acid chain lengths may be involved in interdigitation (Bunow & Levin, 1980; Barenholz & Thompson, 1980). These lipids also exhibit metastable behavior and an exothermic transition by calorimetry (Estep et al., 1980; Bunow, 1979).

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