Hydrophobic and Electrostatic Interactions of Myelin Basic Protein with Lipid. Participation of N-Terminal and C-Terminal Portions†

Joan M. Boggs,* D. Denise Wood, and Mario A. Moscarello

ABSTRACT: The basic protein of myelin may be involved in association of the cytoplasmic surfaces of myelin by a bridging mechanism whereby the N-terminal half of the protein binds to one surface and the C-terminal half binds to the opposing surface. The feasibility of this mechanism was investigated by studying the ability of the N-terminal and C-terminal fragments, prepared by cleavage of the protein at the tryptophanyl residue 116, to interact both electrostatically and hydrophobically with bilayers of acidic lipids. Electrostatic and hydrophobic interactions were monitored from the effect of the protein and fragments on the phase transition temperature and fatty acid spin-label motion in different regions of the bilayer and from their ability to competitively inhibit Mn^{2+} binding to the lipid. Comparison with the effects of polylysine, divalent cations, and protonation (charge neutralization) of the lipid helped to assess the contribution of both hydrophobic and electrostatic interactions to the effect of basic protein and its fragments on the lipid. Both fragments, the N-terminal two-thirds and the C-terminal one-third, and the intact protein had a qualitatively similar effect on the lipid-phase transition temperature, and fatty acid chain packing suggesting that they interact hydrophobically with lipid in a similar way. The C-terminal fragment had quantitatively less effect, however. The amount of lipid protected from Mn^{2+} binding by the intact protein, N-terminal fragment, and C-terminal fragment was 26 ± 5, 17 ± 3, and 11 ± 1.5 molecules of lipid per molecule of protein, respectively. Since the number of positively charged amino acids per mole of protein at pH 7.4 is 31, 20, and 11, respectively, this suggests that nearly all of these residues participate in binding to acidic lipid head groups. It is therefore possible for each half of the protein to bind to opposing lipid bilayers or cytoplasmic surfaces in myelin through similar hydrophobic and electrostatic interactions, suggesting that a bridging mechanism is quite possible.

The forces which induce and maintain the multilamellar structure of myelin are not yet understood. Electron microscopy of myelin in hypotonic solutions indicates that the cytoplasmic regions in myelin are more closely and more tightly associated than the extracellular surfaces (McIntosh & Robertson, 1976). The basic protein of myelin is thought to be located exclusively on the cytoplasmic side of the membrane (Adams et al., 1971; Herndon et al., 1973; Poduslo & Braun, 1975; Golds & Braun, 1976; Crang & Rumsby, 1977) and may be responsible for this tight association of the cytoplasmic surfaces.

The mechanism of this association may be through dimerization of basic protein monomers bound to adjacent lamellae (Golds & Braun, 1978a,b; Smith & MacDonald, 1979; Smith, 1980) or, alternatively, through interlamellar interaction by a monomer of basic protein. A triproline segment almost in the middle of the sequence of basic protein (Brostoff & Eylar, 1971) must lead to a bend at this point, effectively dividing the molecule into two equal segments. Evidence for structured regions in the sequence near the triproline segment has been found by NMR spectroscopy (Chapman & Moore, 1976, 1978; Littlemore, 1978). The structure of the remainder of the protein in solution may be largely random coil (Epand et al., 1974; Krigbaum & Hsu, 1975; Martenson, 1978), but interaction with lipid induces α-helical and β structure (Anthony & Moscarello, 1971; Keniry & Smith, 1979). Interlamellar interaction of each of the segments on either side of the triproline sequence with opposing myelin lamellae would be an effective and nonrandom mechanism for bridging and associating the two surfaces.

It is necessary to understand how both halves of the protein interact with lipid to establish the feasibility of this mechanism. Basic protein can be conveniently cleaved with 2-(2-nitrophenylsulfenyl)-3-methyl-3′-bromoindolenine (BNPS-skatole)[1] at the single tryptophanyl residue at position 116, not far from the triproline sequence and the postulated bend, yielding a large N-terminal fragment, residues 1–116 (F-1), and a smaller C-terminal fragment, residues 117–170 (F-2). A study of the interaction of these fragments with lipid vesicles can help to understand the contribution of both halves of the intact protein. Previous studies with these fragments have led to the impression that the two halves of the protein do not interact in the same way with lipid (London et al., 1973; Jones & Rumsby, 1977).

The interaction of basic protein with lipid is complex since it appears to interact both electrostatically and hydrophobically. It is highly charged with 19% positively charged amino acids at pH 7.4 and interacts primarily with acidic lipids (Palmer & Dawson, 1969; Demel et al., 1973; London et al., 1973). However, inspection of the sequence of basic protein reveals the presence of 52% apolar and/or hydrophobic amino acids distributed throughout its sequence in 14 segments of 4–9 amino acids in length (Eylar et al., 1971; Boggs & Moscarello, 1978a). There is abundant evidence that, once electrostatic interaction has occurred, this protein also can interact hydrophobically with the lipid fatty acid chains, possibly by penetration of the hydrophobic segments into the bilayer and/or deformation of the bilayer (Gould & London, 1972; Papahadjopoulos, et al., 1973, 1975; Demel et al., 1973; London & Vossenberg, 1973; London et al., 1973; Boggs & Moscarello, 1978b; Boggs et al., 1980).

A study of the interaction of the N- and C-terminal fragments with lipid monolayers led to the conclusion that the N-terminal fragment interacted hydrophobically with lipid to a greater extent than the C-terminal fragment (London et al.,

† From the Research Institute, Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8. *Received July 10, 1980.* This investigation was supported by the Multiple Sclerosis Society of Canada and the Medical Research Council of Canada. J.M.B. is the recipient of a Career Development Award from the Multiple Sclerosis Society.

[1] Abbreviations used: BNPS-skatole, 2-(2-nitrophenylsulfenyl)-3-methyl-3′-bromoindolenine; ESR, electron spin resonance; F-1, N-terminal fragment; F-2, C-terminal fragment; Hepes, *N*-2-(hydroxyethyl)-*N*′-2-ethanesulfonic acid; DSC, differential scanning calorimetry.

0006-2960/81/0420-1065$01.25/0

1973) while a study of their interaction with lipids in a biphasic solvent system led to the conclusion that the sites for ionic interaction of the intact protein with lipid were located in the C-terminal region (Jones & Rumsby, 1977). On the basis of these studies, a model for interlamellar association in myelin with the N-terminal half of basic protein interacting hydrophobically with one myelin cytoplasmic surface and the C-terminal half interacting electrostatically with an adjacent cytoplasmic surface was derived (Rumsby & Crang, 1977). However, the basic and hydrophobic residues are distributed fairly evenly throughout the sequence, and indeed there are a nearly equal number of positively charged residues and hydrophobic segments of 5 or more amino acids on each side of the triproline sequence, suggesting that both halves should interact similarly with lipid.

The ability of the intact protein and its two fragments, formed by cleavage with BNPS-skatole, to interact hydrophobically and electrostatically with lipid has been reinvestigated in the present study. The contribution of hydrophobic interaction to the effect of the protein on lipid organization has been assessed by comparing the effects of basic protein and its fragments with the effects of polylysine, lipid protonation, and lipid complexation by Ca^{2+} or Mg^{2+} on the lipid-phase transition temperature and motion of fatty acid spin-labels intercalated into the lipid bilayer. The number of positively charged residues which can interact electrostatically with lipid has been measured by determination of the ability of the intact protein and its fragments to prevent the paramagnetic ion Mn^{2+} from binding to the lipid, using ESR spectroscopy to determine free Mn^{2+} concentration.

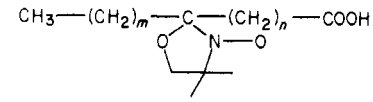
Materials and Methods

Preparation of Basic Protein. Myelin was isolated from normal human white matter and basic protein extracted from it by the method of Lowden et al. (1966). The protein was stored in the lyophilized form.

Cleavage of Basic Protein. Cleavage of the basic protein at the tryptophanyl residue using BNPS-skatole was carried out by the method of Martenson et al. (1975) with minor modifications. To 50 mg of basic protein in 75% acetic acid were added 280 μmol of BNPS-skatole and 400 μmol of excess tyrosine (endogenous tyrosine was 11.3 μmol). The reaction was carried out at 37 °C for 24 h. Reduction of the methionine side chains, which are partially oxidized during the cleavage, was accomplished by reacting the mixture with β-mercaptoethanol (0.3M) for 5 h at 37 °C. The reaction mixture was extracted with ethyl acetate to remove excess reagent. For precipitation of the peptide fragments, 10 volumes of cold acetone was added to the aqueous phase. The resulting precipitate was taken up in 0.01 N HCl and applied to a Sephadex G-75 (superfine) column (2.5 × 80 cm) equilibrated with 0.01 N HCl. The peptide fragments F-1 and F-2 which were eluted from the column were further purified by rechromatography on the same Sephadex G-75 column. After neutralization, the fragments were lyophilized and then desalted on a Bio-Gel P2 column. Amino acid analyses of the fragments were performed after hydrolysis in 5.7 N HCl 18 h at 110 °C and gave the predicted compositions.

Phosphatidic acid and phosphatidylglycerol prepared from egg phosphatidylcholine, phosphatidylserine, dimyristoylphosphatidic acid, and dipalmitoylphosphatidylglycerol were generous gifts from Dr. D. Papahadjopoulos, University of California Medical School, San Francisco. Egg phosphatidylethanolamine was isolated by the method of Hanahan et al. (1957). Cerebroside sulfate was purchased from Supelco (Bellefonte, PA). The lipids were chromatographically pure and were stored in chloroform under N_2 in sealed ampules at −20 °C.

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



I(*m*,*n*)

Polylysine (type VI, M_r 13 000) was purchased from Sigma. BNPS-skatole was purchased from Pierce, *N*-(2-hydroxyethyl)-*N'*-2-ethansulfonic acid (Hepes) was obtained from Calbiochem. $MnCl_2 \cdot 4H_2O$ was purchased from Fisher Scientific.

Preparation of Vesicles. The vesicles containing basic protein, fragments F-1 and F-2, or polylysine, to be used for differential scanning calorimetry (DSC) or labeling with a fatty acid spin-label, were prepared by suspending the dry lipid in buffer by vortex shaking, usually 2 mg/0.5 mL, at room temperature for phosphatidylglycerol and phosphatidic acid, 45 °C for dipalmitoylphosphatidylglycerol, and 55 °C for dimyristoylphosphatidic acid. The protein or fragments were then added in 0.5-mL buffer, and the sample was vortexed for an additional 10 min at the appropriate temperature. The buffer contained NaCl (10 mM), Hepes (2 mM), and EDTA (.1 mM) (except when divalent cations were used) adjusted to pH 7.4. Dipalmitoylphosphatidylglycerol was protonated by dispersing the lipid in 10 mM NaCl at pH 2. For studies of the effect of the divalent cations Mg^{2+} and Ca^{2+}, an excess of cation (lipid to cation mole ratio = 1:2) was added so that the final concentration of cation was 20 mM. The resulting precipitates were sedimented by centrifugation at 13000*g* at room temperature for 5 min and the pellets were used for calorimetry or spin-labeling. The pellets were washed and the lipid to protein ratio was determined. The supernatants were saved for analysis of lipid and protein. The lipid was determined by phosphorus analysis by a modified Bartlett (1959) procedure. Protein content was analysed by 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 vesicles to be used for Mn^{2+} binding were prepared by sonicating the lipid (10 μmol/mL) in Hepes buffer, pH 7.4, without EDTA, until the suspensions were opalescent. The vesicles were sonicated at room temperature for phosphatidic acid, phosphatidylglycerol, and phosphatidylserine and at 55 °C for cerebroside sulfate. Phosphatidylethanolamine was sonicated at pH 9.0 in borate buffer containing 10 mM NaCl according to the method of Stollery et al. (1978). The phosphatidylethanolamine vesicles were then dialyzed against the Hepes buffer at pH 7.4 for 2 h. Protein solutions were made up containing 0.5 μmol/mL in the Hepes buffer. A stock solution of 0.1 M $MnCl_2$ was made up in distilled water.

The protein and Mn^{2+} solutions were mixed together and adjusted to a final volume of 105 μL. The lipid solution (50 μL) was then added and the samples were sonicated in a bath sonicator for 20 min at room temperature to allow access of Mn^{2+} and protein to the inside of the vesicles. In some cases the order of addition of lipid, Mn^{2+} and protein was changed, but this made no difference. Aliquots of the lipid solution were taken for phosphorus analysis and of the protein solution for amino acid analysis and the final ratios of lipid to protein were adjusted. The final concentrations of Mn^{2+} varied from 1.6 mM to 20 mM and the final lipid concentration was 3.2 mM. Samples were taken up in 50-μL micropipets for ESR measurement of the signal due to Mn^{2+} free in solution. Mn^{2+}

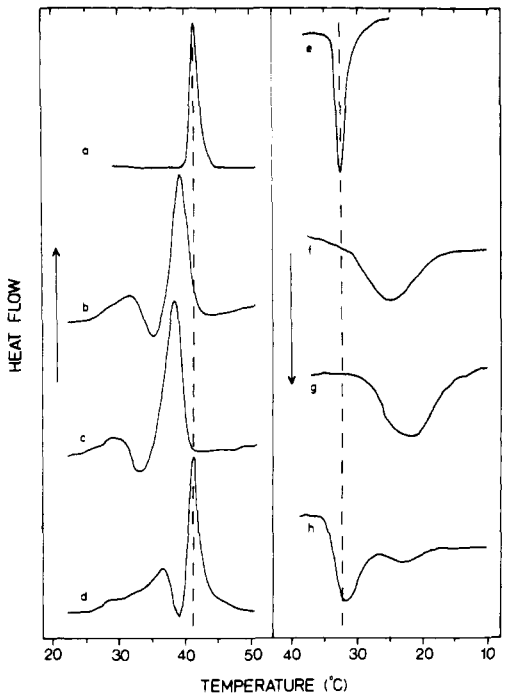


FIGURE 1: Differential scanning calorimetric thermograms of dipalmitoylphosphatidylglycerol vesicles obtained by heating (a–d) and cooling (e–h) at 10 °C/min. (a, e) Lipid only; (b, f) with 37.6% basic protein; (c, g) with 43% F-1; (d, h) with 32.2% F-2. There is normally 9–10 °C hysteresis in the T_c between heating and cooling at a rate of 10 °C/min. The dashed line indicates the T_c of the pure lipid on cooling and heating. The effect of the proteins is similar at slower heating rates, but the exothermic peak for F-2 on heating (d) is more apparent at a slower rate, 2.5 °C/min (not shown).

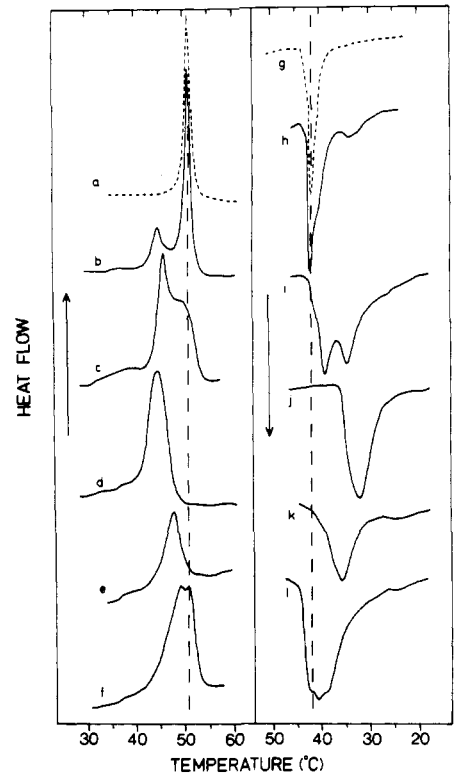


FIGURE 2: Differential scanning calorimetric thermograms of dimyristoylphosphatidic acid vesicles obtained by heating (a–f) and cooling (g–l) at 10 °C/min. (a, g) Lipid only; (b, h) with 15.6% basic protein; (c, i) with 27.4% basic protein; (d, j) with 40.1% basic protein; (e, k) with 38.7% F-1; (f, l) with 24.2% F-2. Other details as for Figure 1.

which is bound to the vesicles does not interfere with the much more intense signal of Mn^{2+} free in solution (Galla & Sackmann, 1975; Puskin, 1977). A standard curve of Mn^{2+} concentration vs. ESR signal height was obtained by using known concentrations of Mn^{2+}.

Differential Scanning Calorimetry. Samples were run on a Perkin-Elmer DSC-2 at heating or cooling rates of 5 or 10 °C/min. Slower cooling rates gave similar results.

Electron Spin Resonance Measurements. The vesicles were labeled with fatty acid spin-labels and the motional parameter, τ_0, and order parameter, S, where $S = (T_\parallel - T_\perp)/(T_{xx} - T_{zz})$ of the fatty acid spin-labels, was measured as described earlier (Boggs & Moscarello, 1978b) on a Varian E-4 spectrometer at 37 °C. $T_\parallel$ was also used as a measure of motion of the probe. The Mn^{2+} signal was measured at room temperature. Free Mn^{2+} in the vesicle suspensions was determined by measuring the height of one of the Mn^{2+} ESR peaks and obtaining the concentration from the standard curve measured under the same conditions.

Results

Effect of Proteins, Divalent Cations, and Protonation on Lipid-Phase Transition. The effect of the intact basic protein on the phase transition temperature of dipalmitoylphosphatidylglycerol and dimyristoylphosphatidic acid is shown in Figures 1 and 2. As reported earlier, basic protein decreases the phase transition temperature, T_c, of both of these lipids (Figure 1b,f, Figure 2b–d,h–j), which was interpreted to indicate hydrophobic interaction of the protein with the lipid (Papahadjopoulos et al., 1975; Boggs & Moscarello, 1978b). In contrast, 25% polylysine increases the phase transition temperature of dipalmitoylphosphatidylglycerol by 7 °C in agreement with a study by Papahadjopoulos et al. (1975) and of dimyristoylphosphatidic acid by 19 °C as indicated in Figure 3c,e and Table I. Higher concentrations have no further effect on the phase transition temperature of dipalmitoylphosphatidylglycerol but abolish the phase transition of dimyristoylphosphatidic acid at temperatures below 100 °C. Mg^{2+} increases the phase transition temperature of dipalmitoylphosphatidylglycerol by 13 °C for a metastable state which goes into a more stable state melting 30 °C higher (Table I), as reported by Van Dijck et al. (1975). However, Mg^{2+} raises the T_c of dimyristoylphosphatidic acid by only 13 °C. Protonation (neutralization) of the lipid raises the T_c of dipalmitoylphosphatidylglycerol by 19 °C (Watts et al., 1978) but decreases the T_c of dimyristoylphosphatidic acid by 7 °C (Table I) (Eibl & Blume, 1979).

Table I: Effect of Proteins, Mg^{2+}, and Protonation on the Phase Transition Temperature of Dipalmitoylphosphatidylglycerol and Dimyristoylphosphatidic Acid

	dipalmitoyl-phosphatidyl-glycerol T_c (°C)	dimyristoyl-phosphatidic acid T_c (°C)
control (pH 7.4)	42	52
basic protein[a]	30, 39	43.8
polylysine[a]	48.7	71.3
Mg^{2+}	55,[b] 72	65
protonation (pH 2)	61[c]	45[d]

[a] Maximum effect produced on heating scans. [b] First transition is metastable. [c] Watts et al. (1978). [d] Eibl & Blume (1979).

The basic protein has a greater effect when added to dipalmitoylphosphatidylglycerol above its phase transition temperature than below and also has a greater effect on cooling scans (Figure 1f) than on heating scans (Figure 1b). This led to the conclusion that the interaction can be reversed by supercooling (Papahadjopoulos et al., 1975; Boggs & Moscarello,

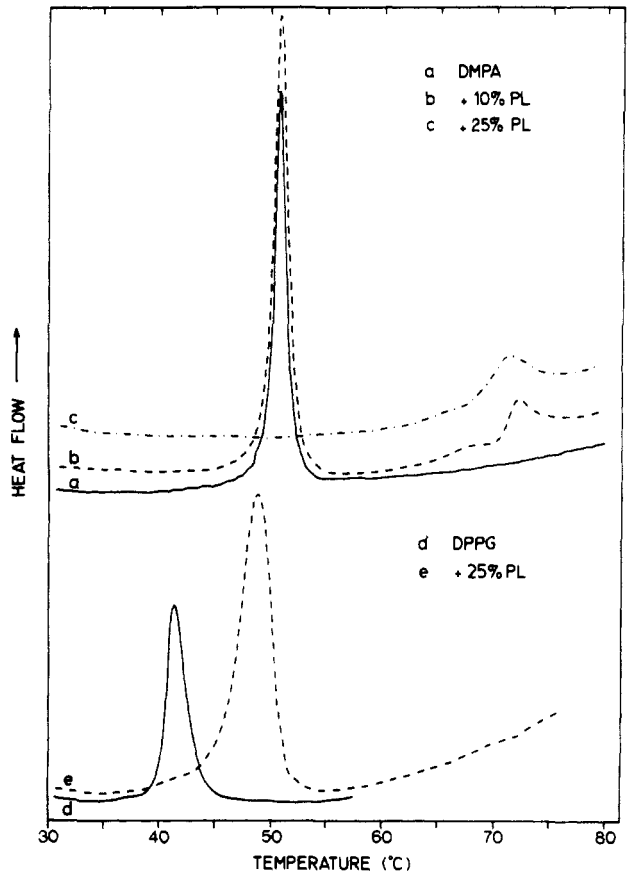


FIGURE 3: Differential scanning calorimetric thermograms of vesicles of dimyristoylphosphatidic acid containing (a) (—) 0, (b) (---) 10% and (c) (·····) 25% polylysine and dipalmitoylphosphatidylglycerol containing (d) (—) 0 and (e) (---) 25% polylysine. Heating rate 10 °C/min.

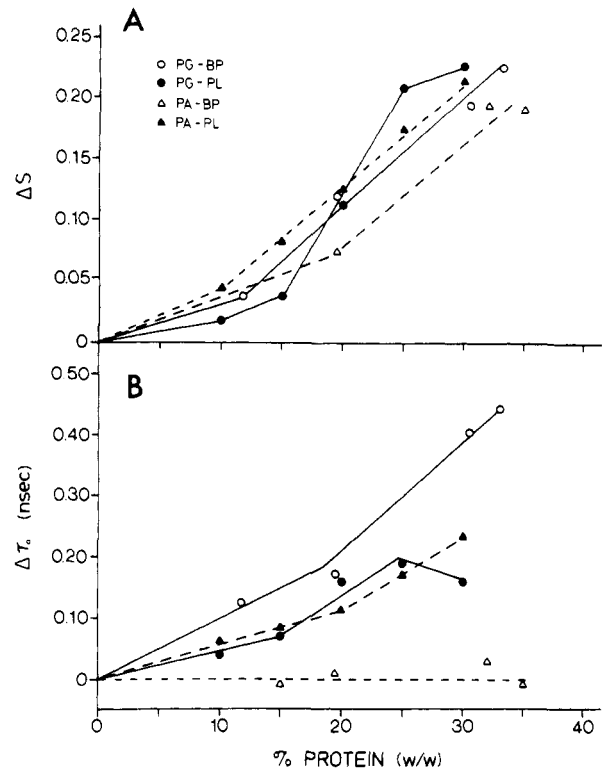


FIGURE 4: Effect of increasing concentration of basic protein or polylysine on (A) change in order parameter, ΔS, of 5-S-SL and (B) change in motional parameter, $\Delta\tau_0$, of 16-S-SL relative to the pure lipid in phosphatidic acid and phosphatidylglycerol vesicles. Phosphatidylglycerol and basic protein (○); phosphatidylglycerol and polylysine (●); phosphatidic acid and basic protein (△); and phosphatidic acid and polylysine (▲).

1978b; Boggs et al., 1980). However, in the case of dimyristoylphosphatidic acid the protein has a similar effect on the heating scans as on the cooling scans (Figure 2b–d,h–j). Thus the hydrophobic interaction may occur to a greater extent with the liquid crystalline phase of dipalmitoylphosphatidylglycerol than with the gel phase but occurs to a similar extent with the gel phase as with the liquid crystalline phase of dimyristoylphosphatidic acid. Basic protein also causes a small exothermic transition on heating for dipalmitoylphosphatidylglycerol (Figure 1b), indicating a change in phase structure to a more stable state (Boggs et al., 1980). This does not occur in dimyristoylphosphatidic acid (Figure 2b–d). These diverse effects of basic protein on these two lipids can be utilized to examine whether the two fragments behave similarly to the intact protein.

Effect of Fragments on Lipid-Phase Transition. F-1 and F-2 have qualitatively similar effects on dipalmitoylphosphatidylglycerol as intact basic protein as shown in Figure 1, parts c,g and d,h, for vesicles containing 43% (wt %) F-1 and 32.2% F-2, respectively, compared to a concentration of basic protein of 37.6% in Figure 1, parts b,f. Like the intact protein, both F-1 and F-2 decrease T_c to a greater extent on cooling (Figure 1g,h) than on heating and both cause the appearance of an exothermic transition on heating. This exothermic transition was even more apparent at a lower heating rate. The greater effect of F-1 compared to BP is probably due to the slightly greater concentration of F-1 in the vesicles. F-2 does not decrease T_c as much as the intact protein even if a similar concentration of BP is used.

The effect of increasing BP content on T_c of dimyristoylphosphatidic acid is shown in Figure 2b–d,h–j. Two components are present for 15.6% (Figure 2b,h) and 27.4% BP (Figure 2c,i), but at 40.1% BP (Figure 2d,j) there is only one component which melts 7–10 °C below the T_c of the pure lipid. Like the intact protein, the fragments also decrease T_c, have similar effects on the heating and cooling scans of this lipid, and do not produce an exothermic transition in dimyristoylphosphatidic acid. The effect of F-1 at a concentration of 38.7% (Figure 2e,k) is between that of a lower and higher concentration of BP (Figure 2, parts c,i and d,j). F-2 at a concentration of 24.2% (Figure 2f,l) does not have as great an effect as a similar concentration of intact protein (27.4% BP in Figure 2c).

Effect of Proteins, Divalent Cations, and Protonation on Fatty Acid Motion. The effect of basic protein on lipid fatty acid motion and orientation near the polar head group and in the interior of the bilayer was monitored by using 5-S-SL and 16-S-SL, respectively. The effect on these spin-labels in the liquid crystalline phase of unsaturated phosphatidylglycerol and phosphatidic acid, both prepared from egg phosphatidylcholine, is shown in Figure 4A,B. Basic protein produces a pronounced increase in the order parameter of 5-S-SL in both lipids (Figure 4A). It also increases the motional parameter of 16-S-SL in phosphatidylglycerol (Figure 4B) but has no effect on 16-S-SL in phosphatidic acid (Figure 4B), as reported earlier (Boggs & Moscarello, 1978b). The motional parameter is a measure of both rotational motion and the amplitude of motion since the motion of 16-S-SL is not very anisotropic.

Comparison with the effects of polylysine, divalent cations, and protonation (neutralization) of the lipid on fatty acid motion in these two lipids helps to understand the effect of basic protein. As shown in Figure 4 and Table II, polylysine increases the order parameter, S, of 5-S-SL (Figure 4A) and

Table II: Effect of Proteins, Divalent Cations, and Protonation on Order Parameter S and Motional Parameter τ_0 of Fatty Acid Spin-Labels in Phosphatidylglycerol and Phosphatidic Acid at 37 °C

	phosphatidyl-glycerol		phosphatidic acid	
	5-S-SL S	16-S-SL τ_0 (ns)	5-S-SL S	16-S-SL τ_0 (ns)
control (pH 7.4)	0.388	0.784	0.460	1.084
30% basic protein	0.584	1.190	0.656	1.084
30% polylysine	0.615	0.944	0.676	1.319
Mg^{2+}, Ca^{2+}	0.524	1.039	0.484	0.909
protonation (pH 2)	0.431	0.990	0.460	0.924

the motional parameter, τ_0, of 16-S-SL (Figure 4B) in both lipids. The effect of polylysine on 5-S-SL is similar to that of basic protein. However, the effect of polylysine on 16-S-SL in phosphatidylglycerol is less than that of basic protein while in phosphatidic acid the effect of polylysine is greater than that of basic protein. Complex formation between phosphatidylglycerol and divalent cations has similar effects on fatty acid motion as basic protein or polylysine, as indicated in Table II. Protonation of phosphatidylglycerol at low pH, which abolishes the repulsion of the negatively charged lipid head group and raises the phase transition temperature [(Watts et al. (1978) and Table I)], has less effect on fatty acid motion (Table II), suggesting that the effect of the proteins is more like complex formation than charge neutralization.

In contrast to phosphatidylglycerol, the order parameter of 5-S-SL in phosphatidic acid is much less affected by complexation with divalent cations than by interaction with the proteins, while the motional parameter of 16-S-SL is decreased rather than increased (Table II). The phase transition temperature of dimyristoylphosphatidic acid is also less affected by divalent cations than by polylysine (Table I). This may be due to disruption of intermolecular hydrogen bonding between adjacent phosphatidic acid molecules or to induction of the hexagonal phase by divalent cations (Papahadjopoulos et al., 1976). Participation of phosphatidic acid in intermolecular hydrogen bonds accounts for its high phase transition temperature (Jacobson & Papahadjopoulos, 1975; Eibl & Blume, 1979) and results in restriction of fatty acid motion in the interior of the bilayer, monitored by 16-S-SL, relative to phosphatidylglycerol, as indicated in Table II. Protonation of phosphatidic acid also has a disordering effect on both 5-S-SL and 16-S-SL (Table II) and has been shown to decrease the phase transition temperature due to disruption of intermolecular hydrogen bonds (Eibl & Blume, 1979).

Since the interaction of basic protein with the gel phase of dipalmitoylphosphatidylglycerol is different from the liquid crystalline phase, its effect on the motion of 16-S-SL in the gel phase and through the phase transition was studied. The increase in motion of 16-S-SL during the phase transitions of dipalmitoylphosphatidylglycerol and dimyristoylphosphatidic acid is indicated by a decrease in $T_\parallel$ at low temperatures and a decrease in τ_0 at high temperatures in Figure 5, parts A and B, respectively. Basic protein has a pronounced immobilizing effect on 16-S-SL in the gel phase of dipalmitoylphosphatidylglycerol (Figure 5A). At the concentration of basic protein used for Figure 5, two components are present in the spectrum at low temperatures. The $T_\parallel$ values for both components are indicated. Basic protein produces an increase in $T_\parallel$ from 22 to 32 G for the more immobilized component at 20 °C. A much smaller ordering effect is produced on the gel phase of dimyristoylphosphatidic acid (Figure 5B). Polylysine has a small disordering effect, protonation has little

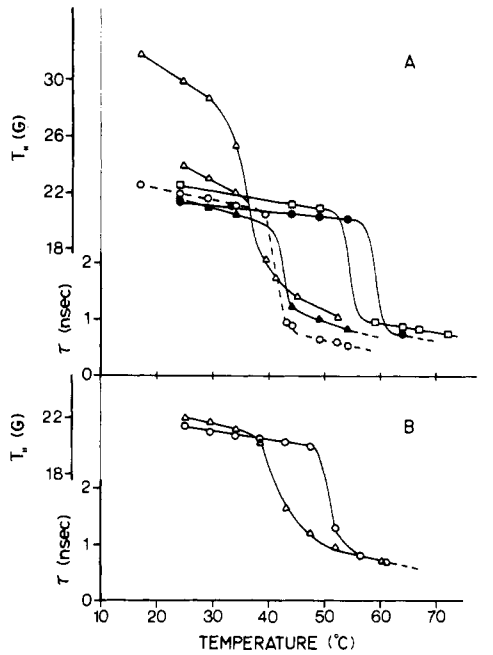


FIGURE 5: (A) Effect of temperature on ESR spectral parameters of 16-S-SL in dipalmitoyl phosphatidylglycerol. Lipid only (○) dashed line; with 28% basic protein (△); with 30% polylysine (▲); with Mg^{2+} (□); at pH 2 (●). (B) Effect of temperature on the ESR spectral parameters of 16-S-SL in dimyristoylphosphatidic acid. Lipid only (○) and with 33% basic protein (△). $T_\parallel$ is plotted at lower temperatures and τ_0 is plotted at higher temperatures where spectrum becomes more isotropic.

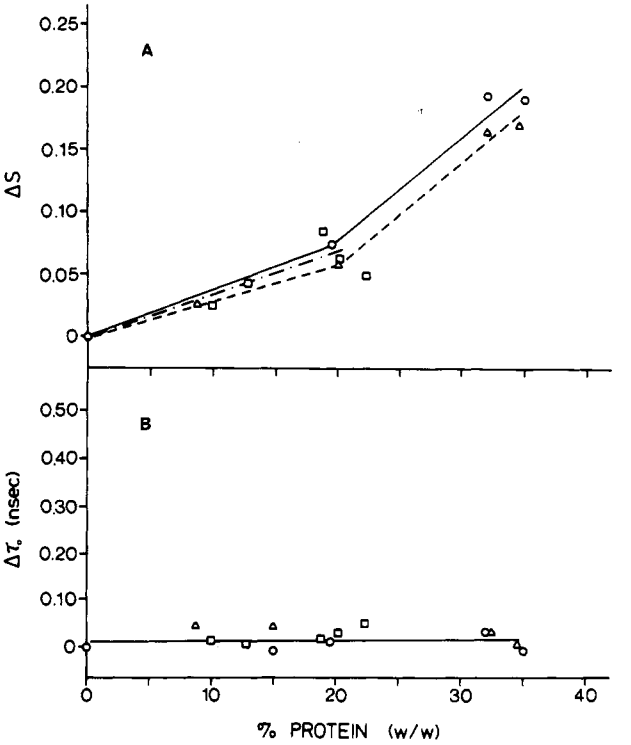


FIGURE 6: Effect of increasing concentration of basic protein and fragments in phosphatidic acid vesicles at 37 °C on (A) change in order parameter, ΔS, of 5-S-SL and (B) change in motional parameter, $\Delta\tau_0$, of 16-S-SL relative to the pure lipid. Basic protein (○) (—); F-1 (△---), F-2 (□·····).

effect, and complexation with divalent cations has only a small ordering effect on the gel phase of dipalmitoylphosphatidylglycerol (Figure 5A). The effects on the phase transition temperature are similar to those observed by DSC.

Effect of Fragments on Fatty Acid Motion. The effects of intact basic protein and its fragments on fatty acid motion in

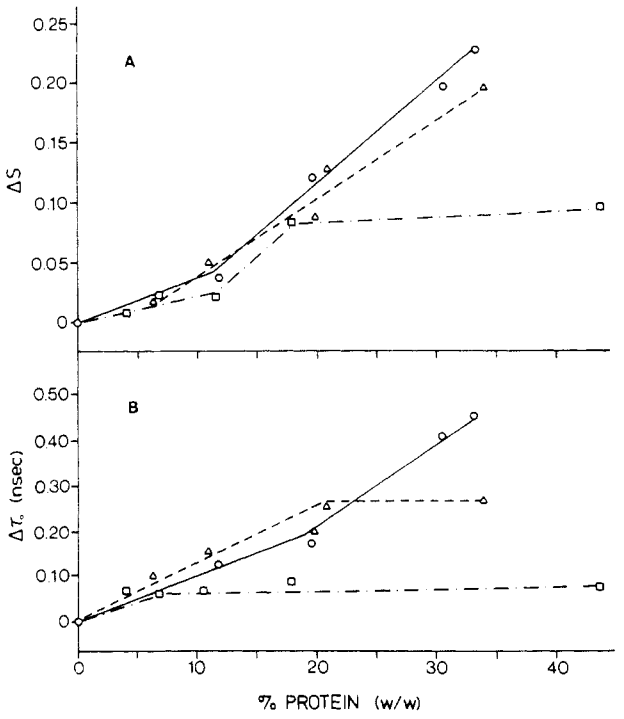


FIGURE 7: Effect of increasing concentration of basic protein and fragments in phosphatidylglycerol vesicles at 37 °C on (A) change in order parameter, ΔS, 5-S-SL, and (B) change in motional parameter, $\Delta\tau_0$, of 16-S-SL relative to the pure lipid. Basic protein (O—), F-1 (Δ- - -); F-2 (□·····).

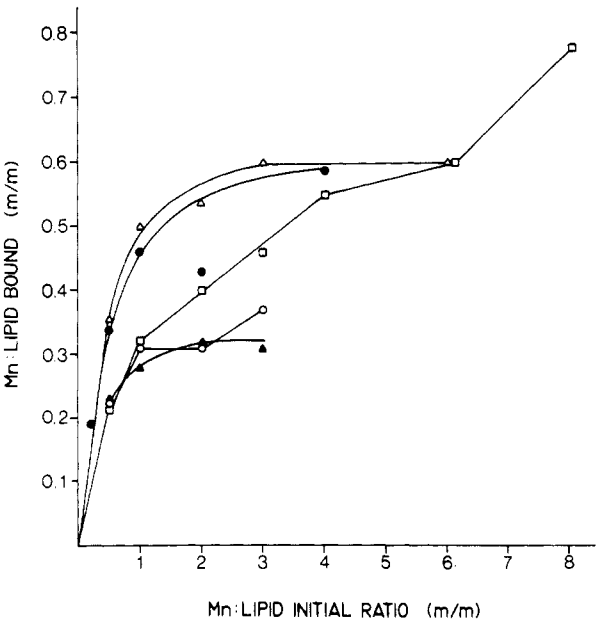


FIGURE 8: Mole ratio of bound Mn^{2+} to lipid plotted against initial mole ratio for phosphatidic acid (Δ), phosphatidylserine (●), phosphatidylglycerol (□), cerebroside sulfate (O) and phosphatidylethanolamine (▲).

phosphatidic acid and phosphatidylglycerol are compared in Figures 6 and 7, respectively. The changes in order parameter (ΔS) of 5-S-SL and the motional parameter ($\Delta\tau_0$) of 16-S-SL, relative to the pure lipid, produced by increasing concentration of protein are shown. The proteins are compared at equivalent weight percentages and therefore equal moles of amino acid, rather than moles of protein.

F-1 has a similar effect to those of BP on 5-S-SL and 16-S-SL in both phosphatidic acid (Figure 6) and phosphatidylglycerol (Figure 7) although the magnitude of the effect is not quite as large as that produced by the intact protein. F-2 has a similar effect to those of BP on 5-S-SL (Figure 6A) and 16-S-SL (Figure 6B) in phosphatidic acid up to a concentration of 22%, which was the highest concentration used. In phosphatidylglycerol, F-2 has a similar effect to those of BP on 5-S-SL (Figure 7A) up to a concentration of 17.5% where the curve then levels off. The effect of F-2 on 16-S-SL in phosphatidylglycerol was not nearly as great as those of F-1 and BP; $\Delta\tau_0$ leveled off at 7.0% F-2 content with no further change.

Competition with Mn^{2+}. The number of basic residues of the protein which can interact with acidic sites on lipids was estimated by determining the amount of Mn^{2+} which could be prevented from binding to the lipid by the protein. In this study it was desired to have enough Mn^{2+} to associate with all the lipid so that the protein would have to compete with Mn^{2+} in order to bind to the lipid. However, a large excess of free Mn^{2+} was undesirable, since this would make the measurements of small increases in free Mn^{2+} less accurate and might also prevent the protein from binding. Therefore the binding of Mn^{2+} to a number of lipids at various initial ratios of Mn^{2+} to lipid was investigated.

The ratio of bound Mn^{2+} to lipid was plotted against the initial ratio in Figure 8. Phosphatidic acid and phosphatidylserine become saturated at an initial ratio of 3:1, resulting in a ratio of bound Mn:lipid of 0.6:1. Phosphatidic acid was studied at pH 6.5 while the other lipids were at pH 7.4. The stoichiometry of binding of Mn^{2+} to phosphatidic acid at pH 5–8 has been shown to be 0.5:1 while at pH 8–10, where the lipid has two negative charges, it is 1:1 (Galla and Sackmann, 1975). Ca^{2+} has been found to bind to phosphatidylserine in a 0.5:1 ratio also (Bangham & Papahadjopoulos, 1966) at a concentration of 1 mM. However, Hauser et al. (1976) reported a ratio of 0.5:1 at concentrations less than 0.1 mM and a ratio of 1:1 at concentrations higher than 0.1 mM. Mn^{2+} binding to phosphatidylserine can cause dissociation of the proton from the amino group, resulting in further Mn^{2+} binding (Puskin & Coene, 1980). Further dissociation can probably occur with phosphatidic acid also. Under the conditions used in the present study the molar ratio of bound Mn^{2+} to lipid is 0.5–0.6:1 at initial ratios of Mn to lipid up to 6:1 (20 mM Mn^{2+}). The level of Mn bound to phosphatidylglycerol did not reach 0.5:1 until an initial ratio of 4:1 was used. Phosphatidylethanolamine was saturated at an initial ratio of 1:1, but the ratio bound was only 0.3:1. The ratio of Mn^{2+} bound to cerebroside sulfate was only 0.37:1 at an initial ratio of 3:1.

Since the lipids were saturated at initial ratios of Mn to lipid of 1:1 to 4:1, these ratios were generally used to determine the ability of basic protein to compete with Mn^{2+} for the acidic lipid head groups. Basic protein was added in ratios of lipid to protein of 125:1 to 80:1 so that there was an excess of lipid to basic residues on the protein. The amount of lipid bound to the protein was determined by multiplying the increase in amount of free Mn^{2+} by 2, assuming that the protein must bind to 2 molecules of lipid to prevent 1 molecule of Mn^{2+} from binding. Thus

$$\text{moles of PL/moles of BP} = 2(\Delta[Mn^{2+}])$$

where

$$\Delta[Mn^{2+}] = [Mn^{2+}]\ (+BP) - [Mn^{2+}]\ (\text{no BP})$$

The results are snown in Table III and indicate that approximately 26 ± 5 molecules of phosphatidic acid and phosphatidylserine are bound per molecule of basic protein at Mn:PL ratios up to 3:1. Above 3:1 the amount of lipid to

Table III: Molar Ratio of Phospholipid Bound to Basic Protein and Its Fragments Determined by Competition with Mn^{2+}

lipid	Mn:PL initial ratio[a]	PL:BP[a,b] bound	PL:F-1[a] bound	PL:F-2[a] bound
PA	1:1	25.6 ± 4.5		
	2:1	20.1 ± 4.6		
	3:1	22.2 ± 2.9		
	4:1	16.9 ± 12.0		
PS	1:1	25.1 ± 5.6	16.7 ± 2.9	10.6 ± 1.5
	2:1	26.1 ± 1.6	12.9 ± 1.1	2.6 ± 1.8
	4:1	9.2 ± 6.1	7.8	0
	6:1	0		
	8:1	0		
PG	1:1	14.1		
	1.5:1	16.4 ± 1.3		
	2:1	16.1 ± 1.7		
	6:1	0		
PE	1.4:1	14.1 ± 3.1		
	2:1	9.4		
	4:1	10.9		
		Cerebroside Sulfate		
	1:1	10.7 ± 3.4		
	2:1	10.5		

[a] Mole/mole. [b] At least six determinations were made at Mn:PL ratios of 1:1 and 2:1 for PA and PS, two determinations at higher ratios for PA and PS and for all other lipids.

BP decreases, indicating that higher concentrations of Mn^{2+} can compete successfully for the lipid and decrease protein binding. Since there are 31 positively charged residues in the intact protein, these results indicate that most of these residues bind to phosphatidic acid and phosphatidylserine vesicles even in the presence of Mn^{2+}. For phosphatidylglycerol, the results indicate that only 16 molecules of lipid bind to 1 molecule of protein. However, phosphatidylglycerol is not saturated at the concentration of Mn^{2+} used, and there may be enough available free lipid to bind some of the protein without preventing Mn^{2+} binding. It is also possible that in phosphatidylglycerol the inner layer of the vesicles is less accessible to Mn^{2+} unless high concentrations are used. Similar reasons may account for the low ratio of lipid to protein, indicated by competition with Mn^{2+}, of 14 and 10.7 for phosphatidylethanolamine and cerebroside sulfate, respectively.

Since the estimates of the number of basic residues bound to lipid was close to the total number available in the intact protein for phosphatidylserine, this lipid was chosen for investigation of the number of residues on the two fragments involved in binding to the lipid head groups. As shown in Table III, at an initial ratio of Mn^{2+} to lipid of 1:1, 16.7 ± 2.9 positively charged residues of the large fragment and 10.6 ± 1.5 positively charged residues of the small fragment can bind to phosphatidylserine. This is nearly equal to the amount of positively charged residues present in each fragment (20 and 11 for F-1 and F-2, respectively), and the sum is also equal to the number involved in the intact protein. At higher Mn^{2+} concentrations, however, the number of residues which participate in lipid binding decreases, even at a 2:1 ratio where no change is observed for the intact protein. This number decreases even more for the small fragment than for the large fragment. Thus Mn^{2+} can compete more effectively for the lipid against F-2 which has only 11 positively charged residues than it can with the intact protein with 31 residues.

Discussion

The effects of basic protein on lipid organization have been interpreted to indicate that hydrophobic regions of the protein can interact with the fatty acid chains of some acidic lipids while the basic residues interact electrostatically with the polar head groups. Comparison with the effects of polylysine, a polypeptide which can probably only interact electrostatically, divalent cations, and protonation reinforces this interpretation. The hydrophobic interactions of basic protein cause, among other effects, a decrease in the phase transition temperature of phosphatidylglycerol and phosphatidic acid while the electrostatic interactions of polylysine cause an increase in the phase transition temperature as shown by Papahadjopoulos et al. (1975) for dipalmitoylphosphatidylglycerol and in the present study for dimyristoylphosphatidic acid. Polylysine has also been found to decrease the surface pressure of lipid monolayers initially, indicating a contraction of the film, although this is later followed by an increase in surface pressure (Shafer, 1974). However, basic protein causes a much greater increase in surface pressure (Demel et al., 1973).

Complexation with divalent cations also causes an increase in the phase transition temperature of phosphatidylglycerol and phosphatidic acid. Neutralization increases the phase transition temperature of phosphatidylglycerol but decreases the phase transition temperature of phosphatidic acid due to loss of intermolecular hydrogen bonding for this lipid. The effect of basic protein on the phase transition temperature of phosphatidic acid is similar to that of neutralization. That its effect is not due to neutralization, however, is evident from the effect of polylysine which can also neutralize the charge on phosphatidic acid, yet raises its T_c. Therefore, although binding of basic residues of proteins to phosphatidic acid does neutralize its charge, this does not result in a decrease in T_c. The electrostatic interaction must be accompanied by a distortion of the lipid packing, due to hydrophobic interaction of segments of the protein with the lipid fatty acid chains, in order to cause a decrease in the T_c.

Hydrophobic interaction and penetration of the protein partially into the bilayer should increase the order parameter and decrease the motion near the polar head group but might be expected to have no effect or to increase the motion (decrease τ_0) in the interior of the bilayer. However, a strong electrostatic interaction and complexing effect will decrease the motion both near the polar head group and in the interior of the bilayer, as is evident from the effects of divalent cations on phosphatidylglycerol and polylysine on phosphatidylglycerol and phosphatidic acid. The effect of basic protein on spin-label motion in phosphatidic acid is consistent with hydrophobic interaction and much different from the effect of polylysine. However, the effect of basic protein on the liquid crystalline phase of phosphatidylglycerol is similar to that of polylysine even though basic protein decreases the T_c of dipalmitoylphosphatidylglycerol while polylysine increases it. Indeed basic protein decreases the motion of 16-S-SL in phosphatidylglycerol even more than polylysine.

This difference is even greater in the gel phase of dipalmitoylphosphatidylglycerol. The DSC results suggested that the hydrophobic interaction is partially reversed below the phase transition and that the interaction of basic protein with the gel phase of this lipid is mainly electrostatic. However, basic protein restricts the motion of 16-S-SL in the gel phase of dipalmitoylphosphatidylglycerol much more than polylysine, divalent cations, or protonation. Therefore, although the effects of basic protein are not the effects expected for hydrophobic interaction, they are also much different from the effects of electrostatic interaction.

This restriction of motion of 16-S-SL in the gel phase may be due to interdigitation of the fatty acid chains of di-

palmitoylphosphatidylglycerol in the presence of basic protein. Expansion of the lipid would be necessary to accommodate hydrophobic segments of the protein, and interdigitation of the terminal ends of the fatty acid chains would allow better packing in the gel phase and restrict the motion of 16-S-SL. In the liquid crystalline state this interdigitation may be considerably reduced, resulting in less restriction of the motion of 16-S-SL compared to the gel phase but more restriction than in the liquid crystalline phase of phosphatidic acid. Interdigitation of the fatty acid chains has been observed by X-ray diffraction in the gel state of dipalmitoylphosphatidylglycerol at low ionic strength (Ranck et al., 1977). Further studies on the phase behavior with basic protein are in progress (J. M. Boggs et al., unpublished results).

The fragments behaved in a qualitatively similar way to the intact protein in their effect on both the lipid-phase transition temperature and fatty acid spin-label motion, suggesting that they also interact hydrophobically with lipid although there were quantitative differences between the effect of F-2 and that of basic protein. The effect of the fragments was compared to the intact protein on the basis of equal moles of amino acid and therefore equal weight. If they are compared on the basis of moles of peptide, the fragments, particularly the smaller C-terminal fragment, have much less effect than the intact protein. London et al. (1973) concluded that the fragments, particularly F-2, caused much less expansion of lipid monolayers than the intact protein, but compared them on the basis of moles of protein. If the data presented in London's study are compared on the basis of equal moles of amino acid, the small fragment increases the surface pressure nearly as much as the large fragment, in agreement with the results presented here.

The effects of F-1 and F-2 on T_c and fatty acid motion reported here and on the area of monolayers reported by London et al. (1973) suggest that both fragments can interact hydrophobically although the small fragment may not do so to as great an extent as the large fragment. These results also indicate that the tryptophan region is not required for this interaction, although it may contribute in the intact protein. An increase in α-helical and β structure upon interaction with lipid, similar to that which occurs with the intact protein, has been observed in both fragments (R. M. Epand et al., unpublished results), indicating that the conformation and interaction with lipid of the isolated fragments may be similar to the behavior of these portions when in the intact protein. Thus, in the intact protein, the hydrophobic segments in the C-terminal portion beyond the triproline segment should be able to participate in hydrophobic interactions to a similar extent as the N-terminal portion.

The number of positively charged residues of each fragment as well as of the intact protein which participate in electrostatic interaction with acidic lipids was measured from their ability to inhibit Mn^{2+} from binding to the lipid polar head groups. Basic protein was able to compete with Mn^{2+} for the lipid polar head groups provided that the ratio of Mn^{2+} to lipid was less than 3:1. Even if Mn^{2+} was allowed to bind to the lipid vesicles first, the protein was able to displace it. However, this was after sonication which may have transiently disrupted the Mn^{2+}–lipid complex. Demel et al. (1973) reported earlier that basic protein could displace Ca^{2+} from cerebroside sulfate monolayers. Since the protein was able to prevent Mn^{2+} binding, the increase in free Mn^{2+} in the presence of protein could be used to quantitate the number of positively charged residues in the protein which participate in electrostatic binding to lipid. This led to an estimate that 26 ± 5 positively charged residues in the intact protein bind to phosphatidylserine and phosphatidic acid. Thus almost all or possibly all of the positively charged residues in the intact protein can bind electrostatically to phosphatidylserine and phosphatidic acid. This is consistent with our earlier estimate based on the extent of phase separation produced by the protein in a mixture of acidic and neutral lipids (Boggs et al., 1977).

Nearly all of the positively charged residues of the N-terminal and C-terminal fragments (17 ± 3 for F-1, 11 ± 2 for F-2) also were able to participate in binding to lipid and prevention of Mn^{2+} binding at Mn:lipid ratios of 1:1. However, higher concentrations of Mn^{2+} were able to compete more effectively with the fragments for lipid than with the intact protein. This was especially true for the small fragment and is presumably due to the lower multivalency which reduces the affinity of the fragments for the lipid.

This study indicates that basic protein is fairly homogeneous throughout its sequence with regard to hydrophobic and electrostatic interactions with lipid. More subtle and specific effects may of course by localized to one or more sites in the protein, but such effects would be related to the specific amino acid composition rather than to the charge or relative polarity or hydrophobicity. Both halves of basic protein on either side of the triproline segment, the postulated hinge region, can interact in a similar way with lipids. This interaction might be with a single bilayer or could also be interlamellar, with one half interacting with one bilayer and the other half interacting with an adjacent bilayer. A model has been presented elsewhere (Boggs et al., 1981). This would be an effective and nonrandom mechanism for association of two lamellae. Other proteins including polylysine and each of the fragments of basic protein can cause aggregation of lipid vesicles, indicating that they too can bridge two bilayers. However such an interaction would be random and possibly not suitable for producing an ordered structure such as myelin. Some specificity of the interlamellar interaction of basic protein compared to its fragments and two other basic proteins, cytochrome *c* and polylysine, has been demonstrated in X-ray diffraction studies (Mateu et al., 1973; Gulik-Krzywicki et al., 1969), where basic protein was shown to bind together alternating layers of cerebroside sulfate and a mixture of other lipids.

Whether or not this mechanism of interlamellar interaction by a monomer of basic protein occurs in myelin cannot be concluded at present. Basic protein also dimerizes, and a dimerization mechanism has been suggested for interlamellar association in myelin (Golds & Braun, 1978a,b; Smith & McDonald, 1979; Smith, 1980). Smith & MacDonald (1979) showed that basic protein in sodium dodecyl sulfate was a dimer at low detergent concentrations but was a monomer at higher detergent concentrations and that it bound detergent micelles together in a ratio of one molecule of basic protein per two micelles. Thus one molecule of basic protein is capable of joining two lamellae together, and the results presented here indicate that both hydrophobic and electrostatic interactions could be involved in the association with each lamella.

Acknowledgments

We thank Dr. D. Papahadjopoulos for generous gifts of most of the lipids used in this study, Dr. N. Z. Stanacev for use of his ESR spectrometer, and L. Kashuba and D. Stamp for expert technical assistance.

References

Adams, C. W. M., Bayliss, O. B., Hallpike, J. F., & Turner, D. R. (1971) *J. Neurochem. 18*, 389.

Anthony, J. S., & Moscarello, M. A. (1971) *Biochim. Biophys. Acta 243*, 429.
Bangham, A. D., & Papahadjopoulos, D. (1966) *Biochim. Biophys. Acta 126*, 181.
Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466.
Boggs, J. M., & Moscarello, M. A. (1978a) *Biochim. Biophys. Acta 515*, 1.
Boggs, J. M., & Moscarello, M. A. (1978b) *J. Membr. Biol. 39*, 75.
Boggs, J. M., Moscarello, M. A., & Papahadjopoulos, D. (1977) *Biochemistry 16*, 5420.
Boggs, J. M., Stollery, J. G., & Moscarello, M. A. (1980) *Biochemistry 19*, 1226.
Boggs, J. M., Moscarello, M. A., & Papahadjopoulos, D. (1981) in *Molecular Biology of Lipid-Protein Interactions* (Griffith, O. H., & Jost, P., Eds.) Wiley-Interscience, New York (in press).
Brostoff, S., & Eylar, E. H. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 765.
Chapman, B. E., & Moore, W. J. (1976) *Biochem. Biophys. Res. Commun. 73*, 758.
Chapman, B. E., & Moore, W. J. (1978) *Aust. J. Chem. 31*, 2367.
Crang, A. J., & Rumsby, M. G. (1977) *Biochem. Soc. Trans. 5*, 110.
Demel, R. A., London, Y., Geurts van Kessel, W. S. M., Vossenberg, F. G. A., & van Deenen, L. L. M. (1973) *Biochim. Biophys. Acta 311*, 507.
Eibl, H., & Blume, A. (1979) *Biochim. Biophys. Acta 553*, 476.
Epand, R. M., Moscarello, M. A., Zirenberg, B., & Vail, W. J. (1974) *Biochemistry 13*, 1264.
Eylar, E. H., Brostoff, S., Hashim, G., Caccam, J., & Burnet, P. (1971) *J. Biol. Chem. 246*, 5770.
Galla, H. J., & Sackmann, E. (1975) *Biochim. Biophys. Acta 401*, 509.
Golds, E. E., & Braun, P. E. (1976) *J. Biol. Chem. 251*, 4729.
Golds, E. E., & Braun, P. E. (1978a) *J. Biol. Chem. 253*, 8162.
Golds, E. E., & Braun, P. E. (1978b) *J. Biol. Chem. 253*, 8171.
Gould, R. M., & London, Y. (1972) *Biochim. Biophys. Acta 290*, 200.
Gulik-Krzywicki, T., Schechter, E., Luzzatti, V., & Faure, M. (1969) *Nature (London) 223*, 1116.
Hanahan, D. J., Dittmer, J. C., & Warshima, E. (1957) *J. Biol. Chem. 228*, 685.
Hauser, H., Darke, A., & Phillips, M. C. (1976) *Eur. J. Biochem. 62*, 335.
Herndon, R. M., Rouch, H. C., & Einstein, E. R. (1973) *Immunol. Commun. 2*, 163.
Jacobson, K., & Papahadjopoulos, D. (1975) *Biochemistry 14*, 152.
Jones, A. J. S., & Rumsby, M. G. (1977) *Biochem. J. 167*, 583.
Keniry, M. A., & Smith, R. (1979) *Biochim. Biophys. Acta 578*, 381.
Krigbaum, W. R., & Hsu, T. S. (1975) *Biochemistry 14*, 2542.
Littlemore, L. A. T. (1978) *Aust. J. Chem. 31*, 2387.
London, Y., & Vossenberg, F. G. A. (1973) *Biochim. Biophys. Acta 307*, 478.
London, Y., Demel, R. A., Geurts van Kessel, W. S. M., Vossenberg, F. G. A., & van Deenen, L. L. M. (1973) *Biochim. Biophys. Acta 311*, 520.
Lowden, J. A., Moscarello, M. A., & Morecki, R. (1966) *Can. J. Biochem. 44*, 567.
Martenson, R. E. (1978) *J. Biol. Chem. 253*, 8887.
Martenson, R. E., Deibler, G. E., Kramer, A. J., & Levine, S. (1975) *J. Neurochem. 24*, 173.
Mateu, L., Luzzatti, V., London, Y., Gould, R. M., Vossenberg, F. G. A., & Olive, J. (1973) *J. Mol. Biol. 75*, 697.
McIntosh, T. J., & Robertson, J. D. (1976) *J. Mol. Biol. 100*, 213.
Palmer, F. B., & Dawson, R. M. C. (1969) *Biochem. J. 111*, 629.
Papahadjopoulos, D., Cowden, M., & Kimelberg, H. (1973) *Biochim. Biophys. Acta 330*, 8.
Papahadjopoulos, D., Moscarello, M., Eylar, E. H., & Isac, T. (1975) *Biochim. Biophys. Acta 401*, 317.
Papahadjopoulos, D., Vail, W. J., Pangborn, W. A., & Poste, G. (1976) *Biochim. Biophys. Acta 448*, 265.
Poduslo, J. F., & Braun, P. E. (1975) *J. Biol. Chem. 250*, 1099.
Puskin, J. S. (1977) *J. Membr. Biol. 35*, 39.
Puskin, J. S., & Coene, M. T. (1980) *J. Membr. Biol. 52*, 69.
Ranck, J. L., Keira, T., & Luzzatti, V. (1977) *Biochim. Biophys. Acta 488*, 432.
Rumsby, M. G., & Crang, A. J. (1977) *Cell Surf. Rev. 4*, 248.
Shafer, P. T. (1974) *Biochim. Biophys. Acta 373*, 425.
Smith, R. (1980) *Biochemistry 19*, 1826.
Smith, R., & McDonald, B. J. (1979) *Biochim. Biophys. Acta 554*, 133.
Stollery, J. G., & Vail, W. J. (1978) *Biochim. Biophys. Acta 471*, 372.
Van Dijck, P. W. M., Ververgaert, P. H. J. Th., Verkleij, A. J., van Deenen, L. L. M., & de Gier, J. (1975) *Biochim. Biophys. Acta 406*, 465.
Watts, A., Harlos, K., Maschke, W., & Marsh, D. (1978) *Biochim. Biophys. Acta 510*, 63.