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

Joan M. Boggs,* John G. Stollery,‡ and Mario A. Moscarello

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.

† From the Department of Biochemistry, The Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada. *Received July 12, 1979; revised manuscript received November 30, 1979.* Supported by a grant (to J.M.B.) from the Multiple Sclerosis Society of Canada and grants (to J.M.B. and M.A.M.) from the Medical Research Council of Canada.

* Correspondence should be addressed to this author. She is a recipient of a Career Development Award from the Multiple Sclerosis Society of Canada.

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The protein increases the permeability of lipid vesicles to glucose (Gould & London, 1972) and Na^+ (Papahadjopoulos et al., 1975), expands lipid monolayers (Demel et al., 1973; Papahadjopoulos et al., 1975), is partially protected from enzymatic hydrolysis when interacting with lipid (London & Vossenberg, 1973; London et al., 1973), decreases the enthalpy and temperature of the lipid phase transition (Papahadjopoulos et al., 1975), and has an immobilizing and disordering effect on fatty acid spin-labels which is much greater near the polar head group than in the interior of the bilayer (Boggs & Moscarello, 1978a).

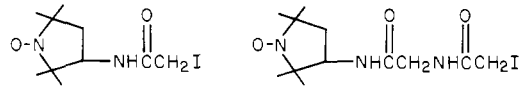
The degree of hydrophobic interaction was also shown to depend on the state of the lipid. It was greater in the liquid-crystalline phase for dipalmitoylphosphatidylglycerol and could be partially reversed on brief cooling and more completely reversed on prolonged cooling (Papahadjopoulos et al., 1975; Boggs & Moscarello, 1978a). It also seemed to depend on the type of lipid (Demel et al., 1973; London & Vossenberg, 1973; Boggs & Moscarello, 1978a; Stollery et al., 1980).

In the present study, in addition to examining the effects of the protein on lipid organization, we have also examined the effect of the lipid upon the protein by spin-labeling the protein with an iodoacetamide spin-label at the two methionines at positions 21 and 167, near the N terminal and C terminal, respectively. The spin-labeled protein has been used at low concentrations, as a probe, to avoid the effects of larger amounts of protein on bulk lipid properties. We are thus able to study the microenvironment of the methionine regions of the protein in the bilayer.

Three different lipids are used on which the protein has differing effects, dipalmitoylphosphatidylglycerol, dimyristoylphosphatidic acid, and dimyristoylphosphatidylethanolamine. The effects of the protein on the phase transition temperature and fatty acid packing in these three lipids suggest that it has the greatest hydrophobic interaction with dipalmitoylphosphatidylglycerol and dimyristoylphosphatidic acid and the least with dimyristoylphosphatidylethanolamine. The hydrophobic interaction with dipalmitoylphosphatidylglycerol is partially reversed on supercooling while that with dimyristoylphosphatidic acid is not. Changes in the mobility of the protein spin-label during phase transitions of these three lipids could be correlated with the evidence suggestive of hydrophobic interaction of the protein with the lipids.

Materials and Methods

Spin-Labels. 3-(2-Iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (I), 3-[2-(2-iodoacetamido)acetamido]-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (II), and 16-doxylstearic acid (III; $m = 1$; $n = 16$) were purchased from Syva (Palo Alto, CA). Doxyl represents the 4′,4′-dimethyloxazolidinyl-1-oxy derivative of the parent ketone.



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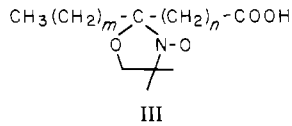

III

Lipids. Dimyristoylphosphatidic acid and dipalmitoylphosphatidylglycerol were generous gifts from Dr. D. Papahadjopoulos (University of California, San Francisco). Dimyristoylphosphatidylethanolamine was purchased from Fluka.

The basic protein was prepared and covalently spin-labeled as described in the preceding paper (Stollery et al., 1980).

Preparation of Lipid–Protein Vesicles. The vesicles for differential scanning calorimetry were prepared by evaporating the lipid under nitrogen and dispersing the lipid (dimyristoylphosphatidic acid and dipalmitoylphosphatidylglycerol) in the buffer (10 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid, and 2 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid adjusted to pH 7.4) at a temperature above the phase transition temperature. A solution of the protein in buffer was then added, and the sample was vortexed for 10 min at 45 °C for dipalmitoylphosphatidylglycerol and 55 °C for dimyristoylphosphatidic acid. Dimyristoylphosphatidylethanolamine vesicles were prepared by dissolving the lipid and protein in 90% 2-chloroethanol and dialyzing against buffer overnight at 55 °C as described earlier (Boggs & Moscarello, 1978a). The vesicles were sedimented by centrifugation in an Eppendorf microcentrifuge at 10000*g* at room temperature for 5 min. The lipid/protein ratio of the vesicles was measured on the washed pellet by determining lipid content by phosphorus assay and protein content 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.

Vesicles containing the spin-labeled basic protein (4% w/w) were prepared by dispersing 1 mg of the lipid in 50 μL of buffer above the phase transition temperature. A solution of the spin-labeled basic protein in water (40 μg in 20 μL) was added above or below the T_c as specified in the text, and the sample was vortexed again. For ESR measurements the vesicles were taken up in 50-μL disposable micropipets and centrifuged at 2000 rpm for 10 min so that signal intensity measurements could be made without having changes due to sedimentation of the vesicles. Dimyristoylphosphatidylethanolamine vesicles were prepared by dialysis from 2-chloroethanol, and the protein was then added in the same way as the other lipids.

Differential Scanning Calorimetry. Samples were run on a Perkin-Elmer differential scanning calorimeter (DSC-2) at heating or cooling rates of 2.5–10 °C/min as specified in the text. Similar results were obtained at all heating rates. The midpoint of each peak was defined as the phase transition temperature (T_c).

Electron Spin Resonance Measurements. Spectra were obtained on a Varian E-4 spectrometer with a Varian temperature control accessory. The microwave power used was 10 mW, the modulation amplitude was 1.6 G, the time constant was 0.3 s, and the scan speed was 4 min.

This study is concerned with relative changes in motion. Therefore, the motion of the spin-label bound to the protein at temperatures where it possessed fast, nearly isotropic motion was monitored by calculating an empirical motion parameter derived from spectral parameters as in (Eletr & Keith, 1972)

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

where $K = 6.5 \times 10^{-10}$ s is fixed arbitrarily at its limiting value in the case of rapid isotropic tumbling. W_0 is the width of the center line, and h_0 and h_{-1} are the heights of the center and high-field first-derivative lines, respectively, measured as described earlier (Boggs & Moscarello, 1978a).

Since an increase in motion, within certain frequency limits, also increases the height of the peaks, the height of the center peak, h_0, normalized to the same instrument sensitivity was also used as a measure of the probe motion and its sensitivity to the lipid phase transition.

Measurements of the dependence of signal intensity and τ_0 on temperature for the spin-labeled protein bound to vesicles were repeated at least twice for each lipid. The order of

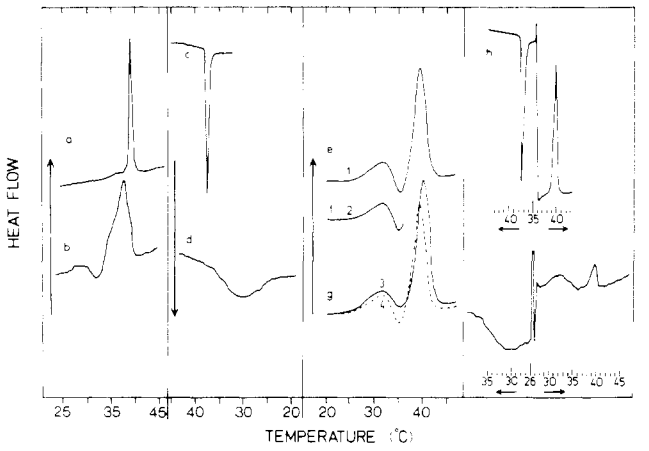

FIGURE 1: Differential scanning calorimetry scans for dipalmitoylphosphatidylglycerol vesicles with or without basic protein. Lipid only: (a), (c), and (h). Vesicles containing 38% basic protein: (b), (d), (e–g), and (i). All samples were run at 2.5 °C/min except (e–g) which were run at 10 °C/min. (a and b) Heating scans; (c and d) cooling scans; (e) sample heated from 7 to 47 °C; (f) sample heated to 36 °C and held for 5 min and then recooled to 7 °C; (g) sample reheated from 7 to 47 °C (—), cooled to 7 °C, and heating repeated (- - -); (h) lipid-only vesicles cooled to 35 °C and immediately reheated with the instrument set on cycle; (i) lipid-protein vesicles cooled to 26 °C and immediately reheated; the peak at 37.9 °C is greatly decreased in intensity and shifted to 39.6 °C. Phase transition temperatures depend on the heating rate and can be compared only to samples measured at the same heating rate. Even at 2.5 °C/min, the lipid melts 2 °C higher than it freezes due to hysteresis of the instrument.

occurrence of the phase transition on first heating, cooling, and second heating scans as shown in Figures 3–5 and 8 was reproducible.

Results

Basic Protein–Dipalmitoylphosphatidylglycerol Vesicles. The effect of basic protein on the phase transition temperature of dipalmitoylphosphatidylglycerol was measured by differential scanning calorimetry. Heating and cooling scans are shown in Figure 1a–d. The protein not only decreases the phase transition temperature to a greater extent on cooling than on heating as described earlier but also causes an exothermic transition in this lipid on heating. This was observed in the earlier studies (Papahadjopoulos et al., 1975; Boggs & Moscarello, 1978a) but was not explored in detail.

The small endothermic transition at ~29 °C, followed by the exothermic transition at ~32 °C (Figure 1b), suggested the presence of a metastable state similar to that produced by Mg^{2+} on dimyristoylphosphatidylglycerol (Ververgaert et al., 1975). However, the following treatment indicates that the phase giving rise to the lower melting endothermic transition does not appear to be the component which gives rise to the exothermic transition. If the sample is heated to a temperature near that of the exothermic transition and held for a few minutes, cooled, and reheated, as shown in Figure 1e–g, the exothermic transition no longer occurs but the lower melting endothermic peak is still present (Figure 1g). The higher melting endothermic peak is shifted to a slightly higher temperature and increased in intensity. After being cooled back to 8 °C, the differential scanning calorimetry scan regains its original appearance on reheating (Figure 1g, dashed line).

The lower melting endothermic transition probably corresponds to the broad transition observed on cooling but is decreased in intensity. Cooling to only 26 °C, followed immediately by reheating (Figure 1i), results in a broad endothermic transition at ~32 °C on heating, with loss of the exothermic transition and almost complete loss of the large endothermic transition at ~38 °C. This indicates that the protein has a similar effect on heating as on cooling provided that the sample has not been cooled to a low temperature (8 °C) before reheating. If the sample is supercooled as in Figure 1b, the lower melting endothermic transition is decreased in intensity and most of the sample melts at only 2 °C below the T_c of pure dipalmitoylphosphatidylglycerol. Figure 1h shows that cooling of pure dipalmitoylphosphatidylglycerol to a few degrees below its T_c, followed immediately by reheating, does not result in any reduction of the endothermic transition of the pure lipid in contrast to its behavior with basic protein.

These observations reinforce the conclusion reached previously that the hydrophobic interaction of the protein occurs with the liquid-crystalline phase lipid, resulting in a decrease in T_c on cooling. Even brief supercooling results in partial reversal of this interaction. However, the sample does not immediately attain its original state when first added to the vesicles at 0 °C since the exothermic transition in Figure 1b is not observed until the sample has been heated once and within a few hours prior to the second measurement. The results in Figure 1e–g also indicate that complete melting of the sample, followed by supercooling, is necessary for the exothermic transition to occur. This suggests that hydrophobic interaction of the protein with the lipid, followed by a partial reversal, results in some new and unstable associations of the protein with the lipid. At 32 °C some change in the lipid, possibly associated with its premelt transition, may occur which allows the protein to interact in a more stable way but without complete hydrophobic interaction. Thus, the complex falls to a lower energy level and heat is released. The bulk lipid is perturbed slightly so that it melts a few degrees lower than normal. Further hydrophobic interaction with greater perturbation of the lipid then occurs, and the lipid melts at an even lower temperature on cooling.

Basic protein covalently spin-labeled at the methionines was then added to dipalmitoylphosphatidylglycerol in order to observe the behavior of the protein in this lipid throughout heating and cooling cycles. The spectra of basic protein spin-labeled at the methionines in solution and in dipalmitoylphosphatidylglycerol vesicles at 15 and at 55.5 °C are shown in Figure 2. The protein in solution gives a single-component mobile spectrum (Figure 2A). At 15 °C in dipalmitoylphosphatidylglycerol, two components are present, one immobilized and one more mobile (Figure 2B). As the temperature is raised the hyperfine splitting of the immobilized component decreases until the immobilized and mobile components eventually merge. At 55.5 °C (Figure 2C) and down to at least 33 °C, the spectrum is primarily one component.

The hyperfine splitting value of 51 °C is 16.5 G, only slightly decreased relative to that for the free protein, 16.64 G (Table I). This value has been shown to depend on the polarity of the environment (Briere et al., 1965) and indicates that the probe itself is in a relatively polar environment at 51 °C. The location of the probe giving rise to the immobilized component at lower temperatures is not yet known. Alkylation of the methionine with the spin-label introduces a positive charge which probably forces the methionine to be located at the polar head group–aqueous interface. However, it is unlikely that the positively charged sulfonium ion is bound to the lipid phosphate since the methyl and spin-label groups on the sulfur would result in steric hindrance to this kind of interaction.

The spectrum becomes sharper and more intense as the temperature increases, indicating an increase in motion. The intensity of the spectrum is plotted against temperature in Figure 3A for the spin-labeled protein in aqueous solution and

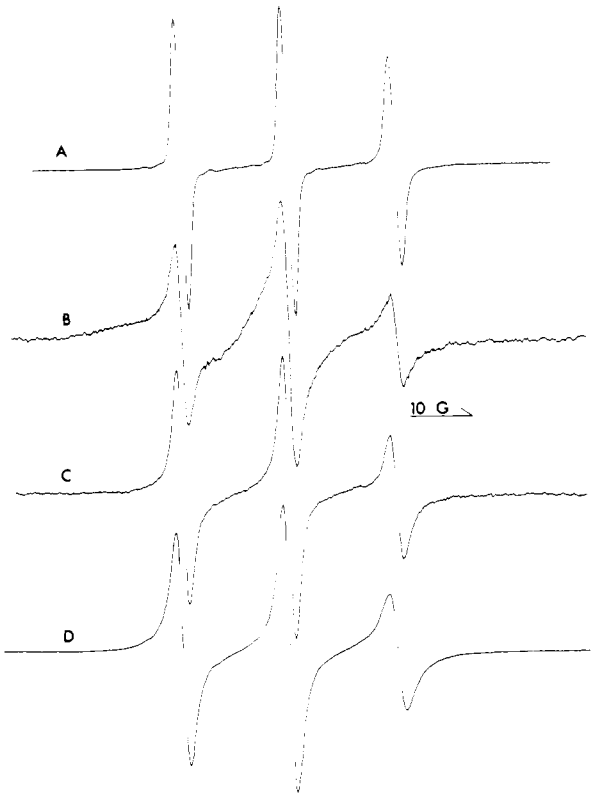

FIGURE 2: Electron spin resonance spectra of iodoacetamide spin-label I covalently bound to methionines of basic protein (A) in aqueous solution at 15 °C, (B) in dipalmitoylphosphatidylglycerol vesicles at 15 °C, (C) in dipalmitoylphosphatidylglycerol vesicles at 55.5 °C, and (D) in dimyristoylphosphatidylethanolamine vesicles at 33 °C.

Table I: Electron Spin Resonance Spectral Parameters of Iodoacetamide Spin-Label Bound to Basic Protein in Solution and in Lipid Vesicles above the Phase Transition

	temp of measurement (°C)	τ_0 (ns)	A_0 (G)
spin-label I			
free spin-label	37.5	0.02	16.85
spin-labeled basic protein	37.5	0.08	16.64
dimyristoylphosphatidic acid	61	0.61	16.22
dipalmitoylphosphatidylglycerol (0 °C)[a]	51	0.25	16.51
dipalmitoylphosphatidylglycerol (45 °C)[a]	51	0.30	16.51
dimyristoylphosphatidylethanolamine	61	0.21	16.51
unsaturated phosphatidic acid[b]	37.5	0.47	16.50
unsaturated phosphatidylglycerol[b]	37.5	0.60	16.41
egg phosphatidylethanolamine[b]	37.5	0.26	16.50
spin-label II			
spin-labeled basic protein	37.5	0.066	16.63
dimyristoylphosphatidic acid	61	0.46	16.35

[a] 0 °C indicates that protein was added at 0 °C. 45 °C indicates that protein was added at 45 °C. [b] Stollery et al. (1980).

in vesicles of dipalmitoylphosphatidylglycerol to which the protein was added at 0 °C. On the first heating scan of the protein in vesicles, the intensity rises sharply, indicating a phase transition at 37 °C (Figure 3A, curve 1). The peak height of the labeled protein in solution increases smoothly with temperature and only by 40%, while in the presence of lipid, the peak height increases by 333% during the phase transition over its value below the phase transition due to the immobilization of the probe by the lipid gel phase. The phase transition of pure dipalmitoylphosphatidylglycerol is monitored by the intensity of the spectra of 16-doxylstearate and occurs at 41.8 °C (Figure 3A, dashed line), similar to the T_c determined by differential scanning calorimetry. Thus, even on the first heating scan, the probe on the protein detects a transition 4.8 °C lower than that of the pure lipid.

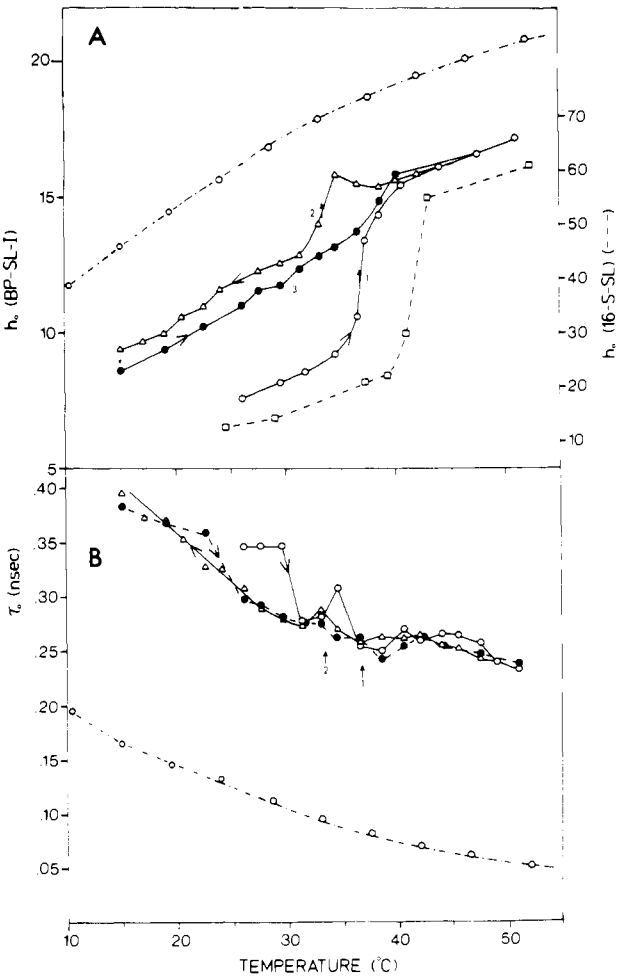

FIGURE 3: Temperature dependence of (A) the height of the center line, h_0, of the electron spin resonance spectrum of iodoacetamide spin-label I covalently bound to basic protein and (B) the motional parameter τ_0 for the spin-labeled protein in aqueous solution (-·-) or in dipalmitoylphosphatidylglycerol vesicles. Curve 1, first heating scan (○); curve 2, cooling scan (△); curve 3, second heating scan (●). In (A), arrows indicate the midpoints of the steepest portion of curves 1 and 2 and have been placed at the same temperatures in (B). In (A), h_0 for 16-doxylstearate in pure dipalmitoylphosphatidylglycerol is also plotted against temperature (---).

On being cooled (Figure 3A, curve 2) the peak height changes with a change in temperature much more gradually than on heating, and the main drop does not occur until 33.5 °C, 4 °C lower than that observed on heating and ~9 °C below the T_c of the pure lipid. The spin-labeled protein is present at a low concentration which is not enough to produce a detectable decrease in T_c of the bulk lipid by differential scanning calorimetry. In this case, the probe is sensitive to the lipid in the microenvironment of the protein. However, in agreement with the differential scanning calorimetry results in Figure 1, these results also indicate a greater effect of the protein on the lipid on cooling than on heating.

The intensity continues to drop gradually on cooling but does not reach its initial value. A wait of about 5 min at 15 °C before starting the second heating scan results in a further drop as indicated in Figure 3A, and prolonged cooling results in a further decrease in intensity. The increased intensity at 15

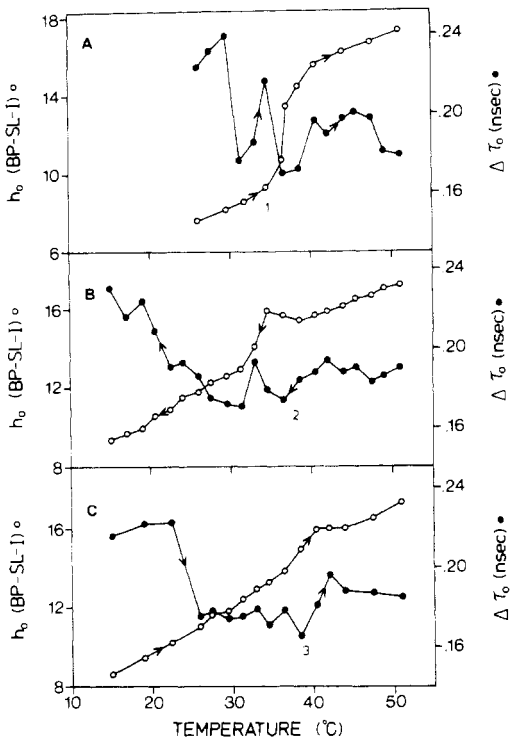

FIGURE 4: Temperature dependence of h_0 (○), the height of the center line of the electron spin resonance spectrum, and $\Delta\tau_0$ (●), the change in the motional parameter of the spin-labeled protein in dipalmitoylphosphatidylglycerol vesicles relative to that in solution, for samples shown in Figure 3. (A) Curve 1, first heating scan; (B) curve 2, cooling scan; (C) curve 3, second heating scan.

°C for the cooling scan relative to the first heating scan suggests that the hydrophobic interaction has not been completely reversed and that the protein still perturbs its environment so that it has more motion than in less perturbed lipid (Figure 3A, curve 1) at 15 °C.

The peak height on the second heating scan (Figure 3A, curve 3), obtained only minutes after the cooling scan, falls between the cooling scan and the first heating scan, indicating that the hydrophobic interaction has been partially but not completely reversed, in agreement with differential scanning calorimetry results published earlier (Boggs & Moscarello, 1978a). Part of the lipid is much more perturbed than on the first heating scan while part melts at the original temperature, around 38 °C.

The motional parameter τ_0 of the protein in dipalmitoylphosphatidylglycerol was calculated at those temperatures at which the spectrum contains primarily one component. τ_0 changes with temperature in a more complex way as shown in Figure 3B, although τ_0 for the protein in solution decreases smoothly with increase in temperature. The decrease in τ_0 for the protein in vesicles follows the curve for the protein in solution with a series of sharp increases and decreases superimposed upon it. Although some of these changes in τ_0 are relatively small, they are not due to experimental error since no such variation is observed for τ_0 of the protein in solution. The changes in motional parameter due to the lipid phase transition can be seen more distinctly if τ_0 for the protein in solution is subtracted from that of the complex to give $\Delta\tau_0 = \tau_{complex} - \tau_{aqueous}$. $\Delta\tau_0$ is plotted against temperature and compared directly to the change in peak height with temperature for the first heating scan, cooling scan, and second heating scan in parts A, B, and C of Figure 4, respectively.

Examination of these curves reveals that in each case after the initial large drop in $\Delta\tau_0$, the most distinct changes in $\Delta\tau_0$ occur over the temperature range at which the phase transition can be detected most sharply, as monitored by the change in intensity of the spectrum of the spin-labeled protein. The changes in $\Delta\tau_0$ are more abrupt and of greater magnitude for the first heating scan (Figure 4A) where the probe senses the phase transition most sharply. On the cooling curve (Figure 4B) and second heating curve (Figure 4C) where the broader changes in signal intensity suggest that the protein is now perturbing the lipid in its microenvironment, the changes in $\Delta\tau_0$ are much more gradual. After being heated, the sample must be cooled to 20 °C (Figure 4B) before $\Delta\tau_0$ increases to its initial value observed on the first heating scan (Figure 4A) at temperatures up to 28 °C. On the second heating scan (Figure 4C) $\Delta\tau_0$ first drops at 22 °C, a much lower temperature than on the first heating scan.

The value of τ_0 above the phase transition for the protein added to dipalmitoylphosphatidylglycerol at 0 °C is 0.25 ns. A somewhat larger value, 0.30 ns, is obtained if the protein is added to this lipid above the phase transition (Table I).

Basic Protein–Dimyristoylphosphatidylethanolamine Vesicles. It was concluded earlier that basic protein did not interact hydrophobically as much with egg phosphatidylethanolamine as with phosphatidylglycerol and other lipids. Even with the maximum amount bound, 25% by weight, it decreases the phase transition by only 1.5–2.6 °C on cooling and 1.2 °C on heating and had only a small immobilizing effect on fatty acid spin-labels incorporated into the vesicles (Boggs & Moscarello, 1978a). The maximum amount of protein which binds to dimyristoylphosphatidylethanolamine is even less than for egg phosphatidylethanolamine. Only 11% by weight could be incorporated into dimyristoylphosphatidylethanolamine, causing a decrease in T_c, measured by DSC, of only 0.5 °C on heating and 1.3 °C on cooling.

The spin-labeled basic protein was added to dimyristoylphosphatidylethanolamine vesicles above the phase transition temperature. Its spectrum in this lipid at 33 °C, below the phase transition, is shown in Figure 2D. A sharp single-component spectrum was obtained at this and all other temperatures. $\Delta\tau_0$ and the peak height are plotted against temperature in parts A and B of Figure 5. The lipid phase transition can be seen clearly from the change in intensity on first heating, cooling, and second heating scans. On the first and second heating scans (curves 1 and 3, Figure 5B), the phase transition is detected 2 °C below that of the pure lipid (measured by differential scanning calorimetry and indicated by a large arrow in Figure 5B). On the cooling scan (curve 2) the phase transition occurs 5.2 °C below that of the pure lipid, suggesting that an increased perturbing interaction has occurred above the phase transition which is partially reversed on cooling. The initial drop in peak height on the first heating scan (curve 1) indicates increased interaction as the lipid begins to melt, resulting in further immobilization. The failure of the peak height to rise to its initial height above T_c on the second heating scan (curve 3) also suggests that increased interaction has occurred after the first heating and cooling cycle. An abrupt increase and then a decrease in $\Delta\tau_0$ occur on all scans over the temperature range at which the phase transition occurs (Figure 5A). This change in $\Delta\tau_0$ is shifted to a lower temperature for the cooling curve. In contrast to the behavior with dipalmitoylphosphatidylglycerol, however, the changes in $\Delta\tau_0$ and signal intensity are equally distinct and of equal magnitude on all heating and cooling scans.

τ_0 for the protein bound to dimyristoylphosphatidylethanolamine above the phase transition is 0.21 ns, less than that for the protein bound to dipalmitoylphosphatidylglycerol (Table I).

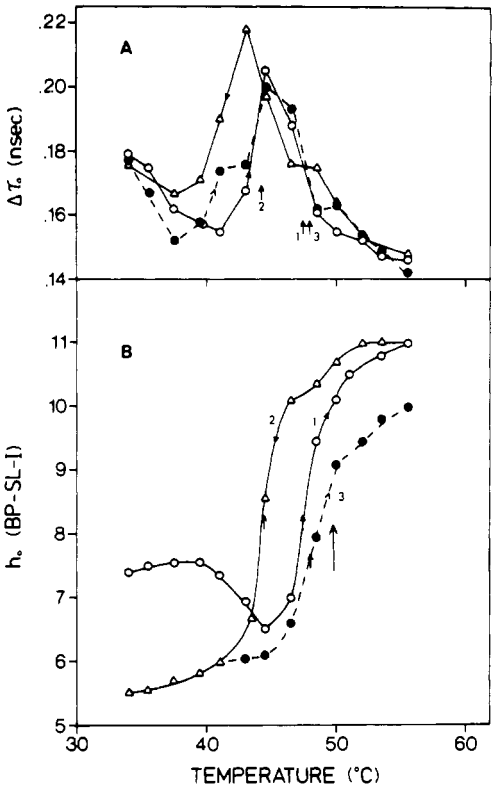

FIGURE 5: Temperature dependence of (A) $\Delta\tau_0$, change in the motional parameter of the spin-labeled protein in dimyristoylphosphatidylethanolamine vesicles relative to that in solution, and (B) the height of the center line, h_0, of the electron spin resonance spectrum of iodoacetamide spin-label I covalently bound to basic protein in dimyristoylphosphatidylethanolamine vesicles. The protein was added to the lipid above its phase transition temperature. (1) Heating scan (○); (2) cooling scan (△); (3) repeated heating scan (●). In (B), a large arrow indicates the T_c of pure dimyristoylphosphatidylethanolamine determined by differential scanning calorimetry. Small arrows indicate the position of the phase transition from the midpoints of the curves. These temperatures are marked by arrows in (A) also.

Basic Protein–Dimyristoylphosphatidic Acid Vesicles. Basic protein decreases the phase transition of unsaturated phosphatidic acid or dimyristoylphosphatidic acid to a similar extent as in dipalmitoylphosphatidylglycerol but has as large an effect on heating as on cooling as shown in parts a and b of Figure 6 for samples of dimyristoylphosphatidic acid containing 27 and 40% basic protein. No exothermic transition is observed for this lipid on heating. Heating and cooling scans for the 27% sample are also shown at a heating rate of 2.5 °C/min (parts c and d of Figure 6), where the sample was supercooled to 20 °C before heating. In Figure 6e, the sample was cooled at 2.5 °C/min to 37 °C, just below the phase transition, and immediately reheated. The heating scan has an identical appearance with that obtained after cooling to 20 °C (Figure 6c). Thus, supercooling does not change the appearance of the heating scan of dimyristoylphosphatidic acid containing basic protein as it did in the dipalmitoylphosphatidylglycerol. This suggests that the hydrophobic interaction with dimyristoylphosphatidic acid is not reversed by cooling.

The ESR spectra of the spin-labeled protein bound to dimyristoylphosphatidic acid vesicles at 34, 48.5, and 59 °C are shown in Figure 7. The protein was added below the lipid phase transition. The spin-label is more immobilized than in dipalmitoylphosphatidylglycerol below the phase transition. There are at least two components at 34 °C, one quite immobilized and the other moderately immobilized (Figure 7A).

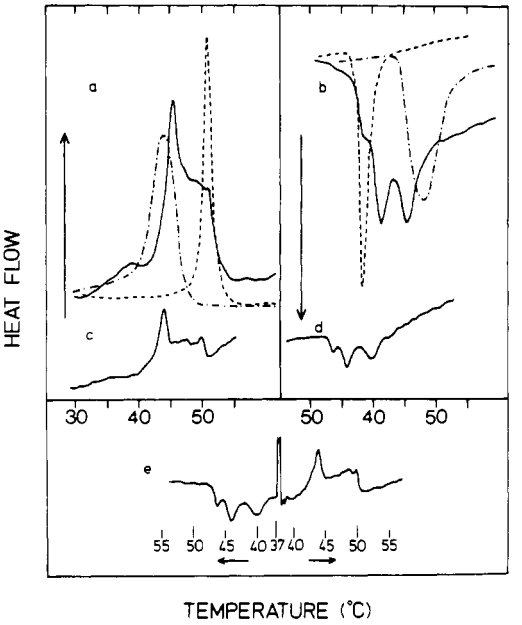

FIGURE 6: Differential scanning calorimetry scans for dimyristoylphosphatidic acid vesicles with or without basic protein on heating (a and c) and cooling (b and d). In (a) and (b), samples containing 0 (---), 27 (—), and 40% basic protein (–·–) were scanned at 10 °C/min. In (c) and (d), samples containing 27% basic protein were scanned at 2.5 °C/min from 17 to 60 °C. In (e), the sample containing 27% basic protein was cooled from 60 to 37 °C and immediately reheated with the instrument set on cycle, as in Figure 1h,i.

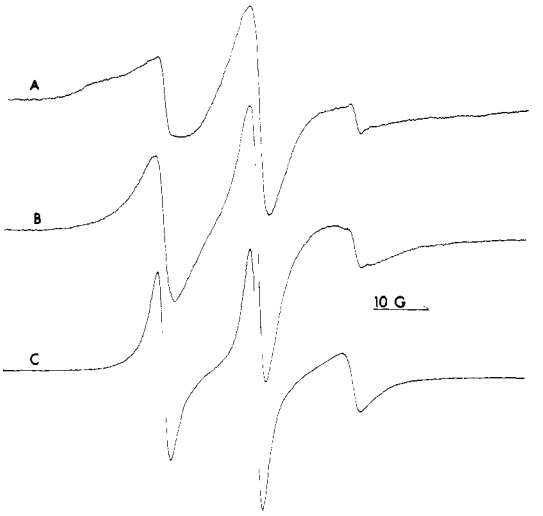

FIGURE 7: Electron spin resonance spectra of iodoacetamide spin-label I covalently bound to basic protein in dimyristoylphosphatidic acid vesicles at (A) 34, (B) 48.5, and (C) 59 °C.

As the temperature increases, the spectrum appears to become a single-component spectrum with increasing mobility (Figure 7C). The motional parameter could not be calculated below the phase transition. However, above the phase transition it is 0.61 ns, greater than that in the other lipids (Table I). Above the phase transition, the hyperfine splitting value is 16.22 G, somewhat less than in the other lipids, indicating that the probe is in a somewhat less polar environment.

On the first heating scan (Figure 8A, curve 1), the signal intensity of the protein spin-label rises a few degrees below the lipid phase transition temperature as monitored by 16-doxylstearate (Figure 8A, dashed line) but less sharply. On being cooled (Figure 8A, curve 2), it drops back a few degrees lower than the first heating scan (curve 1), indicating, as with the other lipids, some perturbing effect of the protein on the

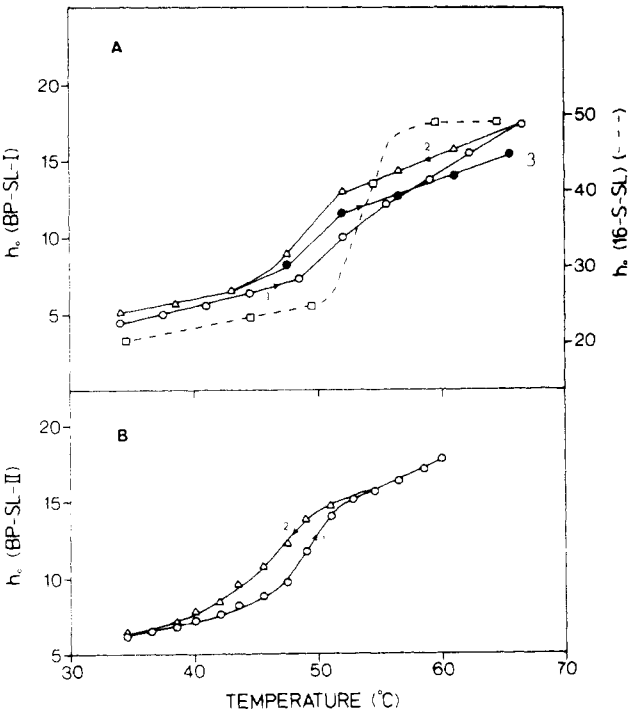

FIGURE 8: Temperature dependence of the height of the center line of the electron spin resonance spectrum of (A) iodoacetamide spin-label I covalently bound to basic protein in dimyristoylphosphatidic acid vesicles on (1) the first heating scan (○), (2) the cooling scan (△), and (3) the second heating scan (●) and the height, h_0, of 16-doxylstearate in pure dimyristoylphosphatidic acid (---). Temperature dependence of the height of the center line of the electron spin resonance spectrum of (B) iodoacetamide spin-label II covalently bound to basic protein in dimyristoylphosphatidic acid vesicles on (1) the first heating scan (○) and (2) the cooling scan (△). The spin-labeled proteins were added below the lipid phase transition temperature.

lipid suggestive of increased hydrophobic interaction. The transition is more gradual than that in dimyristoylphosphatidylethanolamine but not nearly as broad as that in dipalmitoylphosphatidylglycerol. The second heating scan (Figure 8A, curve 3) is shifted upward from the cooling scan (curve 2) by only about 1 °C, in agreement with differential scanning calorimetry results. The peak height does not reach the same value as on the first heating scan (curve 1), suggesting that increased hydrophobic interaction and immobilization have occurred once the sample has been heated.

The motion of the iodoacetamide spin-label II with a longer chain length between the protein and the pyrrolidinyl ring was also sensitive to the phase transition in dimyristoylphosphatidic acid as shown in Figure 8B. As indicated in Table I, τ_0 above the phase transition was less than for spin-label I, 0.46 ns, and the hyperfine splitting was a little larger, 16.35 G, indicating location in a somewhat more polar environment with less restricted motion than spin-label I. However, the motion of even this longer spin-label is more restricted by binding of the protein to dimyristoylphosphatidic acid vesicles and in a less polar environment than spin-label I in the other lipids. An immobilized component was also observed in the spectrum of spin-label II in dimyristoylphosphatidic acid below the phase transition.

Discussion

Studies of the effect of basic protein on lipid organization suggest that hydrophobic segments of the protein are able to interact with the lipid fatty acid chains either by penetration into the lipid bilayer or by deformation of the bilayer or both. This interaction occurs to an even greater degree above the lipid phase transition than below it and is partially reversed by cooling to below the T_c for at least one lipid, dipalmitoylphosphatidylglycerol (Papahadjopoulos et al., 1975; Boggs & Moscarello, 1978a). This is likely to result in a conformation change of the protein. Interaction of basic protein with lipids and detergents has been found to induce 20% α-helical and 12% β structure in the protein, which has a random structure in the absence of lipid (Anthony & Moscarello, 1971; Keniry & Smith, 1979). This study, using spin-label probes bound to the methionines of the protein, represents the first attempt to look at the effect of the lipid environment on the protein throughout the phase transition.

The hyperfine splitting values for the protein spin-label in the lipid vesicles indicate that it is in a relatively polar environment. This is to be expected in view of the positive charge acquired by the methionine after alkylation with the spin-label. The probe giving rise to the immobilized component at lower temperatures in dipalmitoylphosphatidylglycerol and dimyristoylphosphatidic acid is probably also in a polar environment. However, the following facts suggest that the probe is located at the bilayer surface, possibly embedded as deeply as the ester linkage, rather than extending into the aqueous phase: (1) an immobilized component can also be observed with the longer probe, spin-label II, in dimyristoylphosphatidic acid at low temperatures; (2) the hyperfine splitting of both of these probes in all three lipids at higher temperatures is less than that of the protein in solution; (3) both probes are sensitive to the lipid phase transition in all three lipids; (4) these probes have low water solubility and are soluble in apolar solvents.

The greater immobilization of the probe in dipalmitoylphosphatidylglycerol and dimyristoylphosphatidic acid below the phase transition, compared to dimyristoylphosphatidylethanolamine, suggests that the probe is embedded more deeply into the polar region of these two lipids than that of dimyristoylphosphatidylethanolamine. This is also suggested by the lower hyperfine splitting for dimyristoylphosphatidic acid. The immobilization of the protein spin-label in these lipids is unlikely to be due to specific interaction of the probe with the polar head groups since dimyristoylphosphatidic acid has the smallest polar head group and yet immobilizes the probe to the greatest extent.

Sensitivity of the probe to the lipid transition probably reflects motion of the polar head group. ^{31}P NMR studies on a variety of lipids have shown that the motion of the phosphorus in the polar head group of phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylcholine, but not phosphatidic acid, increases during the lipid phase transition (Cullis & de Kruyff, 1976; Kohler & Klein, 1977). The orientation of the phosphorus in phosphatidic acid does reflect the phase transition, however. Changes in the motion of the head groups occurred a few degrees lower than the main transition, which may account for the decrease in T_c sensed by the protein spin-label on the first heating scan in all three lipids. The larger decrease in T_c detected by the protein spin-label on cooling probably indicates increased perturbation of the lipid in the microenvironment of the protein. This perturbation could be due to deeper penetration of the methionine regions into the polar region of the bilayer and to increased hydrophobic interaction of other regions of the protein with the lipid hydrocarbon region which might be sensed by the methionine spin-labels.

Differential scanning calorimetry evidence suggests that the protein can interact hydrophobically with dipalmitoylphosphatidylglycerol to a much greater extent than di-

myristoylphosphatidylethanolamine and also that the interaction is much greater with dipalmitoylphosphatidylglycerol in the liquid-crystalline phase than in the gel phase. The fact that the methionine spin-label no larger detects a sharp phase transition in dipalmitoylphosphatidylglycerol on cooling and second heating while it does in dimyristoylphosphatidylethanolamine suggests that the methionine spin-label is indeed sensitive to the lipid perturbation caused by increased hydrophobic interaction of other segments of the protein in dipalmitoylphosphatidylglycerol and that it can only sense a sharp phase transition when no such perturbation occurs.

The situation with dimyristoylphosphatidic acid is different from dipalmitoylphosphatidylglycerol. The protein has nearly as great an effect on the phase transition temperature as in dipalmitoylphosphatidylglycerol but has a similar effect on heating and cooling, suggesting that the hydrophobic interaction is not reversed on supercooling. The signal intensity of the spin-labeled protein in dimyristoylphosphatidic acid was equally sensitive to the phase transition on first heating, cooling, and reheating in contrast to dipalmitoylphosphatidylglycerol, although the curve was shifted down a few degrees for cooling and reheating. However, the change in signal intensity with temperature was more gradual for dimyristoylphosphatidic acid than for dimyristoylphosphatidylethanolamine but not nearly as broad as in dipalmitoylphosphatidylglycerol on cooling and reheating. This suggests that the perturbation of the lipid in the environment of the protein spin-label in dimyristoylphosphatidic acid is greater than in dimyristoylphosphatidylethanolamine but less than in dipalmitoylphosphatidylglycerol.

The complex way in which $\Delta\tau_0$ changes during the phase transition of dipalmitoylphosphatidylglycerol and dimyristoylphosphatidylethanolamine and the correlation between the differential scanning calorimetry results and the temperature dependence of $\Delta\tau_0$ on first heating, cooling, and second heating suggest that it is also sensitive to changes in the interaction of the protein with the lipid above and below the phase transition and to the perturbing effect due to the hydrophobic interaction of other regions of the protein with the lipid. The changes in $\Delta\tau_0$ in dipalmitoylphosphatidylglycerol and dimyristoylphosphatidylethanolamine can be interpreted in terms of opposing effects which tend to simultaneously increase and decrease $\Delta\tau_0$ as the lipid melts. Thus, a decrease in $\Delta\tau_0$ occurs as the lipid melts due to increased mobility of the spin-labeled protein bound to the more fluid lipid. At the same time, however, increased interaction with the lipid as it melts tends to decrease the mobility of the probe. The net change in $\Delta\tau_0$ above and below the phase transition is therefore small and $\Delta\tau_0$ is higher in the gel phase than in the liquid-crystalline phase. τ_0 measurements for the protein in the liquid-crystalline phase, however, may indicate the degree of hydrophobic interaction of the protein with the lipid. A deeper location of the probe in the polar region in more expanded lipids should increase τ_0. Increased hydrophobic interactions of other regions of the protein might also increase τ_0 through the perturbing effect on the lipid in the environment of the protein spin-label, just as these interactions cause a decrease in the mobility of a fatty acid, spin-labeled near the polar head group (Boggs & Moscarello, 1978a). The same forces which allow deeper penetration of the methionine region of the protein should also allow increased hydrophobic interaction of other regions of the protein with the lipid. In both cases lipid expansion and decreased intermolecular interactions between lipid polar head groups are required. Thus, τ_0 was used in the preceding paper as a measure of the degree of hydrophobic interaction of the protein with different lipids (Stollery et al., 1980).

The values of τ_0 for the protein bound to the different lipids used in this study decreased in the order dimyristoylphosphatidic acid >> dipalmitoylphosphatidylglycerol > dimyristoylphosphatidylethanolamine >> protein in solution, suggesting that the greatest penetration of the methionine region occurred in dimyristoylphosphatidic acid and dipalmitoylphosphatidylglycerol. However, the protein spin-label was least sensitive to the phase transition of dipalmitoylphosphatidylglycerol (after the first heating), followed by dimyristoylphosphatidic acid and then dimyristoylphosphatidylethanolamine, indicating that the greatest perturbation of the lipid, and most likely the greatest hydrophobic interaction of other regions of the protein, occurred with dipalmitoylphosphatidylglycerol.

A comparison of the results obtained with the natural lipids, egg phosphatidylethanolamine, and phosphatidylglycerol and phosphatidic acid derived from egg phosphatidylcholine, used in the preceding paper, with those obtained on the saturated lipids is interesting. For the unsaturated lipids, egg phosphatidylglycerol produced the greatest immobilizing effect, followed by egg phosphatidic acid, while for the saturated lipids the order was reversed. The reversal of the hydrophobic interaction of the protein with dipalmitoylphosphatidylglycerol below the phase transition indicates that the closely packed lipid in the gel state cannot interact hydrophobically with the protein. Above the phase transition dipalmitoylphosphatidylglycerol is more closely packed than egg phosphatidylglycerol and so may not allow as much hydrophobic interaction. The interaction with dimyristoylphosphatidic acid is not reversed below the phase transition, indicating that it can occur even when the lipid is closely packed, although a perturbation of the packing clearly occurs. Thus, the interaction with egg phosphatidic acid which is less closely packed than dimyristoylphosphatidic acid above the phase transition might not be expected to be better than that with dimyristoylphosphatidic acid. In fact, the protein spin-label is immobilized even more in dimyristoylphosphatidic acid than in egg phosphatidic acid which may be due to the closer packing and greater order in dimyristoylphosphatidic acid.

The immobilizing effect of dimyristoylphosphatidylethanolamine was similar to that of egg phosphatidylethanolamine, but the maximum amount of protein which could be bound was only 11% compared to 25% for egg phosphatidylethanolamine. This can be related to the tighter packing of dimyristoylphosphatidylethanolamine. For example, the binding of Ca^{2+} to phosphatidylethanolamine in monolayers decreases as the molecular packing increases (Seimiya & Ohki, 1972). Decreased accessibility of the phosphate as suggested or increased intermolecular binding between the phosphate and the amine of neighboring phosphatidylethanolamine molecules may result in reduced binding of either divalent cations or basic protein.

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Escherichia coli Elongation Factor G Blocks Stringent Factor†

E. Gerhart H. Wagner* and Charles G. Kurland

ABSTRACT: The relationship between the binding domains of elongation factor G (EF-G) and stringent factor (SF) on ribosomes was studied. The binding of highly purified, radioactively labeled, protein factors to ribosomes was monitored with a column system. The data show that binding of EF-G to ribosomes in the presence of fusidic acid and GDP or of the noncleavable analogue GDPCP prevents subsequent binding of SF to ribosomes. In addition, stabilization of the EF-G–ribosome complex by fusidic acid inhibits SF's enzymatic activities. Removal of protein L7/L12 from ribosomes leads to weaker binding of EF-G, while SF's binding and activity are unaffected. In the absence of L7/L12, EF-G-dependent inhibition of SF binding and function is reduced. The data presented in this report suggest that these two factors bind at overlapping, or at least interacting, ribosomal domains.

There are two classes of factor-dependent reactions for nucleoside triphosphates on the ribosome. One of these is the hydrolysis of GTP to form GDP and inorganic phosphate as, for example, in the reactions mediated by the elongation factors (Lucas-Lenard & Lipman, 1971). The other is a reaction of GTP with ATP to form ppGpp[1] and pppGpp, the so-called magic spots MSI and MSII, which are produced by stringent factor (Haseltine et al., 1972; Pedersen et al., 1973; Sy & Lipmann, 1973). The functional relationship between the ribosomal domains responsible for these two kinds of nucleotide reactions is the subject of the present report.

That the ribosomal domains associated with the hydrolytic reactions of GTP are functionally coupled to each other, if not overlapping, is suggested primarily by two lines of evidence. First, the elongation factors G (EF-G) and Tu (EF-Tu) cannot be bound simultaneously to the ribosome (Cabrer et al., 1972; Miller, 1972; Richter, 1972; Modolell & Vazquez, 1973; Richman & Bodley, 1972). Second, the 50S ribosomal protein L7/L12 seems to be indispensable for the hydrolytic reactions mediated by both EF-Tu and EF-G as well as by initiation factor 2 (Fakunding et al., 1973; Kay et al., 1973; Lockwood, 1974; Möller, 1974).

In contrast, it has been argued that a separate, nonoverlapping ribosome domain is associated with (p)ppGpp production by stringent factor (SF). Thus, the protein L7/L12 is dispensable for the SF-dependent reactions on the ribosome (Lund et al., 1973; Richter, 1973). In addition, it has been suggested that SF can be bound to the ribosome simultaneously with elongation factors (Kleinert & Richter, 1975; Richter et al., 1975). Nevertheless, there are other observations that are not wholly compatible with a complete functional separation of the ribosomal domains responsible for the two nucleotide reactions.

Thus, the ribosome-dependent formation of (p)ppGpp by SF requires deacylated tRNA, which is bound codon specifically, and just as with normal A site function, this reaction is sensitive to tetracycline (Pedersen et al., 1973; Haseltine & Block, 1973). If SF is functioning at a site which overlaps or is near to the A site, we might expect the elongation factors to interfere with SF binding to its ribosome domain. Indeed, it has been observed that in the presence of low concentrations of fusidic acid, EF-G inhibits (p)ppGpp production (Lund et

† From the Institute of Molecular Biology, Wallenberg Laboratory, Dag Hammarskjölds väg 21, S-751 22 Uppsala, Sweden. *Received May 15, 1979.* This work was supported by the Swedish Cancer Society and the Natural Sciences Research Council.

[1] Abbreviations used: EF-G, elongation factor G; EF-Tu, elongation factor Tu; SF, stringent factor (pyrophosphoryl transferase); pppGpp, guanosine 5′-triphosphate 3′-diphosphate; ppGpp, guanosine 5′-diphosphate 3′-diphosphate; GDPCP, β,γ-methyleneguanosine 5′-triphosphate; poly(U), poly(uridylic acid); IgG, immunoglobulin G; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate.

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