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Effect of pH and Fatty Acid Chain Length on the Interaction of Myelin Basic Protein with Phosphatidylglycerol†

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ABSTRACT: The basic protein of myelin binds electrostatically to acidic lipids but has several hydrophobic segments which may penetrate into the lipid bilayer. Calorimetric and spin-label evidence suggests that below the phase transition temperature, T_c, several phase states occur in the complex of phosphatidylglycerol with basic protein, possibly due to differences in the degree of penetration of the protein and/or interdigitation of the lipid acyl chains. One of these states is a metastable state which starts to melt 10 °C below the T_c of the pure lipid and then refreezes, with release of heat, into a stable state. The stable state melts near the T_c of the pure lipid but restricts the motion of a fatty acid spin-labeled near the terminal methyl much more than does the pure lipid. The relationship between the rate of conversion to the stable state and the degree of penetration of the protein at varying pH, in the range 4-8, and the lipid acyl chain length, in the range 14 to 18 carbons, was investigated. Altering the pH in this range affects protonation of the histidines of the protein but has no effect on the lipid at pH 4 and above. The rate of conversion of the sample to both the metastable state and the stable state decreased with increase in pH for phosphatidylglycerol with all lipid chain lengths. It also decreased with decreasing chain length at constant pH. This suggested that the lipid could refreeze into the stable state more readily if a smaller proportion of the total bilayer thickness was occupied by the hydrophobic segments of the protein. The consistency of these results with the concept of penetration of portions of the protein partway into the bilayer lends support to this hypothesis.

The basic protein of central nervous system myelin contains 10 histidines, 13 lysines, and 18 arginines and interacts electrostatically with acidic lipids (Palmer & Dawson, 1969; Demel et al., 1973; London et al., 1973; Boggs & Moscarello, 1978). However, it also contains several segments composed of 5-10 hydrophobic and neutral amino acids which may penetrate partway into the hydrocarbon region of the bilayer. This conclusion is based on the perturbing effects of the protein on the lipid (Gould & London, 1972; Papahadjopoulos et al., 1973, 1975; Demel et al., 1973; Boggs & Moscarello, 1978; Boggs et al., 1980, 1981a) as well as evidence for sequestration

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of parts of the protein by the lipid (London & Vossenberg, 1973; London et al., 1973; Boggs et al., 1981b). This evidence has been reviewed recently (Boggs et al., 1981c; Boggs, 1982).

We have recently reported calorimetric and electron spin resonance (ESR)[1] evidence which indicates that at neutral pH the complex of basic protein with dipalmitoylphosphatidylglycerol (DPPG) exists in several phase states depending on sample history (Boggs et al., 1981d; Boggs & Moscarello, 1982). These states undergo transitions between themselves under different conditions as given below:

$$A \xrightarrow[\text{exothermic}]{\text{heat}} B \xrightarrow[\text{endothermic}]{\text{heat}} C$$

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

A, A′, and B are states which exist below the T_c of the complex, and C is the liquid-crystalline state of the complex. When state C is cooled, it initially freezes into an intermediate state, A′, with a reduced enthalpy and at a temperature 5–10 °C below the T_c of pure DPPG. This perturbation of the lipid is consistent wth penetration of the protein into the bilayer. If the sample has not been cooled to a low temperature (below 12 °C), this transition is reversible. If it is supercooled to below 12 °C, however, it goes into state A which is metastable. When subsequently reheated, state A starts to melt at a temperature 5–10 °C below the T_c of pure DPPG as state A′ does but then refreezes with release of heat into state B. State B can also be obtained by incubating the sample below 12 °C for a prolonged time (2–18 h).

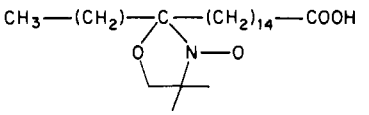
State B then melts with a temperature and enthalpy slightly less than those of the pure lipid. However, the motion of a fatty acid spin-labeled near the terminal methyl, 16-doxylstearate, is restricted in state B much more than in the gel phase of the pure lipid. Its degree of immobilization is similar to that of a fatty acid spin-labeled near the polar head group, 5-doxylstearate. The degree of immobilization of 16-doxylstearate in state A is also greater than in the pure lipid but not as great as in state B. In state C, the protein decreases the motional parameter of 16-doxylstearate, but the probe still has nearly isotropic motion as in the liquid-crystalline phase of the pure lipid.

We suggested that the lipid may refreeze into state B by excluding the protein so that it interacts only electrostatically and/or by interdigitation of its fatty acid chains. The latter mechanism would account for the high degree of immobilization of the terminal ends of the acyl chains (Boggs et al., 1981d). Regardless of the mechanism, the phenomenon appears to depend on the penetration of the protein into the bilayer. In the present study, the relationship between fatty acid chain length, degree of penetration of the protein, and the rate of transition from one state to another was studied, in order to better understand the mechanisms involved. The degree of penetration of the protein was altered by varying the extent of protonation of the histidines over the pH range 4–8. The lipid itself is not affected at pH values of 5 and above (van Dijck et al., 1978). The fatty acid chain length was varied from 14 to 18 carbons by using synthetic species of phosphatidylglycerol.

[1] Abbreviations: ESR, electron spin resonance; DSC, differential scanning calorimetry; PG, phosphatidylglycerol; DMPG, dimyristoylphosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol; DSPG, distearoylphosphatidylglycerol; BP, myelin basic protein; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

Materials and Methods

The fatty acid spin-label 16-doxylstearate (16-S-SL) (I) was purchased from Syva (Palo Alto, CA) where doxyl stands for the 4′,4′-dimethyloxazolidinyl-*N*-oxy derivative of the parent



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ketone. Dipalmitoylphosphatidylglycerol (DPPG) was purchased from Supelco (Bellefonte, PA), and distearoylphosphatidylglycerol (DSPG) was purchased from Avanti (Birmingham, AL). Dimyristoylphosphatidylglycerol (DMPG) was prepared enzymatically from dimyristoylphosphatidylcholine (purchased from Sigma) by the method of Papahadjopoulos et al. (1973). The DSPG was found to be chromatographically pure but melted at too high a temperature, 81 °C, suggesting that it contained bound divalent cations. This was removed by dissolving the lipid in a Bligh & Dyer (1969) monophasic solvent system containing 1 M NaCl and 0.1 M EDTA at pH 7.5 in the aqueous component and adding additional chloroform and aqueous phase to convert to a biphasic system. After this treatment, the lipid melted at the correct temperature. The other lipids were also chromatographically pure and melted at the correct temperatures. Basic protein was extracted from isolated myelin by the method of Lowden et al. (1966) and stored in the lyophilized form.

Preparation of Lipid–Protein Vesicles. Spin-labeled vesicles were prepared by evaporating a chloroform–methanol (2:1) solution of the lipid and spin-label together at a molar ratio of 350:1 and also used for DSC studies in order that identical samples could be studied by both techniques. At this concentration, the spin-label had no discernible effect on the T_c and ΔH of the lipid or interaction with the protein. The lipid was dispersed by vortex shaking at a concentration of 4 mg/mL in aqueous buffer containing 10 mM NaCl and 1 mM EDTA for DPPG and DSPG, and 100 mM NaCl and 1 mM EDTA for DMPG, at 45 °C for DMPG and DPPG and at 75 °C for DSPG. The buffer was 10 mM acetate for pH 4 and 5, 10 mM Mes for pH 6, 10 mM Hepes for pH 7 and 7.4, and 20 mM Hepes for pH 8. The protein was dissolved in the appropriate buffer at a concentration of 5 mg/4 mL, and the pH was measured and adjusted in some cases or used as found. The protein solution was added to the lipid suspension and dispersed again at the appropriate temperature for 10 min. The suspensions were centrifuged at 2000 rpm for the lipid–protein complexes and at 10000 rpm for 5 min in an Eppendorf microcentrifuge for the pure lipid. The pellets were used for calorimetry or ESR spectroscopy. The pH of the supernatant was checked and was always found to be identical with that of the protein solution used. 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. The weight percent of protein incorporated at different pH values and at different initial values of protein concentration is indicated in Table I. There is considerable variability in the amount incorporated even when identical amounts of protein were added. This may be due to errors in analysis. The amount of protein incorporated may have decreased slightly at lower pH in spite of the fact that the protein is more highly charged.

Differential Scanning Calorimetry. Samples were run on a Perkin-Elmer DSC-2 at heating or cooling rates of 1.25–20

Table I: Weight Percent Protein Incorporated at Different pH Values

lipid	initial wt % protein added	wt % protein incorporated[a] at pH					
		4	5	5.8	6.8	7.4	8
DPPG	45			44.8	38.8	40.5	
DPPG	50	30.4		35.8	48.6	44.8	54.7
	50						39.0
DSPG	50	37.0		35	39.6	40.4	38.6
	50	37.8					34.5
	50						42.7
DMPG	40	29	35.1				
DMPG	45			40.1	37.3		36.8
DMPG	50	31.5					

[a] In some cases, several samples were made at the same pH. Percent protein is given for each sample.

°C/min. The midpoint of each peak was defined as the phase transition temperature (T_c). The temperature of the exothermic transition was defined as T_{ex}.

Electron Spin Resonance Measurements. The motional parameter, τ_0, was measured as described earlier (Boggs & Moscarello, 1978). $T_\parallel$, the maximum outer hyperfine splitting, was also determined as a measure of the amplitude of motion of the molecular long axis about the average orientation of the fatty acid chains in the bilayer. Spectra were measured on a Varian E-104 spectrometer, at a microwave power of 10 mW, equipped with a Varian temperature control accessory.

Results

Dipalmitoylphosphatidylglycerol. The effect of pH on the DSC thermograms of the complex of DPPG with 50% BP (weight percent added, see Table I for amount bound) at a heating rate of 10 °C/min is shown in Figure 1. The thermograms of the pure lipid at pH 4 and 7 are shown in scans b and d, respectively (dashed lines), of Figure 1. Thermograms of DPPG at pH 5–8 are identical with that at pH 7. However, the T_c of DPPG is higher at pH 4 (~50 °C) due to partial protonation of the lipid. Complete protonation raises the T_c to 61 °C (Watts et al., 1978). Increasing the pH from 5 to 8 only affects the ionizable groups of the protein.

The perturbing effect of the protein on the melting transition (Figure 1a–g) increases with increasing pH. At pH 4 (Figure 1a), the T_c of the DPPG–BP complex on heating is similar to that of the pure lipid in its unprotonated state at pH 7 (Figure 1d) and to that of the DPPG–BP complex at pH 5.8 (Figure 1c) but much less than that of the lipid at pH 4, indicating that the protein displaces protons from the lipid. It lowers the pK_a of phosphatidylglycerol as monovalent and divalent cations do, by lowering the surface change density of the lipid bilayer (Traüble et al., 1976). Basic protein also displaces Mn^{2+} from acidic lipids (Boggs et al., 1981a). Thus, the effect of pH 4 on the lipid itself can be ignored, and the effect of the protein on the lipid at this pH can be compared to its effects at other pH values.

At pH 5.8 (Figure 1c), the protein still has little effect on the T_c determined from the heating scan although there is a shoulder at a lower temperature. At pH 6.8 (Figure 1e), the lower temperature endothermic peak at 29 °C due to initial melting of state A and the exothermic transition described in our earlier studies are most apparent. At pH 7.4 (Figure 1f), the amount of the lower temperature component is greater relative to the upper temperature component, and at pH 8 (Figure 1g), only the lower temperature component is present.

On being cooled from the liquid-crystalline phase, the thermograms consisted primarily of a single broad peak 4–10 °C below the T_c of the pure lipid (scans not shown).[2] The temperature of this peak was higher at pH 4 and 5 but was not well correlated with pH and may also depend on the protein content or on sample homogeneity. At pH 4, the T_c was less than that of the pure lipid in its unprotonated state at pH 7 as well as at pH 4. Thus, even at pH 4, the protein has a greater effect on the freezing transition than on the melting transition.

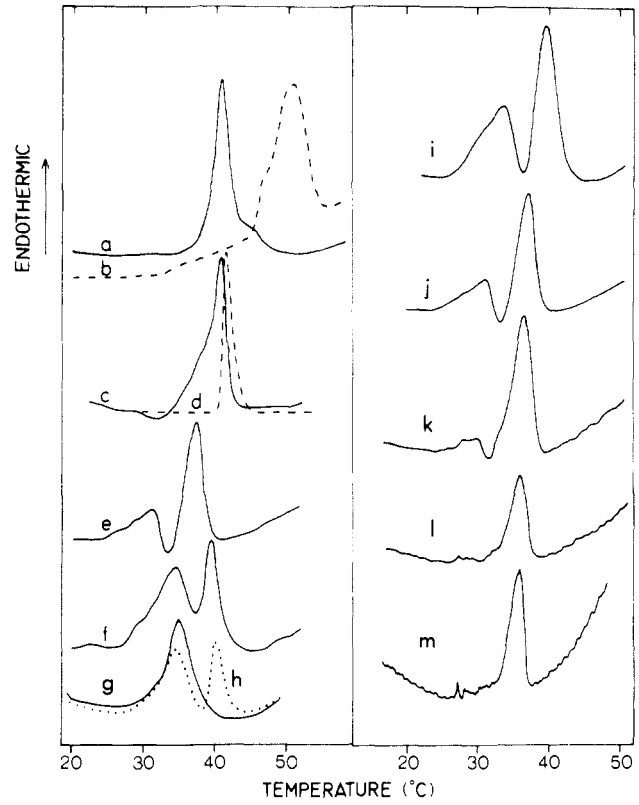

FIGURE 1: Differential scanning calorimetric thermograms of DPPG alone (- - -) at pH 4 (b) and pH 7 (d) and complexed with 50 wt % BP (initial amount) (—) at pH 4 (a), 5.8 (c), 6.8 (e), 7.4 (f), 8 (g and h), and 6.8 (i–m). Heating rate was 10 (a–h), 20 (i), 10 (j), 5 (k), 2.5 (l), and 1.25 °C/min (m). All scans were heated from below 12 °C. The samples were heated at least once prior to the scans shown, and the scans were reproducible through at least five heating and cooling cycles. The dotted line curve in (h) was produced by incubating the pH 8 sample at 32 °C overnight. In scans i–m, the sensitivity was adjusted to maintain a reasonable peak height so that the sensitivity relative to that in (i) is (j) 1×, (k) 2×, (l) 2×, and (m) 5×. The amount of protein incorporated was (a) 30.4%, (c) 35.8%, (e) 48.6%, (f) 44.8%, (g and h) 54.7%, and (i–m) 48.6%.

The DSC heating scans shown in Figure 1 were produced by supercooling to well below the T_c (below 12 °C) which is necessary to convert the sample to state A at neutral pH. If the samples at pH 6.8 and 7.4 are cooled to only 17 °C, they remain in state A′. A subsequent scan consists of a single broad peak 6–10 °C below the T_c of the pure lipid (scans not shown),[2] as observed on cooling and resembling the heating scan at pH 8. At pH 8, the thermogram is identical regardless of whether or not the sample is supercooled.

The exothermic transition in the curves produced by supercooling is not significantly below the base line in these particular samples at this heating rate (10 °C/min) even at pH 6.8 (Figure 1e). However, its occurrence diminishes the area of the lower melting endothermic peak in the scans in Figure 1e,f. The greater height of the lower relative to the

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

Table II: Effect of pH on Spectral Parameters of 16-S-SL in Lipid and Lipid–Basic Protein Complexes Above and Below the Phase Transition Temperature, T_c

		below T_c			above T_c	
sample	pH	temp[a] (°C)	$T_{\parallel}$ (G)[b]	state[c]	temp[a] (°C)	τ_0 (ns)
DPPG	7	9	25.0		59	0.56
DPPG–BP50	4	9	32.4	B	59	0.64
	5.8	9	32.6	B	59	0.76
	6.8	9	32.9	B	59	0.92
	7.4	9	32.9	B	59	0.92
	8	9	30.9	A′ or A	59	0.94
DSPG	7	14	25.3		63	0.42
DSPG–BP50	4	14	32.4	B	63	0.61
	6.8	14	32.5	B	63	0.88
	7.4	14	32.2	B	63	0.75
	8	14	32.3	B	63	0.71
DMPG	5	4	25.2		34	0.91
	7	4	25.2		34	0.88
DMPG–BP50	4	4	~25.5, ~32.0[d]	A, B	34	1.20
DMPG–BP40	5	4	33.0	B	34	1.21
DMPG–BP45	5.9	4	23.3	A′	34	1.26
DMPG–BP45	6.9	4	22.7	A′	34	1.24
	8	4	24.7, 29.0[d]	A′, A	34	1.46

[a] Temperature of measurement. [b] Maximum value of $T_{\parallel}$ which could be produced by incubation at T_{ex}. [c] Probable state of sample below T_c deduced from $T_{\parallel}$ and calorimetric behavior. [d] Two components present. Estimated values of $T_{\parallel}$.

upper temperature endothermic peak at pH 7.4 compared to pH 6.8 is due to less complete conversion to state B at pH 7.4 during the scan. These two samples can be converted completely to state B by incubation at a temperature near that of the exothermic transition, T_{ex}, for a few minutes, resulting in a single peak of increased height at the upper temperature.

At pH 8, it was necessary to incubate the sample at or near T_{ex} for 16 h in order to generate even a small upper temperature peak as shown in Figure 1h (dotted line). 16-S-SL was not immobilized below the T_c as much as at lower pH (Table II), and at T_{ex}, the spin-label was more mobile than in the pure lipid, suggesting that the sample was still in state A or A′. This suggested that the rate of conversion to state B is slower at higher pH. At pH 7.4, incubation at the low temperature (below 12 °C) for a few minutes decreased the amount of time required at T_{ex} for conversion to state B while only transient incubation at the low temperature was necessary at pH 6.8. This indicates that the rate of conversion to state A also decreased with increasing pH.

At pH 5.8, no lower temperature transition could be detected even if the sample was cooled to only 22 °C and reheated (not shown). This difficulty in trapping the sample in state A′ and the similarity of the heating scan (Figure 1c) to that of the pure lipid suggested that at pH 5.8 the sample was converted to state B very soon after the cooling transition was completed. This was confirmed by the behavior of the fatty acid spin-label 16-S-SL in this sample. The immobilized powder-type spectrum characteristic of state B (Boggs et al., 1981d) occurred immediately on cooling to 9 °C (Table II), while completion of the exothermic process was required to generate this type of spectrum for samples at pH 6.8 and 7.4. When the pH 5.8 sample was reheated to a temperature near T_{ex}, the ESR spectrum consisted entirely of a component characteristic of greater immobilization than in the pure lipid (not shown).[2] No component characteristic of greater motion could be observed even momentarily at T_{ex}, in contrast to the results at higher pH.

The incubation time at T_{ex} required in order to observe the changes in the DSC scans or ESR spectra indicative of state B was greater at higher pH. However, the rate of conversion to state B depended greatly on the temperature chosen, and the optimum temperature varied from one sample to another as did T_c and T_{ex}. Thus, the rate of change in the DSC scans or ESR spectra at different pH values could not be compared quantitatively.

More quantitative comparison could be made by comparing the effect of the heating rate on the DSC scans at different pH values as shown in Figure 1i–m for the pH 6.8 sample at heating rates of 20 °C/min down to 1.25 °C/min. The recorder speed was adjusted in order to maintain a constant temperature interval per length of chart paper. The T_c normally decreases with decrease in heating rate as shown, but the enthalpy of a pure lipid remains constant over the range of heating rates used. The height of the transition also normally decreases since the energy is being absorbed over a longer time span and the instrument sensitivity must be increased at slower heating rates (see legend to Figure 1 for relative differences in instrument sensitivity for scans i–m). However, in the case of the samples with protein, the height of the upper temperature transition may also increase with the decrease in heating rate as the exothermic process has more time to occur and partially counteract the normal decrease in height. In order to resolve the increase which occurs due to conversion to state B form the normal decrease which occurs with decrease in heating rate, the change in height with scan rate for the lipid–protein complex must be compared to that which occurs for the pure lipid.

As shown in Figure 1i–m, the relative height of the lower temperature transition compared to that of the upper temperature transition decreases with decreasing heating rate until it and the exothermic transition have disappeared at a rate of 2.5 °C/min (Figure 1l). The ratios of the heights of the upper temperature transition at different heating rates, $hr_1/(hr_2)$ (normalized to the same instrument sensitivity), of the lipid–protein complex are compared to those of the pure lipid in Table III. Thus, the height of the peak for a pure lipid at 10 °C/min is normally 62–70% of its value at 20 °C/min. However, for the DPPG–BP sample at pH 6.8 (48.6 wt % BP), it is greater, 77% of its value at 20 °C/min due to the greater conversion to state B at the lower rate.

The degree of completion of the exothermic process and conversion to state B at different heating rates can also be estimated from the ratio of the peak height at each rate, hr_1, to the maximum height obtained by incubating the sample at T_{ex}. Since these scans were all made at 10 °C/min, this height is denoted as h_{10ex}. This ratio, hr_1/h_{10ex}, must be compared

Table III: Ratios of the Heights of the Upper Temperature Transition at Different Heating Rates

	% BP incorporated	$hr_1/(hr_2)^a$				$hr_1/h_{10ex}{}^b$				minimum rate[c] (°C/min)
		h_{10}/h_{20}	h_5/h_{10}	$h_{2.5}/h_5$	$h_{1.25}/h_{2.5}$	h_{10}/h_{10ex}	h_5/h_{10ex}	$h_{2.5}/h_{10ex}$	$h_{1.25}/h_{10ex}$	
expected[d]		0.62–0.70	0.58–0.67	0.50–0.59	0.48–0.56	1.0	0.59–0.67	0.30–0.38	0.14–0.21	
DPPG–BP										
pH 5.8	31.0	0.76	0.61	0.55	0.58	1.0	0.61	0.34	0.20	>20
pH 5.8	35.8	0.65	0.62	0.61	0.54	0.95	0.58	0.36	0.19	1.25–2.5
pH 5.8	44.8	0.72				1.0				>20
pH 6.8	38.8	0.71	0.62	0.66	0.54	1.0	0.56	0.37	0.20	>20
pH 6.8	48.6	0.77[e]	0.60	0.62	0.56	0.53[e]	0.32[e]	0.20[e]	0.11[e]	1.25–2.5
pH 7.4	40.5	0.74	0.62	0.48		0.37[e]	0.23[e]	0.11[e]		~1.25
pH 7.4	44.8	0.80[e]	1.04[e]	0.67[e]	0.65[e]	0.27[e]	0.28[e]	0.19[e]	0.12[e]	~1.25
pH 8.0	39.0	*f*	*f*	*f*	*f*	0	0	0	0	<<1.25
pH 8.0	54.7	*f*	*f*	*f*	*f*	0	0	0	0	<<1.25
DSPG–BP										
pH 6	35.0	0.67	0.69	0.66	0.57	0.93	0.67	0.45	0.26	>20
pH 6.8	39.6	0.79[e]	0.61	0.54	0.49	0.54[e]	0.36[e]	0.19[e]		1.25–2.5
pH 7.4	40.4	0.96[e]	0.56	0.58	0.58	0.77[e]	0.54[e]	0.31[e]	0.19[e]	2.5–5
pH 8	38.6	1.2[e]	1.0[e]	0.72[e]	0.61[e]	0.54[e]	0.54[e]	0.39	0.24	2.5
pH 8	34.5	*g*	*g*	*g*	*g*	0	0	0	0.07	<1.25
pH 8	42.7		0.58	0.90[e]	1.16[e]	0.23[e]	0.13[e]	0.08[e]	0.09[e]	<1.25
DMPG–BP										
pH 5	35.1		0.73[e]	0.68[e]						~1.25
pH 6		*f*	*f*	*f*	*f*	0	0	0	0	<<1.25

[a] r_1 and r_2 are heating rates used for supercooled samples (°C/min). [b] h_{10ex} is peak height of scan at 10 °C/min after incubation at temperature near T_{ex} and conversion to state B. [c] Minimum rate necessary for maximum conversion to state B. [d] Expected ratio determined from pure lipids. hr_1/h_{10ex} is simply hr_1/h_{10}. [e] Indicates values which differ significantly from expected values. [f] Too slow to measure – no upper peak. [g] No upper peak until 1.25 °C/min.

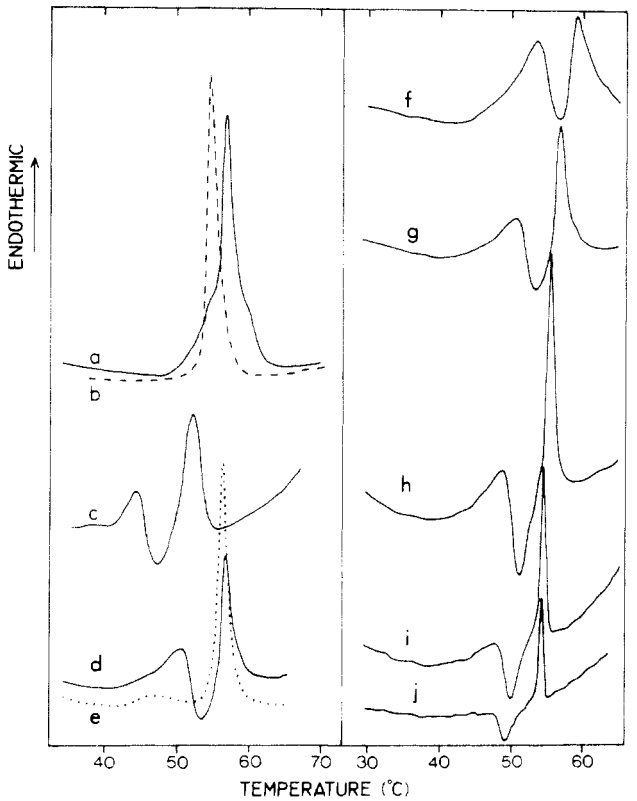

FIGURE 2: Differential scanning calorimetric thermograms of DSPG alone at pH 7 (---, b) and complexed with 50 wt % BP (initial amount) (—) at pH 6 (a), 6.8 (c), and 8 (d–j). Heating rate was 10 (a–e), 20 (f), 10 (g), 5 (h), 2.5 (i), and 1.25 °C/min (j). In (e), the dotted line curve was produced by incubating the sample at 50 °C for 10 min. All scans were produced by heating from below 17 °C. Other details as in Figure 1. Amount of protein incorporated was (a) 35%, (c) 39.6%, and (d–j) 38.6%. In (f–j), the instrument sensitivity was adjusted to maintain a reasonable peak height so that the sensitivity relative to that in (f) is (g) 1×, (h) 2×, (i) 2×, and (j) 2×.

to the ratio of peak heights at different heating rates to that at 10 °C/min for the pure lipid, hr_1/h_{10}. Thus, at 5 °C/min, h_5/h_{10ex} is 0.32 for the lipid–protein complex while the expected ratio h_5/h_{10} determined for the pure lipid is 0.59–0.67, indicating that a rate of 5 °C/min is not slow enough for maximum conversion to state B. As the heating rate is decreased, hr_1/h_{10ex} approaches hr_1/h_{10} for the pure lipid until at a rate of 1.25 °C/min at pH 6.8 it is nearly equal to the value for the pure lipid, indicating that this rate is slow enough for maximum conversion to state B. The process was faster in a sample containing less protein (38.8%) (Table III).

At pH 5.8 for the DPPG–BP complex, $hr_1/(hr_2)$ and hr_1/h_{10ex} are similar to the values for the pure lipid at all heating rates (Table III), indicating that complete conversion to state B occurs even at a rate of 20 °C/min. At pH 7.4 for a sample with 44.8% BP, the exothermic transition is detectable even at a heating rate of 1.25 °C/min while at 20 °C/min the scan consisted almost entirely of the broad lower temperature peak (not shown). Values of $hr_1/(hr_2)$ and hr_1/h_{10ex} deviate more from the expected values than at pH 6.8, particularly at heating rates greater than 2.5 °C/min due to the slower rate of the exothermic process at pH 7.4. However, it probably goes to completion at 1.25 °C/min at pH 7.4 as at pH 6.8. At pH 8, the rate is so slow that no difference in the DSC scans occurs or is expected over this range of heating rates (Table III).

The effect of the protein at different pH values on the motion of 16-S-SL above the T_c was also measured. As shown in Table II, the protein increased the motional parameter τ_0 at all pH values, but the increase is greater at higher pH. Changes in pH over the range 4–8 had no significant effect on τ_0 at 59 °C or $T_{\parallel}$ at 9 °C in the pure lipid.

Distearoylphosphatidylglycerol. DSC heating scans for pure DSPG (dashed lines) and DSPG complexed with 50% BP (see Table I) at pH 6–8 are compared in Figure 2a–d. The behavior is similar to that of DPPG–BP at pH 6–7 except that at pH 6 the T_c of the complex with DSPG (Figure 2a) is 2 °C higher than that of the pure lipid (Figure 2b) rather than somewhat less as with DPPG. At pH 4, the effect of BP on DSPG was also similar to its effect on DPPG (not shown).

At higher pH, the exothermic transition is more apparent than in the DPPG complex and, in contrast to DPPG, occurs in some samples (Figure 2d) at a heating rate of 10 °C/min even at pH 8. In other samples at pH 8, an exothermic transition and an upper temperature endothermic transition occurred only at heating rates of 5 °C/min or less (Table III). These results suggested that the exothermic process is faster in DSPG than in DPPG. The behavior on cooling scans was similar to that with DPPG (not shown).[2]

As with DPPG, supercooling was necessary to obtain the metastable state A (to below 17 °C in the case of DSPG). However, it was more difficult to catch the DSPG samples in state A′ (a small upper temperature peak was always present), further indicating that they went into states A and B faster than DPPG. Incubation of the DSPG–BP samples near T_{ex} (45–50 °C) for a few minutes converted them to state B which melted at the upper temperature and gave a scan of greatly increased peak height, even at pH 8, on subsequent reheating (Figure 2e, dotted line), in contrast to DPPG–BP at pH 8. The behavior of 16-S-SL in the DSPG–BP complex at pH 6.8 and 8 was similar to that in DPPG–BP at pH 6.8 and 7.4. However, the maximum hyperfine splitting of the more immobilized component at T_{ex}, obtained after incubation at T_{ex}, was less than that in the DPPG–BP complex. This may be due to the higher temperature of T_{ex} for DSPG or to the fact that the fatty acid spin-label chain length is better matched to the lipid chain length in DSPG than DPPG. After incubation at 41 °C and subsequent cooling to a low temperature, 14 °C, the spectrum of 16-S-SL was similar to that in state B of DPPG–BP.

The DSC heating scans are compared at different heating rates for the DSPG–BP complex (38.6% BP) at pH 8 in Figure 2f–j. The exothermic transition occurs at all heating rates and is significantly below the base line even at 1.25 °C/min. At pH 6.8, the exothermic transition could be seen distinctly only at heating rates of 20 and 10 °C/min while at pH 7.4 it could be seen even at a rate of 1.25 °C/min (not shown). The exothermic transition was not observed at any heating rate at pH 6. As shown in Table III, at pH 6 $hr_1/(hr_2)$ and hr_1/h_{10ex} are similar to the expected values at all heating rates. At pH 6.8 and 7.4, h_{10}/h_{20} was greater than expected while the ratios at other heating rates were normal in contrast to the results with DPPG (44.8% BP) at pH 7.4. This is consistent with the suggestion that the rate of the exothermic process is faster in DSPG. The rate of the exothermic process also depends somewhat on protein concentration.

The difference in rate between the DPPG and DSPG samples is even more apparent at pH 8. The values of $hr_1/(hr_2)$ and hr_1/h_{10ex} for the DSPG sample with 38.6% BP indicate that a heating rate of 2.5 or 1.25 °C/min may be slow enough for maximum conversion to state B in contrast to DPPG which required overnight incubation for partial conversion. Some DSPG samples at pH 8 had a slower rate, but complete conversion to state B could still be achieved in less than 1 h. As with DPPG, however, the rate for DSPG decreases with increasing pH.

The protein also decreased τ_0 of 16-S-SL above the T_c of DSPG although the τ_0 values in both the pure lipid and lipid–protein complex are less than those in DPPG (Table II).

Dimyristoylphosphatidylglycerol. DSC heating and cooling thermograms for the complex of DMPG with the basic protein at pH 4–7 are compared in Figure 3. The effect of the protein at pH 4 is similar to its effect on DPPG and DSPG. At pH 4 and 5 (Figure 3b, h, c, and j), the thermograms resemble those for DPPG at pH 6. However, at pH values 5.9–8 (Figure

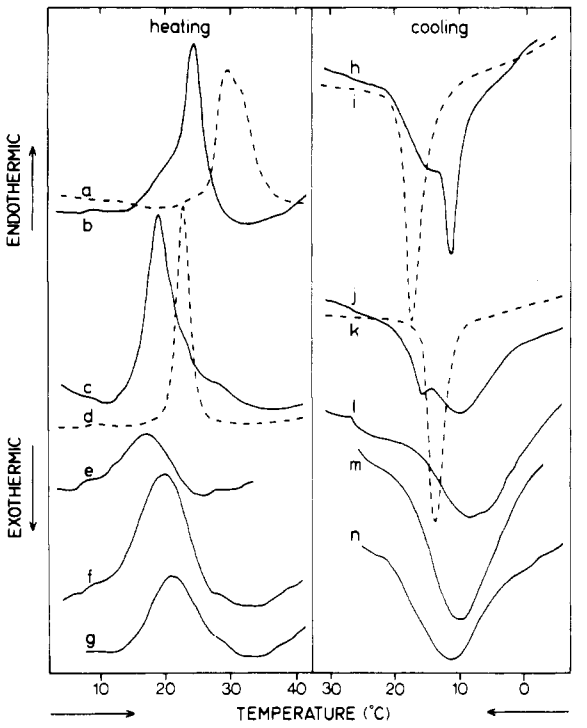

FIGURE 3: Differential scanning calorimetric thermograms of DMPG alone (- - -) at pH 4 (a and i) and at pH 7 (d and k) and of DMPG complexed with 31.5% BP (—) at pH 4 (b and h), 35.1% BP at pH 5 (c and j), 40.1% BP at pH 5.9 (e and l), 37.3% BP at pH 6.9 (f and m), and 36.8% BP at pH 8 (g and n). (a–g) Heating scans from −13 °C; (h–n) cooling scans produced by scanning at 10 °C/min. Other details as in Figure 1.

3e–g and l–n), the protein has a greater effect on the heating scans in the case of DMPG than was observed for DPPG and DSPG even though less protein was used for DMPG (45% at pH 5.9–8; see Table I). The protein broadens the peak and decreases the T_c by about 3.5–5 °C to a similar extent on both heating (Figure 3e–g) and cooling (Figure 3l–n) scans at pH 5.9–8. No exothermic transiton or higher temperature endothermic transition is seen in the heating scans. The behavior on heating at pH 5.9–8 is similar to that of DPPG at pH 8. This suggested that either the formation of state A or the exothermic process or both are much slower in DMPG even at pH 5.9 than in DPPG or DSPG at pH 8 or less.

Incubation of the DMPG samples at various temperatures near that of the endothermic transition of its complex with BP for prolonged perioids of time had no effect on the subsequent thermogram. Values of $hr_1/(hr_2)$ at pH 5 indicate the sample is probably maximally converted to state B even at 10 °C/min (Table III). These values could not be measured at higher pH since no upper temperature peak occurred, but the scans appeared similar at all heating rates. The ESR spectrum of 16-S-SL at 4 °C in the DMPG–BP complex at pH 5 was a powder-type spectrum like that for state B of DPPG–BP and DSPG–BP. At higher pH values, however, the spectrum had both an immobilized component and more mobile component (like that of the pure lipid), suggesting that it may not have gone completely into state A.

Values of τ_0 of 16-S-SL above the T_c were much greater in the DMPG samples than in DPPG or DSPG, but as with the other lipids, the protein increased τ_0 at all pH values. The values were similar from pH 4–7 in contrast to DPPG but increased further at pH 8. The fatty acid spin-label used has an 18-carbon chain and thus may interdigitate in the DMPG and DPPG bilayers even under conditions where the acyl chains of the lipid do not. The increase in τ_0 above the lipid T_c in both the pure lipid and the lipid–protein complex (in the

order DMPG > DPPG > DSPG) with decrease in chain length suggests that this may occur. Below T_c, the maximum hyperfine splitting of 16-S-SL was similar in all three lipids and much less than that in state B of the complex with basic protein.

Discussion

The various phase states of the DPPG–BP complex at neutral pH have been explained on the basis of varying degrees of penetration of the protein into the bilayer, possibly accompanied by interdigitation of the lipid acyl chains, particularly in state B. (Boggs et al., 1981d). There is no direct evidence that interdigitation occurs in the lipid–protein complex, but it has been detected by X-ray diffraction studies of pure DPPG in the absence of added electrolytes (Ranck et al., 1977). It would explain the high degree of immobilization of 16-S-SL in state B and would allow refreezing of the sample into a stable state through the increased van der Waals interactions between the acyl chains. A lesser degree of interdigitation in state C would also explain the increase in τ_0 of 16-S-SL caused by the protein. This increase is difficult to explain by a mechanism of either penetration without interdigitation or electrostatic interaction without penetration (Boggs et al., 1981a).

Whether refreezing into the stable state B occurs by a mechanism in which the hydrophobic segments of the protein are squeezed out of the bilayer or by interdigitation of the acyl chains, it should depend on how deeply the protein penetrates relative to the length of the acyl chain. The effects of pH and acyl chain length on the rate of conversion to state B were consistent with this suggestion. The rate of conversion of the lipid–protein complex to state A or B decreased with increase in pH in all three lipids. Variation of the pH over the range of 4–8 primarily affects the ionizable groups of the protein and principally the histidine residues of which the imidazole pK is 6.5 (Smith, 1980). At least six of the histidines of basic protein are located in the middle of four hydrophobic or neutral segments of basic protein. Two of these segments also have acidic and other basic residues which are close enough that they may neutralize each other. Protonation of the histidines could decrease the number of hydrophobic segments which penetrate by two or three at most and would also decrease the length of two of them.

However, the magnitude of the maximum decrease in T_c determined from cooling scans did not seem to depend significantly on pH, suggesting that protonation of the histidines does not decrease the degree of penetration (depth and/or number of segments) enough to alter the perturbing effect on the lipid. The decrease in T_c does depend on protein concentration, however (Boggs & Moscarello, 1978). It is primarily the ability of the lipid to refreeze into the metastable state A and the stable state B which is affected by the protonation of the histidines. It is possible that as the protein, in a less protonated state, dips more deeply into the bilayer, the free segments of the lipid acyl chains are too short to contribute sufficient van der Waals interactions for the lipid to refreeze even if the chains interdigitate. The protein segments are also less likely to be frozen out.

A decrease in the fatty acid chain length under conditions where the degree of penetration remains constant should have a similar effect. Indeed, it was found that at similar pH values the rate of the exothermic process (conversion to state B) and also conversion to state A decreased in the order DSPG > DPPG > DMPG.

There is no direct proof that segments of basic protein penetrate into the bilayer. It has been suggested that proteins such as basic protein which seem to interact both electrostatically and hydrophobically may remain on the surface but disorder and deform the bilayer, allowing the lipid acyl chains to interact with a hydrophobic surface or pocket of the protein (Gulik-Krzywicki et al., 1969; Kimelberg & Papahadjopoulos, 1971). However, the consistency of the results presented here and previously on the effect of pH and fatty acid chain length on the interaction of basic protein with lipid, the dependence of this interaction on the lipid phase, and the different states of the complex which occur after various treatments, with the concept of penetration, help to support this hypothesis.

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