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Variable Interaction of Spin-Labeled Human Myelin Basic Protein with Different Acidic Lipids†

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ABSTRACT: Myelin basic protein from the human central nervous system has been covalently spin-labeled with an iodoacetamide spin-label at its two methionyl residues at positions 21 and 167 from the N terminus of the protein. The spin-labeled protein, which is thought to bind electrostatically to acidic lipids followed by interaction of some hydrophobic segments with the lipid bilayer, was added to lipid vesicles in order to monitor the behavior of the protein in the lipid environment. A variety of lipids were used with which the degree of hydrophobic interaction seems to vary. On the basis of the effect of the protein on the organization of these lipids, the hydrophobic interaction seems to be greatest for phosphatidylglycerol and phosphatidic acid and decreases in the order phosphatidylserine > cerebroside sulfate ≥ phosphatidylethanolamine. The ESR spectra of the spin-labeled protein in lipid vesicles of phosphatidylglycerol and phosphatidic acid possessed two or three components at low temperatures, one immobilized and other more mobile components. At higher temperatures in these two lipids and in the other lipids at all temperatures, a single component mobile spectrum was observed with hyperfine splitting indicative of a relatively polar environment. However, the motional parameter τ_0 of the protein spin-label in vesicles was greater than that for the protein in solution and varied depending on the lipid. The greatest reduction of motion was observed with phosphatidylglycerol, followed in order by phosphatidic acid, cerebroside sulfate, phosphatidylserine, cardiolipin, and phosphatidylethanolamine. Measurement of the protein spin-label mobility at temperatures where the lipids possessed identical order parameters ($S = 0.4$) suggested that the order of the hydrocarbon chains of the different lipids was not the main factor in determining the probe motion. We conclude that the motion of the spin-label on the methionyl residues reflects different degrees of hydrophobic interaction of some regions of the protein with the bilayer and attribute this variability in hydrophobic interaction to the occurrence of intermolecular electrostatic and hydrogen bonding for some of the lipids.

In recent years, considerable work has been directed toward characterizing the interaction of the peripheral membrane proteins with different lipid species. Several types of interaction have so far been demonstrated (Papahadjopoulos et al., 1975; Susi et al., 1979). One type is characterized by the interaction of polylysine with lipids (Papahadjopoulos et al., 1975; Susi et al., 1979; Hartmann & Galla, 1978; Hammes & Schuller, 1970). In this case, the interaction involves an electrostatic binding of the polymer to the lipid head group with little or no hydrophobic interaction. A second type of interaction also involves electrostatic binding between protein and lipid, but the protein also appears to interact hydrophobically with the lipid bilayer, either by penetrating partway into the bilayer or by deforming the bilayer (Papahadjopoulos et al., 1975; Hartmann et al., 1978). There is considerable

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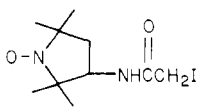
evidence to suggest that myelin basic protein is such a protein. It has been shown that the protein can increase the permeability of liposomes to glucose (Gould & London, 1972) and Na^+ (Papahadjopoulos et al., 1975). Furthermore, the protein expands lipid monolayers (Demel et al., 1973; London et al., 1973) and portions of the protein are protected from enzymatic hydrolysis in the presence of lipid (London & Vossenberg, 1973; London et al., 1973). Using differential scanning calorimetry, Papahadjopoulos et al. (1975) were able to show that the basic protein reduced the enthalpy and temperature of the lipid phase transition when combined with the acidic phospholipid dipalmitoylphosphatidylglycerol. Furthermore, it has been demonstrated by using fatty acid spin-label probes that the basic protein distorts the packing of fatty acid chains by decreasing the motion of the lipid near the polar head group while having little or no effect in the interior of the bilayer (Boggs & Moscarello, 1978a).

Coincident with these studies, it has been noted that the protein interacts to a different degree with each lipid studied (London & Vossenberg, 1973; Demel et al., 1973; London et al., 1973; Boggs & Moscarello, 1978a). It is likely that differences in the lipid packing characteristics and polar head group interactions can influence the extent to which the hydrophobic regions of the protein can interact with the lipid fatty acid chains. Since this protein induces a demyelinating disease in animals in which an immune response against myelin occurs (Kies, 1965; Eylar, 1972) and may also be involved in multiple sclerosis, it is important to understand how its interaction with lipid and its conformation can depend on lipid composition.

In order to demonstrate more fully the nature of this complex interaction of myelin basic protein with different lipids, we have labeled the two methionines found at positions 21 and 167 from the N terminus of the protein with a spin-label probe and incorporated this labeled protein into liposomes composed of different acidic phospholipids. A low concentration of protein was used so that all of the protein was bound electrostatically to each of the lipids used. Variation in the degree of the immobilizing effect of the lipids on the spin-label probes is interpreted in terms of differences in the degree of hydrophobic interaction of the protein with the lipid bilayer. The degree of hydrophobic interaction is also correlated with the chemical properties of the polar head groups.

Materials and Methods

Materials. Spin-labels 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (I) and 5-doxylstearic acid (II; $m = 12$; $n = 3$) were purchased from Syva (Palo Alto, CA). Doxyl represents the 4′,4′-dimethyloxazolidinyl-1-oxy derivative of the parent ketone.



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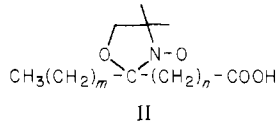

II

Phosphatidic acid and phosphatidylglycerol (synthesized from egg phosphatidylcholine) and bovine brain phosphatidylserine were generous gifts from Dr. D. Papahadjopoulos (University of California, San Francisco). Phosphatidylethanolamine was isolated from hen's egg yolk by the method of Bangham et al. (1974) and purified on silicic acid by the method of Hanahan et al. (1957). Bovine cardiolipin and cerebroside sulfate were purchased from Supelco (Bellefonte, PA).

The fatty acid compositions of egg phosphatidylcholine, from which the phosphatidic acid and phosphatidylglycerol were derived, egg phosphatidylethanolamine, and bovine brain phosphatidylserine have been published by Papahadjopoulos & Miller (1967). These lipids contain primarily 16:0, 18:0, and 18:1. The cardiolipin contains 90% 18:2 (information supplied by Supelco) while cerebroside sulfate contains primarily 24:0, 24:1, 24h:0, and 24h:1 (Svennerholm & Stallberg-Stenhagen, 1968).

Preparation and Spin-Labeling of Basic Protein. Human myelin was purified from normal white matter, and basic protein was extracted from it by the method of Lowden et al. (1966). For spin-labeling, the basic protein (1 μmol) was dissolved in 4.0 mL of glass-distilled water, pH 5.5. To this was added a 30-fold molar excess of the iodoacetamide spin-label. The solution was flushed with nitrogen and incubated at 40 °C for 24 h. One drop of chloroform was added to prevent bacterial contamination. The excess spin-label was removed by exhaustive dialysis against distilled water (4 °C). The modified protein was lyophilized and stored as a dry powder at −20 °C.

By use of [^{14}C]iodoacetamide to monitor the extent of the reaction, it was found that 1.9 mol of iodoacetamide per mol of protein was transferred. The amount of iodoacetamide spin-label bound was quantitated by hydrolyzing an aliquot of the protein solution in 1 M NaOH at 60 °C for 24 h to release the spin-label (Wien et al., 1972). The concentration of spin-label in the hydrolyzed sample was obtained by comparing the signal intensity to a standard curve obtained from known concentrations of a water-soluble spin-label. Another aliquot was hydrolyzed in 5.7 N HCl in an evacuated tube at 110 °C for 20 h to determine the protein concentration by amino acid analysis.

In order to determine which amino acids in the protein had been labeled, we oxidized a sample of the spin-labeled basic protein with performic acid by the method of Hirs (1956) in order to oxidize unmodified methionyl residues. Since the protein does not contain cysteine, failure to isolate methionine sulfone on amino acid analysis is evidence that the methionines have been modified. Samples of unmodified basic protein and the spin-labeled protein were dissolved separately in distilled water. An equal volume of performic acid (87% formic acid–30% H_2O_2, 19:1) was added to each protein solution. After 2.0 h, at 4 °C the reactions were stopped by dilution with distilled water and the reagents were removed by lyophilization. The amount of methionine sulfone generated in each sample was determined by amino acid analysis on a Durrum D500 amino acid analyzer after hydrolysis with 5.7 N HCl in an evacuated tube at 110 °C for 20 h. Alkylation of any histidine residues by the spin-label can be detected by the presence of (carboxymethyl)histidine in the protein hydrolysates. In order to explore this possibility, we prepared 1- and 3-(carboxymethyl)histidine and bis(carboxymethyl)-histidine by the method of Crestfield et al. (1963), and their elution profiles were determined on the Durrum D500 amino acid analyzer.

Preparation of Protein–Lipid Vesicles. For the preparation of multilayered vesicles from phosphatidylglycerol, phosphatidic acid, phosphatidylserine, and cerebroside sulfate, 50 μL of buffer (10 mM NaCl, 2 mM Hepes,[1] and 0.1 mM EDTA, pH 7.4) was added to 1.0 mg of lipid which had been dried previously under nitrogen and placed in a vacuum desiccator for 0.5 h. Liposomes were prepared above the phase transition of the lipid by warming to 37 °C (55 °C for cerebroside

[1] Abbreviations used: ESR, electron spin resonance; DSC, differential scanning calorimetry; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

sulfate) and vortexing for 2 min in order to form multilamellar vesicles. The covalently modified protein (40 μg) in 20 μL of water was added to each vesicle preparation, and the sample was vortexed again. Fifty microliters of the vesicle suspensions was taken up in 50-μL disposable micropipets to obtain a sample for electron spin resonance measurements.

Phosphatidylethanolamine does not readily swell in water to form phospholipid vesicles (Stollery & Vail, 1977), and some difficulty was also experienced in suspending the cardiolipin. Therefore, vesicles were prepared from phosphatidylethanolamine and cardiolipin by the method of Boggs & Moscarello (1978a). Briefly, 5 mg of each lipid was dissolved in 1 mL of 2-chloroethanol, and the vesicles were formed by dialysis of this solution against buffer (10 mM NaCl, 2 mM Hepes, and 0.1 mM EDTA, pH 7.4). The vesicles were sedimented by centrifugation in an Eppendorf microcentrifuge at 10000 rpm for 5 min. Protein was then added as described above.

All of these lipids form large multilayered vesicles (Papahadjopoulos & Miller, 1967). Freeze–fracture electron microscopy showed that phosphatidylethanolamine vesicles prepared by dialysis from 2-chloroethanol formed large spherical vesicles which were probably multilayered (J. G. Stollery and W. J. Vail, unpublished observations). The sodium form of cardiolipin is lamellar (Rand & Sengupta, 1972). The cardiolipin vesicles prepared by dialysis from 2-chloroethanol sedimented readily on standing, indicating that they were large and multilayered. High concentrations of basic protein aggregate and fuse the vesicles but retain the multilayered lamellar structure.

To test the interaction of myelin basic protein with isolated myelin, we added the spin-labeled basic protein (50 μL containing 40 μg) to a suspension of myelin prepared by adding 1.0 mg of myelin to 80 μL of the Hepes buffer described above, and the suspension was warmed at 37 °C.

ESR Measurements. An empirical motion parameter τ_0 (Eletr & Keith, 1972) was derived for the modified protein and the protein–lipid complexes from the spectral parameters as an estimate of the relative rotational correlation time from the expression

$$\tau_0 = KW_0[(h_0/h_{-1})^{1/2} - 1]$$

where $K = 6.5 \times 10^{-10}$ s, W_0 is the width of the center line, and h_0 and h_{-1} are the heights of the center and high-field lines, respectively.

The order parameter S is a measure of the amplitude of the motion of the molecular long axis about the average orientation of the fatty acid chains in the lipid bilayer. It was obtained from the anisotropic hyperfine splittings $T_{\parallel}$ and $T_{\perp}$, measured as described previously (Boggs & Moscarello, 1978a), by using the equation (Seelig, 1970; Hubbell & McConnell, 1971)

$$S = (T_{\parallel} - T_{\perp})/(T_{ZZ} - T_{XX})$$

where T_{ZZ} and T_{XX} are the rigid lattice principal hyperfine values obtained from single crystal spectra (Seelig, 1970).

Order parameter measurements were made on each of the vesicle preparations with and without protein by incorporating the 5-doxylstearic acid spin-label into liposomes as described previously (Boggs & Moscarello, 1978a). Spectra of the spin-label in some of these lipids have been published (Boggs & Moscarello, 1978a).

Results

(I) Effect of Basic Protein on the Order of 5-Doxylstearate in Vesicles. It was reported earlier that basic protein had a much greater effect on the lipid packing near the polar head group than in the interior of the bilayer using fatty acid spin-labels with the nitroxide group at various positions along the chain (Boggs & Moscarello, 1978a). It also appeared that the magnitude of this effect varied with the type of lipid. The effect of increasing concentration of basic protein on the order parameter (S) of 5-doxylstearate in phosphatidylglycerol, phosphatidic acid, phosphatidylserine, phosphatidylethanolamine, and cerebroside sulfate is shown in Figure 1.

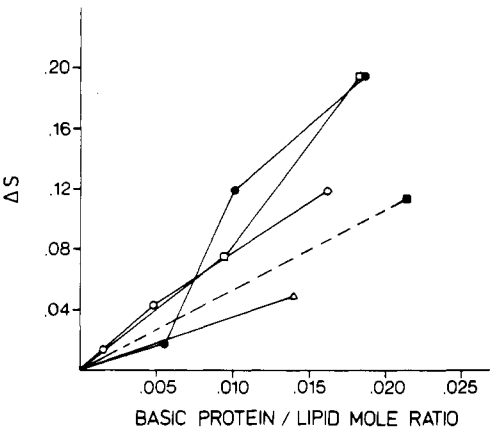

FIGURE 1: Change in the order parameter [$\Delta S = S$(lipid–protein vesicles) – S(pure lipid)] of 5-doxylstearate in vesicles with increasing mole ratios of protein to lipid for phosphatidylglycerol (●), phosphatidic acid (□), phosphatidylserine (○), cerebroside sulfate (■), and phosphatidylethanolamine (△), all at 37 °C except cerebroside sulfate which was at 50 °C. The plot for cerebroside sulfate has been extrapolated to zero.

The nonlinear increase in the order parameter for PG and PA and the nearly linear increase for phosphatidylserine with increasing concentration of basic protein are reproducible and were observed earlier. The small increase in S for phosphatidylethanolamine is also reproducible and also appears to be linear up to a protein to lipid ratio of 0.014. This is the maximum amount of protein which can be incorporated into phosphatidylethanolamine. The effect of only a high protein concentration on cerebroside sulfate has been determined and is extrapolated linearly to zero in Figure 1. At low protein concentrations there may be little significant difference in the effect on the order parameter in the various lipids. At high concentrations, however, the protein has a much greater effect on phosphatidylglycerol and phosphatidic acid, followed by phosphatidylserine and cerebroside sulfate, and has the least effect on phosphatidylethanolamine.

(II) Spin-Labeled Basic Protein. Basic protein was covalently spin-labeled and the effect of the same lipids on the mobility of the spin-labeled protein was investigated.

The amino acid sequence of the human myelin basic protein shows that two methionines are present at positions 21 and 167 from the N terminus and the molecule is devoid of cysteine (Dayhoff, 1972). It is therefore an excellent candidate for specific labeling of the methionine residues by alkylation with an iodoacetamide nitroxide spin-label in a manner similar to that reported already for specific $^{13}CH_3$ enrichment of the methionine residues (Deber et al., 1978).

Removal of the spin-label by NaOH hydrolysis showed that 2 mol of spin-label per mol of protein was present. Performic acid oxidation of the spin-labeled protein failed to produce methionine sulfone while similar treatment of the unlabeled protein yielded 2.4 mol of methionine sulfone. Futhermore, neither 1- nor 3-(carboxymethyl)histidine nor 1,3-bis-(carboxymethyl)histidine could be detected in the amino acid hydrolysates of the spin-labeled protein. Thus, we conclude

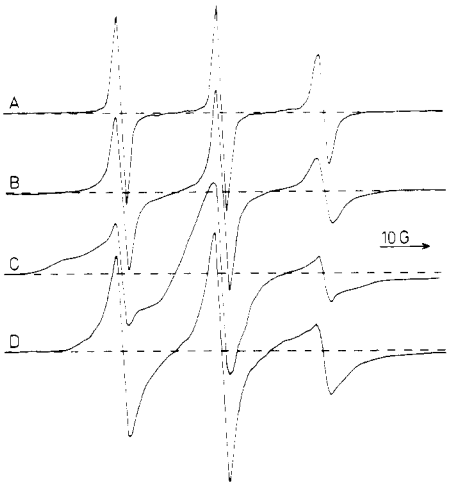

FIGURE 2: ESR spectra of iodoacetamide spin-labeled basic protein (A) in aqueous solution at 37 °C, (B) in phosphatidylethanolamine at 37 °C, (C) in phosphatidylglycerol vesicles at 15 °C, and (D) in phosphatidylglycerol at 37 °C.

Table I: Interaction of Spin-Labeled Myelin Basic Protein with Various Lipids at 37.5 °C

sample	T_c[c] (°C)[a]	S[c]	τ_0[c] (ns)	τ_0(vesicles)/τ_0(soln)[b]	A_0[c] (G)
phosphatidylglycerol	ca. −15	0.4	0.60	7.22	16.41
phosphatidic acid	17	0.425	0.47	5.66	16.50
phosphatidylserine	5	0.47	0.31	3.73	16.53
cardiolipin	30	0.48	0.27	3.25	16.53
phosphatidylethanolamine	10.5	0.59	0.26	3.13	16.50
myelin		0.77	0.38	4.58	16.50
basic protein			0.08		16.64
free probe			0.024		16.85

[a] T_c of egg phosphatidylglycerol $\cong$ T_c of egg phosphatidylcholine (Ladbrooke & Chapman, 1969). T_c's of other lipids are from Boggs & Moscarello (1978a) or measured as described therein by differential scanning calorimetry. [b] Ratio of τ_0 for the spin-labeled protein in vesicles to that of the protein in solution measured at 37.5 °C. [c] T_c, transition temperature; S, order parameter; τ_0, motional parameter; A_0, hyperfine splitting.

that 2 mol of spin-label was specifically bound to the methionine residues of 1 mol of basic protein.

The ESR spectrum of the iodoacetamide spin-labeled basic protein is shown in Figure 2A and is typical of fast motion, suggesting that the spin-label bound to the protein has significant motion with respect to the protein. The motional parameter τ_0 is 0.08 ns (Table I) and is somewhat greater than that for the spin-label itself (0.024 ns), indicating that binding to the protein does slow its motion slightly.

Interaction of the Modified Protein with Lipids. Lipid vesicles containing spin-labeled myelin basic protein and various lipids including phosphatidic acid, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, cerebroside sulfate, and cardiolipin were prepared either by dispersion or by dialysis from 2-chloroethanol. By use of these lipids, basic protein added in solution has been shown to be almost completely bound to the vesicles at concentrations at least up to 30% (w/w) (Boggs & Moscarello, 1978a, unpublished data). In this report we have used low concentrations of protein, 3–4%, and therefore can assume that all of the protein is bound to the vesicles.

Alkylation of the methionines with the iodoacetamide spin-label results in a positively charged sulfonium ion which may alter the interaction of the methionine regions of the protein with the lipid bilayer. However, it was found by Deber et al. (1978), using basic protein labeled with $^{13}CH_3$ on the methionines, that there was no significant difference in the line width of the ^{13}C NMR spectra of the S-methylated and the native form of the protein (obtained by reduction), when bound to lipid vesicles, suggesting that the presence of the sulfonium ion does not significantly perturb the lipid–protein interaction. The positively charged sulfur may be shielded by the methyl and spin-label groups so that it does not bind electrostatically to the lipid phosphate. In any case the sulfonium ion should not affect the interaction of other regions of the protein with the lipid. Variations in the interaction of this spin-labeled protein with different lipids, monitored by the mobility of the spin-labeled methionines, can be extrapolated to the native form of the protein.

The ESR spectra of representative samples are shown in Figure 2. Interaction of the spin-labeled protein with phosphatidylethanolamine (Figure 2B) results in a single-component spectrum characterized by a slight slowing of the motion of the probe (τ_0 = 0.25 ns). This type of spectrum was also observed with phosphatidylserine, cardiolipin, cerebroside sulfate, and myelin although the degree of slowing of the probe motion varied. Interaction of the protein with phosphatidylglycerol and phosphatidic acid at low temperatures, however, resulted in a multicomponent spectrum with one or two more immobilized components present as well as a mobile one similar to that in the other lipids, as shown for phosphatidylglycerol at 15 °C (Figure 2C). Phosphatidylglycerol is above its phase transition at this temperature although phosphatidic acid is not (Table I). A motional parameter was not calculated for the more immobilized component; however, it must be greater than 10 ns provided that the motion is not anisotropic (Freed, 1976). Raising the temperature results in faster motion of the lipid, and the immobilized components tend to merge with the more mobile component as can be seen by comparing spectra for the spin-labeled protein in phosphatidylglycerol at 15 and 37 °C (parts C and D of Figure 2). Examination of the upfield portion of the spectrum at 37 °C (Figure 2D) reveals that there are still two components. This suggests that there are still two populations of probes present but their motion more closely resembles each other at higher temperatures.

The hyperfine splitting value A_0 was measured for each sample at 37 °C as shown in Table I. This value has been shown to depend on the polarity of the environment (Briere et al., 1965). Only a small decrease from the value for the spin-labeled protein in aqueous solution (16.64 G) was seen upon incorporation into lipid vesicles, indicating that the spin-label is still in a polar environment. It is possible that the probe giving rise to the immobilized component in phosphatidylglycerol and phosphatidic acid could be in a hydrophobic environment although it is more likely that the positively charged sulfonium ion would force the methionine to be located at the polar head group–aqueous interface. In any case, when the temperature was raised, the A_0 of the resultant spectrum indicates that the probe is now in a polar environment.

Variable Interaction of Basic Protein with Different Lipids. It was clear from both the relative intensity of the spectra and the calculated motional parameters that the spin-label on the protein was slowed to a differing extent by the various lipids. The motional parameter of the samples at 37.5 °C is shown

in Table I. Those lipids which yielded a two-component spectrum at lower temperatures (phosphatidylglycerol and phosphatidic acid) resulted in the greatest motional parameters at 37.5 °C. The remaining lipids each slowed the probe to a different degree in the order phosphatidylserine > cardiolipin ≥ phosphatidylethanolamine. The myelin membrane slowed the probe to a greater degree than phosphatidylserine. Cerebroside sulfate was not included in this table because its phase transition temperature is greater than 37.5 °C. The hyperfine splitting was least for phosphatidylglycerol, 16.41 G, suggesting location of the probe in a somewhat less polar environment than for the other lipids.

A comparison of the order parameter values, S, in Table I for each of the lipids and the motional parameters, τ_0, of the lipid–protein complexes at 37.5 °C suggested that there might be a relationship between lipid order and the motion of the protein spin-label. It appeared that less ordered lipids slowed the motion more than well-ordered ones. In order to examine the possibility that only the order of the lipid fatty acids determined the motion of the spin-labeled protein, we measured the motional parameters of the spin-labeled protein and the order parameter of 5-doxylstearate in the vesicles at 5 °C intervals from 10 to 60 °C. The motional parameter was measured for those spectra which contained only a mobile component: this was true in all cases above 30 °C. Plots of the motional parameter and the order parameter against temperature were constructed. The motional parameter of the protein in vesicles at temperatures at which all the lipids possessed an equal order parameter was extracted from the plots of τ_0 and S vs. temperature. An order parameter of 0.4 was arbitrarily chosen since all the lipids except phosphatidylethanolamine attained this order parameter above their phase transition temperature and in the temperature range studied. Phosphatidylethanolamine, however, is so highly ordered that even at a temperature of 50 °C it has an order parameter of 0.55. The motional parameter of the protein in phosphatidylethanolamine vesicles was therefore measured at 50 °C. Myelin is also so highly ordered that an order parameter of 0.4 could not be achieved. It was therefore not measured at a temperature above 37 °C. The correlation times of the protein spin-label in lipid vesicles at temperatures corresponding to an order parameter of 0.4 for each lipid are shown in Table II.

Since the motion of the spin-labeled protein in aqueous solution increases with temperature as shown in the following paper (Boggs et al., 1980), the ratio of τ_0 for the protein in vesicles to that of the protein in solution at each temperature was determined in order to account for the temperature effect on the motion. A comparison of this ratio at 37.5 °C in Table I with that at a higher temperature for each lipid in Table II indicates that increased lipid disorder results in an increased immobilizing effect of the lipid on the protein. However, the immobilizing effect still depends strongly on the type of lipid even when the order parameters are identical. Indeed, the order of slowing of the probe at 37.5 °C by different lipids is retained, suggesting that the ordering of the lipid chains at the fifth carbon is not the overriding influence on the ability of the lipid to slow down the spin-label bound to the protein. From the data presented in Tables I and II, the order of ability of the lipids to slow the spin-label is phosphatidylglycerol ≥ phosphatidic acid ≫ myelin > cerebroside sulfate ≥ phosphatidylserine ≃ cardiolipin > phosphatidylethanolamine.

Table II: Interaction of Spin-Labeled Myelin Basic Protein with Lipids of Equal Order Parameters

sample	T_c[c] (°C)[a]	temp of measurement (°C)	S[c]	τ_0[c] (ns)	τ_0(vesicles)/τ_0(solution)[b]	A_0 (G)
phosphatidylglycerol	ca. −15	37.5	0.4	0.60	7.23	16.41
phosphatidic acid	17	43.5	0.4	0.49	7.10	16.44
phosphatidylserine	5	49	0.4	0.27	4.58	16.49
cerebroside sulfate	50	56	0.4	0.26	5.30	16.60
cardiolipin	30	47.5	0.4	0.26	4.26	16.49
phosphatidylethanolamine	10.5	50	0.55	0.20	3.57	16.46
basic protein		37.5		0.08		16.64
free probe		37.5		0.024		16.85

[a] As described in Table I. [b] Ratio of τ_0 for the spin-labeled protein in vesicles to that of the protein in solution, both measured at the same temperature as indicated. [c] T_c, transition temperature; S, order parameter; τ_0, motional parameter; A_0, hyperfine splitting.

Discussion

Myelin basic protein interacts with lipids primarily through electrostatic interactions (Palmer & Dawson, 1969). However, once this interaction has occurred there arises the possibility that the protein may be able to interact hydrophobically with the lipid bilayer, depending upon the ability of the protein to present its hydrophobic segments to the hydrocarbon region of the lipid bilayer. An examination of the sequence of the basic protein reveals that segments of hydrophobic and/or uncharged residues (14–21, 26–30, 35–38, 44–47, 60–64, 66–74, 85–90, 92–96, 98–104, 108–112, 113–117, 123–129, 146–150, and 163–168) do exist despite the fact that the protein is highly basic (Eylar et al., 1971; Boggs & Moscarello, 1978b). These segments may be regions of the protein which can penetrate into the lipid bilayer or, by distorting the bilayer, interact with the fatty acid chains.

The work of London & Vossenburg (1973), London et al. (1973), and Demel et al. (1973) suggested that the degree of hydrophobic interaction varied with different lipids. Judging by the amount of protection of the protein from tryptic hydrolysis afforded by different lipids and expansion of lipid monolayers by the protein, the protein appeared to interact hydrophobically to the greatest extent with the cerebroside sulfate, an acidic lipid extract from myelin, and cardiolipin and, to a lesser extent, with phosphatidylserine, phosphatidylethanolamine, and a total lipid extract from myelin. In an earlier DSC study from this laboratory (Boggs & Moscarello, 1978a), the protein was found to decrease the phase transition temperature in the order phosphatidic acid ≃ phosphatidylglycerol > phosphatidylserine > phosphatidylethanolamine. Basic protein has no effect on the phase transition temperature of cerebroside sulfate (unpublished results).

In this study we have shown that high concentrations of the protein decrease the mobility of fatty acids near the polar head group to a decreasing extent in the order phosphatidic acid ≃ phosphatidylglycerol > phosphatidylserine > cerebroside sulfate > phosphatidylethanolamine. We have also studied the effect of these lipids on the protein and found that binding of basic protein to lipid vesicles results in a slowing of the motion of a spin-label covalently bound to the methionines and that the degree of this immobilization also varies with different lipids in the order phosphatidylglycerol ≥ phosphatidic acid > myelin > cerebroside sulfate ≥ phosphatidylserine ≃ cardiolipin ≥ phosphatidylethanolamine.

There are several ways to explain how the motion of the spin-label probe could be slowed by interaction with a membrane surface. The first is that the spin-label probe on the protein penetrates into the hydrophobic regions of the bilayer. This may be true in the case of phosphatidylglycerol and phosphatidic acid which resulted in the appearance of an immobilized component in the spectrum which might indicate that a fraction of the probes is embedded in the bilayer. However, this has not yet been established and is unlikely in view of the positive charge on the spin-labeled methionine. The hyperfine splitting values recorded for the other protein–lipid complexes (phosphatidylserine, cardiolipin, cerebroside sulfate, phosphatidylglycerol, and myelin) indicate that the probe is in a polar environment. Therefore, it is more likely that the probes are located at the polar head group–aqueous interface, possibly penetrating as deeply as the ester linkage of the fatty acid chains. Differences in the degree of penetration of the spin-labels in the polar head group region may indicate that hydrophobic segments of the protein can also penetrate and interact hydrophobically to different degrees with different lipids. Differences in the probe mobility may also result from conformational changes in the protein associated with the hydrophobic interaction of other regions of the protein with the bilayer. Such interaction may also perturb the lipid, particularly near the polar head groups as discussed for dipalmitoylphosphatidylglycerol in the following paper, so that a decrease in the mobility of the methionine spin-label occurs.

We have shown in the following paper (Boggs et al., 1980) that changes in the motional parameter can be correlated with other evidence which suggests increased hydrophobic interaction of the protein with the bilayer as the lipid melts and partial reversal of this interaction as the lipid freezes. Thus, the spin-label on the methionines appears to be sensitive to the state of the lipid and to the degree of hydrophobic interaction of some portions of the protein with the lipid bilayer even though the spin-label itself is in a polar environment. Therefore, differences in the degree of immobilization of the probe by different lipids may indicate differences in the degree of hydrophobic interaction of the protein with the lipid bilayer.

Thus, the immobilizing effect of different lipids on the spin-labeled protein suggests that the degree of hydrophobic interaction of the protein with these lipids decreases in the order phosphatidylglycerol $\geq$ phosphatidic acid $>$ myelin $>$ cerebroside sulfate $\geq$ phosphatidylserine $\simeq$ cardiolipin $>$ phosphatidylethanolamine.

Results presented here and earlier (Boggs & Moscarello, 1978a) of the effect of the protein on fatty acid packing and on the phase transition temperature indicate that the degree of hydrophobic interaction decreases in a similar order, phosphatidylglycerol $\simeq$ phosphatidic acid $>$ phosphatidylserine $>$ cerebroside sulfate $>$ phosphatidylethanolamine, with the exception of cerebroside sulfate and phosphatidylserine. However, the hydrophobic interaction with these two lipids is fairly similar by the techniques used here and much less than that with phosphatidylglycerol and phosphatidic acid. The relative order of phosphatidylserine, cerebroside sulfate, and cardiolipin is in some disagreement with the earlier studies of London & Vossenburg (1973), London et al. (1973), and Demel et al. (1973). Some of this disparity may be due to different tissue sources of cerebroside sulfate and cardiolipin. Furthermore, although all of the techniques which have been used to study the interaction of this protein with lipid are suggestive of hydrophobic interaction with lipid, they may vary in sensitivity to the interaction of different regions of the protein. Thus, cerebroside sulfate may immobilize the protein slightly more than phosphatidylserine while the protein has a greater effect on the lipid packing in phosphatidylserine than in cerebroside sulfate. Further study with the protein spin-labeled at different sites may help to clarify this point and to determine exactly which regions of the protein interact hydrophobically with different lipids.

In any event, it must be the properties of the lipid that control the degree of hydrophobic interaction and thus control the slowing of the ESR probe covalently attached to the protein. A comparison of the lipids used in these experiments can shed light on the possible nature of the forces in the lipid that influence the protein interaction. All of these lipids possess a negative charge, a prerequisite for the electrostatic binding to basic protein. The fatty acid compositions of phosphatidylglycerol, phosphatidic acid, phosphatidylserine, and phosphatidylethanolamine are not sufficiently different to account for the differences in order parameter of these lipids or differences in their immobilizing effect on the protein. Cerebroside sulfate has longer fatty acid chains than the other lipids which partially accounts for its high order parameter. Therefore, differences in the head group region must be responsible for the degree of hydrophobic interaction of this protein with the lipid.

That different intermolecular forces are present due to different polar head group structures for the different lipids is apparent from a consideration of the phase transition temperatures for the lipids possessing identical fatty acids: dimyristoylphosphatidic acid (52 °C), dimyristoylphosphatidylethanolamine (50 °C), dimyristoylphosphatidylserine (37 °C), and dimyristoylphosphatidylglycerol (22 °C) [as measured in our laboratory except dimyristoylphosphatidylserine (Mombers et al., 1979)]. There is a great deal of evidence which suggests that phosphatidylethanolamine and phosphatidylserine can interact intermolecularly via hydrogen-bond interactions between adjacent polar head groups (Hitchcock et al., 1974; MacDonald et al., 1976). Capacity for intermolecular hydrogen bonding has been demonstrated for sphingolipids such as cerebroside sulfate (Pascher, 1976). These intermolecular interactions account for the high phase transition temperatures of dimyristoylphosphatidylethanolamine, dimyristoylphosphatidylserine, and cerebroside sulfate (50 °C) and the high order parameters of phosphatidylserine and phosphatidylethanolamine compared to phosphatidylglycerol and phosphatidic acid at 37.5 °C in the liquid-crystalline phase (Table I). Such interactions would result in closer packing of the lipid molecules and may restrict protein-induced expansion of the bilayer and hydrophobic interaction with the lipid fatty acid chains.

The reason for the high phase transition of phosphatidic acid also resides in its ability to hydrogen bond. It has one negative charge at pH 7.4 like phosphatidylglycerol and yet melts 30 °C higher. However, phosphatidic acid has a secondary pK of approximately 8.5 and therefore possesses a proton capable of hydrogen bonding at pH 7.4 (Papahadjopoulos, 1968; Trauble & Eibl, 1974). Intermolecular hydrogen bonding between adjacent phosphatidic acid molecules would result in an increased phase transition temperature (Jacobson & Papahadjopoulos, 1975; Eibl & Blume, 1979). However, the protein is apparently able to interact hydrophobically with phosphatidic acid.

Electrostatic interaction between the negatively charged phosphate groups of phosphatidic acid and basic residues on the protein will neutralize phosphatidic acid and disrupt the hydrogen bonding between adjacent phosphatidic acid molecules in the vicinity of the protein, thus allowing hydrophobic

interaction to occur. Hydrogen bonding between phosphatidic acid molecules may also be much weaker in the liquid-crystalline phase than in the gel phase where the polar head groups are closer together. This is indicated by the fact that the order parameter of phosphatidic acid in the liquid-crystalline phase at 37.5 °C is lower than that of phosphatidylserine even though the T_c of phosphatidic acid is higher (Table I). Thus, hydrophobic interaction of the protein with phosphatidic acid is nearly as great as that for phosphatidylglycerol at an equal order parameter and is much greater for these two lipids than for phosphatidylserine, cerebroside sulfate, phosphatidylethanolamine, and cardiolipin.

Phosphatidylethanolamine binds less basic protein than all of the other lipids; it is saturated at 25–30% (approximately 81 lipid molecules to 1 molecule of protein) (Boggs & Moscarello, 1978a). The protein may also bind to phosphatidylethanolamine by utilizing less than its total 31 basic residues. Thus, most of the phosphatidylethanolamine may be involved in intermolecular electrostatic interactions rather than electrostatic interactions with the protein, and the intermolecular network which prevents hydrophobic interaction with the protein will be maintained.

Phosphatidylserine has two negatively charged groups and one positively charged group and thus has one negatively charged group to bind basic protein while the other can participate in intermolecular interactions. Thus, phosphatidylserine can bind more basic protein than phosphatidylethanolamine and still prevent it from expanding the lipids and interacting hydrophobically to a significant extent. The intermolecular hydrogen bonding which occurs for cerebroside sulfate involves the sphingosine amide moiety and fatty acid hydroxyl groups and probably not the negatively charged sulfate, so that it is possible for the protein to bind to this lipid without disrupting the intermolecular hydrogen bonding. Cardiolipin should not be capable of intermolecular hydrogen bonding unless the glycerol hydroxyls are involved. However, the phase transition temperature of this lipid is higher than that for the other natural lipids including phosphatidic acid (unsaturated) in spite of the fact that it contains primarily diunsaturated fatty acids, indicating that some factor which causes a high degree of order in this lipid restricts hydrophobic interaction of the protein, resulting in only a small immobilizing effect on the protein spin-label.

It is interesting that the immobilization of the protein spin-label is greater in myelin than any of its major acidic lipids, phosphatidylethanolamine, phosphatidylserine, and cerebroside sulfate. Any intermolecular interactions which may occur will be different in a mixture of lipids than in a pure lipid. There is the possibility of disrupting intermolecular interactions between lipids of one species and creating new ones between different species, including cholesterol. The effect of mixtures of lipids on the penetration of the protein and its conformation is being investigated.

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Effect of Lipid Environment on the Motion of a Spin-Label Covalently Bound to Myelin Basic Protein†

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ABSTRACT: The interaction of human central nervous system myelin basic protein with lipid has been studied by determining the effect of the protein on lipid organization by using differential scanning calorimetry (DSC) and also by monitoring the influence of the lipid environment on the protein by covalently spin-labeling the protein at its two methionine residues at positions 21 and 167 with iodoacetamide spin-labels. Three lipids were used on which the protein has differing effects: dipalmitoylphosphatidylglycerol, dimyristoylphosphatidic acid, and dimyristoylphosphatidylethanolamine. Previous studies have indicated that hydrophobic segments of the protein can interact hydrophobically with the hydrocarbon region of the bilayer and distort the lipid packing. The degree of this interaction depends on the lipid polar head group. DSC results presented here suggest that basic protein interacts hydrophobically to the greatest extent with dipalmitoylphosphatidylglycerol and dimyristoylphosphatidic acid and to a much lesser extent with dimyristoylphosphatidylethanolamine. The interaction is different for dipalmitoylphosphatidylglycerol and dimyristoylphosphatidic acid also since supercooling appears to partially reverse the interaction for dipalmitoylphosphatidylglycerol but not dimyristoylphosphatidic acid. The spin-labeled protein was used at low concentrations, as a probe for the microenvironment of the methionine regions of the protein in the lipid bilayer. The mobility of the iodoacetamide spin-label was monitored in these three lipids throughout the phase transition on heating and cooling. The spin-labels on the methionines are for the most part in a polar environment, but their mobility is sensitive to the phase transition in a complex way. Hydrophobic interaction of the protein with the liquid-crystalline phase of dipalmitoylphosphatidylglycerol results in perturbation of the lipid gel phase in the immediate environment of the spin-labeled regions so that the phase transition can no longer be clearly seen on cooling and reheating from the mobility of the iodoacetamide spin-labels. The phase transition can be clearly detected for dimyristoylphosphatidic acid and dimyristoylphosphatidylethanolamine, suggesting that the protein either does not interact hydrophobically to the same degree in these two lipids or does not perturb the lipid in the environment of the methionines. Occurrence of the phase transition has opposing effects on the mobility of the spin-label. As the lipid melts, the mobility of the probe increases due to the increased motion of the lipid. At the same time increased interaction of the protein with the more fluid lipid tends to decrease the probe mobility. The results indicate that the mobility of the spin-label on the methionines of basic protein is sensitive to the degree of hydrophobic interaction of the protein with the lipid bilayer even though the spin-labeled methionines are probably located in the polar head group region.

The interaction of proteins with lipids is not yet well understood on a molecular level. Membrane proteins have been classified as intrinsic or extrinsic based on whether they interact electrostatically or hydrophobically with lipids (Vanderkooi, 1972). Papahadjopoulos et al. (1975) has further classified them into three groups based on their properties and effect on lipid organization: (1) primarily electrostatic, (2) electrostatic and hydrophobic, and (3) primarily hydrophobic. Susi et al. (1979) have extended this last group to include "orderly hydrophobic binding" and "disorderly hydrophobic binding". As well as having distinct effects on lipid organization, the conformation of many membrane proteins probably also depends on the phase state and type of lipid. This is suggested by the dependence of enzymatic activity of membrane proteins on the fluidity or type of lipid polar head group although there is little direct evidence.

The basic protein of myelin appears to be of the group 2 classification of Papahadjopoulos et al. (1975). It has 19% basic amino acids and only binds to acidic lipids (Palmer & Dawson, 1979; Demel et al., 1973; Steck et al., 1976). However, it also possesses 52% hydrophobic or apolar amino acids which are distributed throughout its sequence in regions of four to nine amino acids long (Eylar et al., 1971; Boggs & Moscarello, 1978b). There is abundant evidence that some of these hydrophobic segments may be able to interact with the hydrocarbon region of the bilayer either by penetrating partway into the bilayer or by deforming the bilayer such that hydrophobic contacts at the lipid–water interface can occur.

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