Study by Infrared Spectroscopy of the Interaction of Bovine Myelin Basic Protein with Phosphatidic Acid†

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ABSTRACT: The effect of bovine myelin basic protein (MBP) on dimyristoylphosphatidic acid (DMPA) and phosphatidic acid prepared from egg yolk phosphatidylcholine (EPA) has been investigated by transmission and attenuated total reflectance (ATR) Fourier transform infrared spectroscopy. Interaction of MBP with DMPA and EPA dispersions decreases the lipid acyl chain conformational disorder as a consequence of hydrophobic interactions of the protein with the lipids. Since these effects are more important for EPA dispersions than for DMPA, MBP is believed to penetrate more into EPA bilayers. This could be due to the fact that the hydrogen bond network formed by the charged polar headgroups of EPA is weaker than that of DMPA. This is supported by the spectra of the phosphate region showing that the phosphate groups of EPA are less hydrogen bonded than DMPA. In the presence of MBP, the hydrogen bond network is replaced by electrostatic interactions of the protein with the polar headgroups of the lipid. Infrared spectra of the polar headgroup region also show evidence that MBP enhanced the second ionization state of the phosphate group at neutral pH, this effect being more important for EPA than for DMPA bilayers. Also, infrared spectra of the lipid carbonyl stretching region show evidence that MBP limits the accessibility of water molecules to the interfacial part of the lipid bilayer. Finally, ATR measurements on oriented films of lipid/protein complexes indicate that the penetration of the protein into the lipid bilayer is followed by a reorientation of the lipid acyl chains toward the normal to the bilayer in the case of EPA. Assuming a narrow distribution of conformation, we calculated that the mean angle of the C=O amide bonds of the protein with regard to the bilayer normal is around 65°. Since the conformation of MBP is rather complex and composed of several secondary structure elements, it is difficult to associate this angle with an overall orientation of the protein.

Myelin basic protein (MBP)[1] accounts for about 10 wt % of myelin isolated from brain white matter. It may be involved in the pathogenesis of demyelinating diseases such as multiple sclerosis. Although its function is not completely understood, it is thought to be responsible for the tight association of the cytoplasmic surfaces of myelin, through electrostatic interactions with lipids [reviewed by Boggs et al. (1982)] and possibly by dimerization of the protein (Golds & Braun, 1978). In addition to its high content of basic amino acids, it also has a certain degree of apolar character similar to a group of other extrinsic proteins such as the cytolytic peptide mellitin, toxins, apocytochrome *c*, and viral fusion peptides, which seem to be able to interact with the lipid acyl chains in some way, perhaps transiently, giving these proteins a dynamic function.

The perturbing effects of MBP on the lipid bilayer include such effects as increasing the permeability of the bilayer, fusion of lipid vesicles, expansion of lipid monolayers, and a decrease in the temperature and enthalpy of the phase transition of acidic lipids (Gould & London, 1972; Papahadjopoulos et al., 1973, 1975; Demel et al., 1973; London & Vossenberg, 1973; London et al., 1973; Boggs & Moscarello, 1978; Bogs et al., 1981a). Furthermore, MBP can be labeled from the lipid bilayer with the hydrophobic photolabel TID (Boggs et al., 1988). Although it does not bind to a significant extent to bilayers of phosphatidylcholine (PC) (Palmer & Dawson, 1969; Demel et al., 1973; London et al., 1973; Boggs & Moscarello, 1978), it has recently been shown to disrupt PC bilayers into small lipoprotein particles (Roux et al., 1994) similar to mellitin (Dufourc et al., 1986) and apolipoproteins (Brouillette et al., 1984), suggestive of an interaction with the acyl chains of PC.

In spite of its perturbing effect on the lipid, suggestive of a fluidizing effect, it has an immobilizing and ordering effect on fatty acid spin-labels down to the 12th or 14th carbon but little or no effect on the 16th carbon near the terminal methyl group of the acyl chain in both the gel and liquid-crystalline phases of phosphatidylglycerol (PG) (Boggs & Moscarello, 1978; Boggs et al., 1981a; Sankaram et al., 1989). Large effects on the motion of a fatty acid labeled at the 16th carbon in the gel phase of PG are due to

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[1] Abbreviations: ATR, attenuated total reflection; DMPA, dimyristoylphosphatidic acid; DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetic acid; EPA, egg phosphatidic acid; ESR, electron spin resonance; FTIR, Fourier transform infrared; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MBP, myelin basic protein; PA, phosphatidic acid; PC, phosphatidylcholine; R_i, lipid-to-protein molar ratio.

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interdigitation (Boggs et al., 1981a), which does not occur in PA.

In the present work, we have used Fourier transform infrared (FTIR) spectroscopy in order to determine effects of the protein on the acyl chains of phosphatidic acid (PA) using a nonprobe technique. This technique also permits observation of the characteristic bands of other groups of the lipid such as the carbonyl region and the phosphate headgroup. The effect of MBP on the thermotropic phase transition of synthetic saturated dimyristoylphosphatidic acid (DMPA) and unsaturated phosphatidic acid prepared from hen egg yolk phosphatidylcholine (EPA) was investigated. Although FTIR has been used previously to study the interaction of MBP with PG (Surewicz et al., 1987), as noted above, MBP behaves differently with PA than with PG. Interdigitation of PG prevents the MBP-induced decrease in transition temperature, and thus the effects of MBP on the lipid chains related to this decrease cannot be investigated using saturated PG. The interaction with PA is also of interest because it can be affected by the intermolecular hydrogen bonding properties of this lipid (Boggs et al., 1986). In addition, the orientation of the different functional groups of the lipid bilayer in the presence and absence of the protein as well as the orientation of the amide C=O groups of the protein has been determined by polarized attenuated total reflectance (ATR) spectroscopy. This study allows deeper insight into the nature of the interaction of MBP with phosphatidic acid.

MATERIALS AND METHODS

Materials. The disodium salts of dimyristoylphosphatidic acid (DMPA) and egg yolk phosphatidic acid (EPA) were purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. Myelin basic protein was extracted from bovine white matter according to the method described by Cheifetz and Moscarello (1985).

Sample Preparation. Appropriate amounts of solid lipid and protein were mixed at a lipid-to-protein molar ratio (R_i) of 30. Two milligrams of powder was then dispersed in 100 μL of an aqueous buffer solution containing 10 mM HEPES, 10 mM NaCl, and 1 mM EDTA and adjusted to pH 7, which caused immediate precipitation of the lipid/protein complexes. Samples were then heated and cooled to 10 °C above and below the gel-to-liquid-crystalline phase transition temperature. The samples were vortexed between each temperature cycle. This operation was repeated 3 times in order to ensure good homogeneity of the sample, and the precipitate was washed 2 times with the buffer solution in order to eliminate the noncomplexed protein in the suspension.

Infrared Measurements. Approximately 1 mg of lipid−protein wet pellet was added to about 20 μL of buffer and placed between two BaF_2 windows separated by a 12 μm Mylar spacer of a homemade transmission cell. The cell was placed in a computer-controlled thermoelectrically regulated sample holder as described elsewhere (Pézolet et al., 1983); the equilibration time between each temperature was 2 min. Spectra were recorded on a Bomem DA3-02 or a Nicolet Magna 550 Fourier transform infrared spectrophotometer equipped with a narrow-band mercury−cadmium−telluride detector and a germanium-coated KBr beam splitter. A total of 250 scans were averaged at 2 cm^{-1} resolution after triangular apodization.

Oriented films of multilayers of anionic lipids and lipid/protein complexes were obtained by slowly spreading the wet pellet, prepared as described above, with a Teflon bar on one side of germanium attenuated total reflectance (ATR) plates (50 × 20 × 2 mm 45° parallelograms) until dry films were obtained. Prior to the sample deposition, the ATR plates were cleaned with a plasma cleaner (Harrick, NY) in order to make the surface more hydrophilic. The plates supporting the dry films were placed in a variable-angle ATR unit (Harrick, NY) preceded by a rotating wire-grid polarizer (Specac, Orpington, U.K.). Dichroic ratios (R) were determined from either peak heights or integrated intensities of the bands measured with the incident radiation polarized parallel ($A_{\parallel}$) and perpendicular ($A_{\perp}$) with respect to the normal of the ATR plate ($R = A_{\parallel}/A_{\perp}$) (Fringeli & Günthard, 1981). Assuming an uniaxial fiber-type distribution of orientation with respect to the normal to the ATR crystal, the order parameter, $f(\theta)$, related to the orientation of the fiber axis and the normal to the ATR crystal, was calculated using the formula (Hübner & Mantsch, 1991):

$$f(\theta) = \frac{R-2}{R+1.45}\,\frac{2}{3\cos^2\alpha - 1}$$

where α is the angle between the transition moment of a given vibration and the fiber axis. The angle α was set to 90° for the CH_2 stretching and bending modes, and to 0° for the wagging progression bands, and the bands due to the phosphate and the carbonyl groups of the lipid. For the protein, α angles of 15°, 75°, and 10° between the transition moment of amide I, amide II, and amide A vibrations and the peptide C=O bond were used, respectively, according to Rothschild and Clark (1979). The order parameter $f(\theta)$ was then calculated using the formula:

$$f(\theta) = \frac{3\cos^2\theta - 1}{2}$$

RESULTS

Acyl Chain Region. The 2700−3100 cm^{-1} region of the infrared spectra of phospholipids is characterized by two strong bands at 2850 and 2920 cm^{-1} due to the symmetric and antisymmetric CH_2 stretching vibrations, respectively. The frequency of these bands is sensitive to the conformational disorder of the acyl chains and to the *trans-gauche* isomerization of the lipids (Snyder et al., 1978; Umemura et al., 1980). The temperature-induced gel-to-liquid-crystalline phase transition of phospholipids can thus be followed easily from the frequency of the acyl chain bands. In this study, only the band near 2850 cm^{-1} was analyzed, since the protein spectral interference is negligible in this spectral region.

Figure 1A shows the effect of temperature on the frequency of the CH_2 symmetric vibration for a pure DMPA dispersion, and for DMPA in the presence of myelin basic protein. As seen in this figure, the gel-to-liquid-crystalline transition of pure DMPA occurs at 50 °C at pH 7.0, in agreement with previous studies (Copeland & Anderson, 1982). In the presence of MBP at a lipid-to-protein molar ratio of 30 and pH 7.0, the transition temperature shifts down to about 43 °C. Such a decrease of the phase transition temperature of DMPA induced by the protein has been previously observed by calorimetry (Boggs & Moscarello,

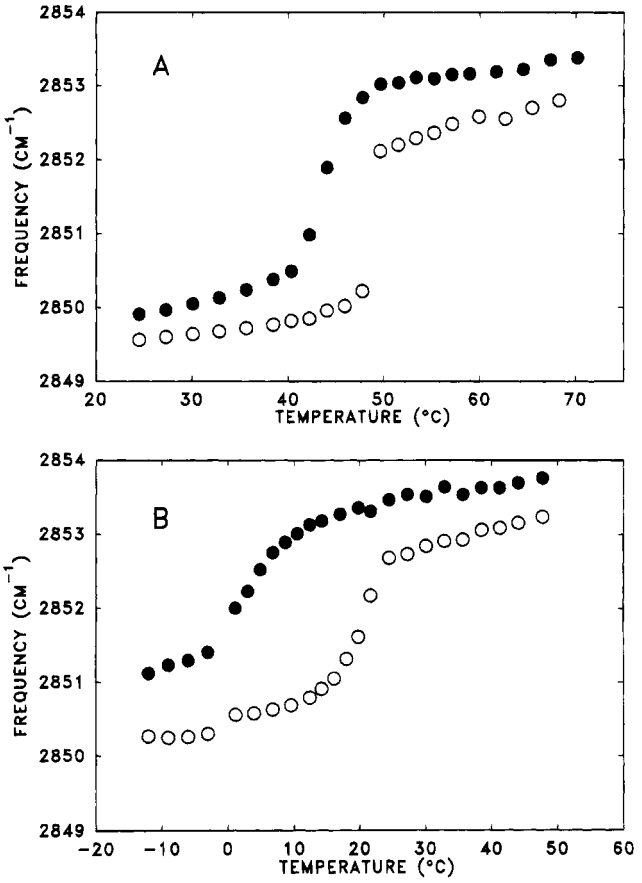

FIGURE 1: Temperature dependence of the acyl chain CH_2 symmetric stretching vibration of (A) DMPA dispersions (○) and DMPA/MBP complexes (●), and (B) EPA dispersions (○) and EPA/MBP complexes (●).

1978) and by electron spin resonance spectroscopy (ESR) (Boggs et al., 1981a). At pH 7.0, myelin basic protein increases slightly the frequency of the 2850 cm^{-1} band in both the gel phase and the liquid-crystalline phase. This result indicates that at a lipid-to-protein molar ratio of 30, MBP induces some conformational disorder in the bilayer core.

Figure 1B shows the temperature dependence of the 2850 cm^{-1} band of EPA, in the absence and presence of MBP at $R_i = 30$ at pH 7.0. The phase transition of this natural lipid occurs at approximately 19 °C and is significantly broader than that of DMPA because of the presence of acyl chains of varying length and the degree of unsaturation. In the presence of MBP, the phase transition temperature of the lipid shifts down from 19 °C to almost 0 °C, in agreement with the differential scanning calorimetry results of Boggs and Moscarello (1978). In addition, the transition occurs over a broader range of temperature, and, as for DMPA, the acyl chains for both the gel and the liquid-crystalline phases are slightly more disordered in the presence of the protein. The effect of the protein on the phase transition and on the frequency of the 2850 cm^{-1} band is greater for EPA than for DMPA.

Interfacial Region. Infrared spectra of phospholipids normally show two bands at 1740 and 1725 cm^{-1} due to C=O stretching modes. The high-frequency component has been associated with free C=O groups, while the low-frequency band is due to C=O groups hydrogen bonded to water molecules (Hübner et al., 1994; Blume et al., 1988). The ratio of the intensities of these two bands, I_{1740}/I_{1725}, can thus give information on the degree of hydration of the bilayer interfacial region. Figure 2 shows the C=O stretching mode region of DMPA and EPA at pH 7.0 in the absence and in the presence of MBP at reduced temperatures of 15 °C below or above the phase transition temperature for the gel and the liquid-crystalline phases, respectively. As seen in this figure, for the pure lipids the proportion of hydrogen-bonded carbonyl groups increases when they undergo the gel-to-liquid-crystalline phase transition, in agreement with previous studies on other phospholipids (Blume et al., 1988). In the presence of MBP, the carbonyl stretching mode region of both DMPA and EPA is slightly affected. In the gel phase, a slight but reproducible decrease of the I_{1740}/I_{1725} ratio is observed in the presence of the protein, suggesting that more carbonyl groups are hydrated when the protein is bound to the lipid. On the other hand, in the liquid-crystalline phase, less carbonyl groups are hydrated in the presence of the protein, the effect being more important for EPA than for DMPA. Similar results were also observed by Surewicz et al. (1987) upon binding of myelin basic protein to dimyristoylphosphatidylglycerol bilayers.

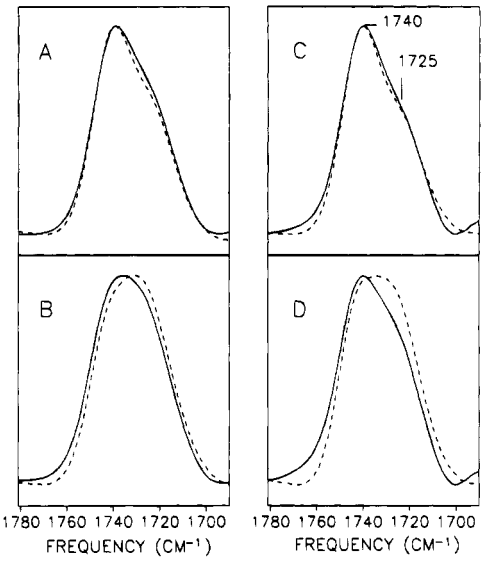

FIGURE 2: Infrared spectra of the lipid carbonyl stretching mode region of pure DMPA (- - -) and DMPA/MBP complexes (—) (left), and pure EPA (- - -) and EPA/MBP complexes (—) (right), in the gel phase (A, C) and in the liquid-crystalline phase (B, D) at the same reduced temperatures of 15 °C below and above the phase transition temperatures of the samples.

Polar Headgroup Region. The phosphate stretching mode region of the infrared spectra of phospholipids, from 900 to 1300 cm^{-1}, is very useful for monitoring electrostatic interactions, hydrogen bonding, and ionization of the headgroup. Figure 3 shows the phosphate spectral region of DMPA and EPA with and without MBP at pH 7.0 at the same reduced temperatures in the gel and liquid-crystalline phases, after subtraction of the spectral contribution of the buffer. In order to compare the relative intensities of the phosphate bands for the free and protein-bound lipid, spectra of this region have been normalized using the area of the lipid carbonyl stretching band. DMPA in the gel phase (Figure 3A) has two strong bands at 1173 and 1071 cm^{-1} assigned to the antisymmetric and symmetric stretching modes of the PO_2^- group, respectively (Thomas & Chittenden, 1970). The P−O−(C) and P−O−(H) stretching modes give bands at 989 and 927 cm^{-1}, respectively (Thomas & Chittenden, 1964). Between 1200 and 1300 cm^{-1}, bands

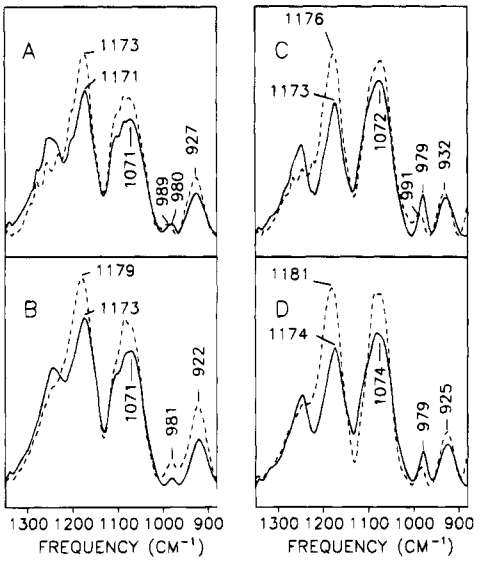

FIGURE 3: Infrared spectra of the phosphate stretching mode region of DMPA (- - -) and DMPA/MBP complexes (—) (left), and pure EPA (- - -) and EPA/MBP complexes (—) (right), in the gel phase (A, C) and in the liquid-crystalline phase (B, D) at the same reduced temperatures of 15 °C below and above the phase transition temperatures of the samples.

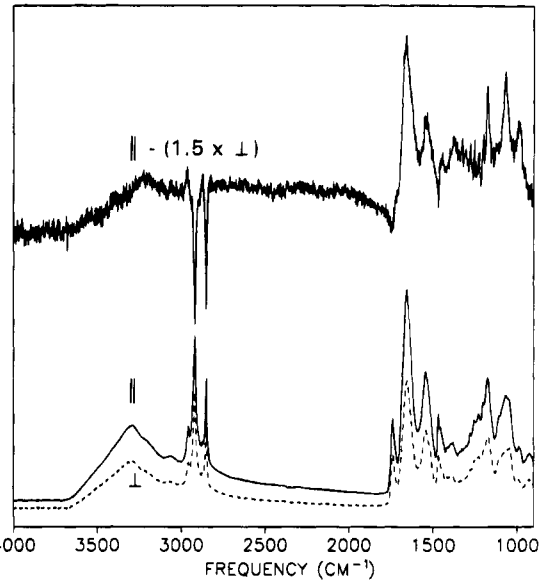

FIGURE 4: Polarized ATR infrared spectra of an oriented DMPA/MBP dry film measured with the polarization of the incident light parallel (∥) and perpendicular (⊥) to the crystal normal. The difference spectrum [∥ − (1.5 × ⊥)] is also shown in this figure (top curve).

due to the CH_2 wagging progression of the phospholipid acyl chains in the *all-trans* conformation are also observed in this spectral region (Casal & Mantsch, 1984). In the case of the DMPA/MBP complex, these bands are, however, partly covered by the amide III band of the protein around 1250 cm^{-1}.

For pure DMPA, the frequency of the PO_2^- symmetric stretching band increases from 1173 cm^{-1} in the gel phase to 1179 cm^{-1} in the liquid-crystalline phase. Studies on PC and phosphatidylserine bilayers have shown that an increase of the frequency of this phosphate band is associated with dehydration of the lipid headgroups (Arrondo et al., 1984; Dluhy et al., 1983; Casal & Mantsch, 1984). However, no such shift is reported during the phase transition of DPPC (Arrondo et al., 1984). Indeed, as shown by the spectra of the C=O stretching modes, in the liquid-crystalline phase, the bilayer surface becomes more fluid and allows water molecules to enter the interfacial polar/apolar region, making it unlikely that dehydration is the explanation for the increase in frequency in the liquid-crystalline phase of DMPA. It is more likely that an intermolecular hydrogen bond network formed by the lipid headgroups at the bilayer surface (Blume & Eibl, 1979) is weakened in the liquid-crystalline phase, resulting in the increase in frequency of the phosphate band. In the case of EPA, the frequency of this band is at 1176 cm^{-1} in the gel phase, higher than for DMPA, suggesting that the intermolecular hydrogen bonding interactions are weaker for the gel phase of EPA due to its heterogeneous acyl chain composition resulting in a greater molecular surface area for this lipid. The frequency increases further in the liquid-crystalline phase of EPA as for DMPA.

Myelin basic protein induces a shift toward lower frequency of this band for both DMPA and EPA, the shift being greater in the liquid-crystalline phase than in the gel phase. This shift is most likely due to the partial neutralization of the charged phosphate groups by the positively charged residues of the protein by electrostatic binding and/or hydrogen bonding of residues of MBP with the lipid phosphates (Désormeaux et al., 1992).

In the liquid-crystalline phase of DMPA, the weak band at 989 cm^{-1}, assigned to the P−O−(C) stretching mode, decreases, and a small band appears at around 980 cm^{-1}, due to the symmetric stretching mode of the PO_3^{2-} group (Désormeaux et al., 1992), indicating that the phase transition has an effect on the equilibrium between the singly and doubly ionized phosphate group populations. Upon binding of MBP to the gel phase of DMPA, the 980 cm^{-1} feature also increases, indicating that MBP increases the degree of ionization of the phosphate at pH 7.0. In the liquid-crystalline phase, MBP has little effect on the intensity of the band at 980 cm^{-1}, but it decreases the intensity of the band at 922 cm^{-1} due to the P−O−(H) stretching mode, indicating that it increases ionization in this phase also.

Comparison of the phosphate region of EPA and DMPA shows that the relative intensity of the band at 1176 cm^{-1} compared to the band at 1072 cm^{-1} is smaller for EPA than for DMPA in both the gel and liquid-crystalline phases. Furthermore, the band at 932 cm^{-1} due to the P−O−(H) stretch is weaker for EPA than for DMPA. Thus, it seems that DMPA and EPA do not have the same ionization state at pH 7.0. In the presence of MBP, the band at 979 cm^{-1} becomes much more pronounced, indicating that more PO_3^{2-} groups are present. It is noteworthy that the increase in intensity of this band is greater for the EPA/MBP complex than for DMPA/MBP.

ATR Measurements. In order to obtain information about the orientation of the protein and the lipid molecules in the PA/MBP complexes, oriented dry films of DMPA/MBP in the gel phase and EPA/MBP in the liquid-crystalline phase were investigated by polarized ATR spectroscopy. Figure 4 shows the infrared spectra obtained for the lipid/protein complex with the incident radiation polarized parallel and perpendicular to the plane of incidence, as well as the difference spectrum. Since the transition moment of the CH_2 wagging vibrations is parallel to the acyl chain axis while those of the CH_2 symmetric and antisymmetric stretching modes are perpendicular to the lipid chain axis, the subtraction coefficient used for the difference spectrum has been

Table 1: Values of Dichroic Ratios, R, Order Parameters, $f(\theta)$, and Average Angles, θ, for Selected Absorption Bands of Oriented Films of Pure DMPA and EPA Dispersions, and DMPA/MBP and EPA/MBP Complexes

		DMPA			DMPA/MBP (R_i 30)			EPA			EPA/MBP (R_i 30)		
assignment	frequency (cm^{-1})	R	$f(\theta)$	θ (deg)[a]	R	$f(\theta)$	θ (deg)[a]	R	$f(\theta)$	θ (deg)[a]	R	$f(\theta)$	θ (deg)[a]
νCH_2 antisym	2920	1.07	0.74	**24**	1.29	0.52	**35**	1.46	0.37		1.34	0.46	
νCH_2 sym	2850	1.03	0.78	**22**	1.27	0.54	**34**	1.44	0.38		1.34	0.46	
δCH_2	1465	1.12	0.68	**27**	1.48	0.44	**38**	1.52	0.32		1.35	0.45	
CH_2 wagging	1230	6.77	0.58	**32**									
	1195	15.49	0.79	**22**									
$\nu C{=}O$	1740	1.15	−0.33	**70**	1.37	−0.22	**65**	1.51	−0.19	**63**	1.36	−0.21	**64**
$\nu C{=}O{\cdots}H$	1720	1.67	−0.11	**59**	1.81	−0.09	**57**	−1.80	−0.06	**57**	−1.87	−0.08	**58**
νPO_2^- antisym	1177	1.89	−0.03	**56**	1.62	−0.12	**60**	1.42	−0.20	**63**	1.31	−0.25	**66**
νPO_2^- sym	1046	2.18	−0.05	**53**	1.61	−0.13	**60**	1.18	−0.31	**69**	1.30	−0.26	**66**
$\nu P{-}O{-}(C)$	990	2.41	0.11	**51**	1.72	−0.09	**58**						
νPO_3^- sym	980										1.50	−0.17	**62**
$\nu P{-}O{-}(H)$	920	1.89	−0.03	**56**	1.27	−0.27	**67**	1.18	−0.30	**68**	1.32	−0.25	**66**
amide A	3283				1.54	−0.16	**62**				1.40	−0.21	**65**
amide I	1660				1.64	−0.13	**60**				1.40	−0.21	**65**
amide II	1530				1.61	0.32	**43**				1.41	0.50	**35**

[a] θ angles are calculated by considering an infinitely narrow distribution.

adjusted in order to observe positive wagging bands and a negative stretching band in the difference spectrum. These spectra show that the lipid molecules, as well as the protein, are oriented since the CH_2 lipid bands at 2920 and 2850 cm^{-1} appear negative on the difference spectrum while the amide I band at 1660 cm^{-1} is positive. Values of calculated dichroic ratios (R), order parameters [$f(\theta)$], and the average θ angle for the major bands of the spectra in Figure 4 are given in Table 1. From this table, the average value of the order parameter for the bands due to the *all-trans* acyl chains in the film of pure DMPA (1200, 1230, 1470, 2850, and 2920 cm^{-1}) is 0.71, showing that the acyl chains of the lipid are tilted with an average angle of 26° with respect to the ATR crystal normal, assuming an infinitely narrow distribution of orientation (C.-P. Lafrance, A. Nabet, R. E. Prud'homme, and M. Pézolet, unpublished results). This value is in agreement with previous orientation measurements by infrared spectroscopy (Désormeaux et al., 1992). Polarized spectra of pure lipid bilayers show that the oriented film on the ATR crystal is not completely dry, since the carbonyl band still gives a strong component at 1740 cm^{-1} due to the lipid-free C=O ester groups and a weak component 1720 cm^{-1} due to the vibration of carbonyl groups hydrogen bonded to water molecules. The measurement of the orientation of these two components after curve fitting (results not shown) reveals that the order parameter for the free carbonyl groups is −0.32, compared to −0.11 for the hydrogen-bonded C=O groups, suggesting that the hydrogen-bonded carbonyl groups are much less oriented than the free ones. For DMPA/MBP complexes, the order parameter calculated from the bands due to acyl chain CH_2 stretching vibrations decreases from 0.71 to 0.50, while the orientation of the carbonyl groups of the phospholipid does not seem to be affected by the protein. The decrease of the order parameter for the methylene vibrations is most likely due to the presence of *gauche* conformers in the presence of the protein.

The order parameter $f(\theta)$ calculated from the amide I and amide A bands is approximately equal to −0.15, indicating that the amide C=O axis is oriented at 61° with respect to the film normal. However, the order parameter $f(\theta)$ calculated from the amide II band is 0.31, giving an orientation of the amide C=O axis of 42° from the crystal normal. Disagreement between the θ angle calculated from the amide II vibration and those calculated from the other amide vibrations has been observed previously (Désormeaux et al., 1992). It may be partly due to the spectral contribution of other protein vibrations such as residue side-chain vibrations (Venyaminov & Kalnin, 1990).

For EPA-oriented bilayers, the average order parameter of the lipid acyl chains equals 0.34, which is lower than that of DMPA. Since EPA is a natural lipid with chains of varying length and unsaturation, it is expected that its conformational disorder is greater. Moreover, since EPA dispersions and EPA/MBP complexes are in the liquid-crystalline phases at room temperature during the deposition of the germanium ATR crystal, the order parameter calculated for the lipid acyl chain axis is expected to be less than that of DMPA due to the presence of *gauche* conformers. Therefore, the average tilt angle of the acyl chains of EPA was not calculated due to the heterogeneity and conformational disorder of the acyl chains. As opposed to the DMPA/MBP gel phase system, the protein increases the order parameter of the EPA acyl chains, in the liquid-crystalline phase. As seen in Table 1, the average orientation of the amide C=O groups of the protein calculated from the amide I and amide A vibrations is at 65° to the bilayer normal, which is close to the value observed for the DMPA/MBP complexes.

DISCUSSION

The temperature dependence of the frequency of the acyl chain CH_2 symmetric stretching vibration of DMPA and EPA indicates that MBP induced a decrease of the gel to liquid-crystalline phase transition temperature of both lipids, with a greater effect on EPA than DMPA. This agrees with calorimetric and spin-label studies of these lipids (Boggs & Moscarello, 1978; Boggs et al., 1981a). In all of these studies, MBP was present on both sides of the bilayer in multilamellar lipid/protein dispersions. However, a recent DSC and infrared study of single bilayers of mixtures of acidic lipids with PC formed on a solid support, with MBP asymmetrically bound to only one side of the bilayer, found that MBP increased the transition temperature in all cases (Reinl & Bayerl, 1993). Comparison with the latter study is difficult because of the asymmetry, the possibility of phase separation in the lipid mixtures, and the fact that the mixtures contained only 10 mol % acidic lipid.

For both the gel and liquid-crystalline phases, MBP caused an increase in the frequency of the CH_2 symmetric stretching vibration, indicating increased *gauche* conformations and thus conformational disorder of the lipid acyl chains. This is supported by the ATR measurements on oriented films of MBP/DMPA in the gel phase, which show that the order parameter of the lipid acyl chains was decreased by the presence of the protein. This decrease of the order parameter can be interpreted as either an increase of the acyl chain axis tilt or an increase of the conformational disorder. The increase of the frequency of the CH_2 stretching bands observed from transmission measurements suggests that the decrease of the order parameter in the case of DMPA is most likely due to the introduction of conformational disorder in the presence of MBP. In contrast, in the case of MBP/EPA, MBP increased the order parameter of the acyl chains. Since transmission measurements show that the conformational disorder of EPA also is increased by MBP, the increase of the order parameter of the acyl chains induced by the protein may then be interpreted as a reorientation of the chain axis toward the bilayer normal. The resulting order parameter of the EPA chains in the complex with MBP is then less than that of DMPA/MBP.

The strong electrostatic interactions of MBP with acidic lipids (Boggs et al., 1981a), combined with the absence of any hydrophobic segments of sufficient length to span the bilayer, suggest that the protein primarly lies along the surface of the bilayer interacting with the lipid headgroups. This is supported by the ATR results which indicate an average angle of the protein C=O amide groups in the presence of DMPA or EPA of about 65° with respect to the bilayer normal, and by X-ray diffraction results (MacNaughton et al., 1985). The disordering effects of MBP on the acyl chains may then be due to partial penetration of the hydrophobic amino acid side chains of the protein, or the hydrophobic surface of amphipathic α-helical segments of the protein, partway into the bilayer. Alternatively, it may be due to lateral separation of the lipid molecules as a result of electrostatic interactions with the protein so that the fluctuation of the acyl chains and the distribution of orientations increase. The MBP-induced increase in the order parameter of spin-labeled acyl chains (Boggs & Moscarello, 1978; Boggs et al., 1981a; Sankaram et al., 1989) and of acyl chains of EPA, detected in the present study by ATR measurements, is more consistent with the former model. These results are consistent with the FTIR results indicating conformational disorder of the acyl chains, if steric hindrance by contact with protein side chains restricts the motion and increases the orientation of the spin-label while reduced van der Waals interactions between the lipid acyl chains cause conformational disorder. However, the spin-label results and the increased order parameter of EPA found by ATR are difficult to reconcile with the second model. This partial penetration could account for the decrease in the phase transition temperature and other perturbing effects of the protein on lipid bilayers and with labeling by a hydrophobic photolabel in the bilayer. Possibly deeper penetration can occur transiently, allowing the protein to have effects such as disruption of PC bilayers into small lipoprotein-like particles (Roux et al., 1994).

The significant effects of the protein on the acyl chain conformation and the lipid order parameter in the gel phase indicate that the protein also partially penetrates into the gel phase, perhaps into defects in the gel phase bilayers (Jain et al., 1985). This is consistent with studies of spin-labeled MBP in which a spin-label covalently bound to MBP is immobilized in the gel phase of DMPA and sensitive to the lipid phase transition (Boggs et al., 1980). Similar partial penetration in the gel phase of saturated PG allows the protein to cause interdigitation of PG, resulting in significant immobilization of the acyl chains spin-labeled near the terminal methyl group (Boggs et al., 1981b) and a decrease in the frequency of the acyl chain CH_2 stretching vibration of PG in the gel phase (Surewicz et al., 1987). The very different effects on DMPA indicate that MBP does not induce interdigitation of this lipid. This has been attributed to the participation of the unbound PA molecules in intermolecular hydrogen bonding, probably mediated by water molecules, which stabilizes the noninterdigitated bilayer (Boggs et al., 1981b).

Since effects of the protein on the phase transition temperature and the frequency of the CH_2 symmetric stretching vibration were greater for EPA than for DMPA, both in the gel and in the liquid-crystalline phases, the protein seems to perturb the conformational order of the EPA acyl chains more than those of DMPA. Partial penetration of the protein into EPA with its more heterogeneous acyl chain composition and greater lipid molecular surface area can probably take place more readily than into saturated DMPA. This is supported by greater effects of the protein on the spectra of the interfacial region of EPA than DMPA. The effect on the carbonyls indicates that the protein limits the accessibility of the bilayer surface to water molecules in the liquid-crystalline phase. Decreased hydration of the lipid bilayer on binding of MBP was also indicated by an X-ray diffraction study (Sedzik et al., 1984) and from an increase in the p*K* of spin-labeled fatty acids (Sankaram et al., 1990).

The greater penetration into EPA may also be due to a weaker, less stable hydrogen bonding network compared to DMPA. This is supported by spectra of the lipid phosphates since the band due to the antisymmetric PO_2^- appears at a higher frequency for EPA than DMPA. An increase in frequency also occurs during the transition from the gel to liquid-crystalline phase, suggesting that it is caused by weakening of the intermolecular hydrogen bonding interactions. Thus, there is a correlation between the stability of the lipid hydrogen bonding network and the ability of the protein to penetrate the bilayer. The perturbing effect of MBP on saturated PA has been shown to be sensitive to its ionization state and intermolecular hydrogen bonding properties (Boggs, 1986). In the presence of the protein, the frequency of the antisymmetric PO^{2-} band decreases, indicating that the hydrogen bond network is replaced by hydrogen bonding or electrostatic interactions of the protein with the phosphate headgroup of the lipid.

The protein also causes the appearance of the band due to the PO_3^{2-} stretching vibration, providing clear evidence that the protein binds to and affects the p*K* of the ionizable groups as does Ca^{2+} (Laroche et al., 1991) and cardiotoxin (Désormeaux et al., 1992). A similar effect has been reported previously for PS and PA (Boggs et al., 1986; Boggs, 1986) from release of protons upon binding MBP, resulting in a decrease in pH of the aqueous phase, when unbuffered.

The increase of the conformational disorder of lipid acyl chains is characteristic of hydrophobic interactions between a protein or a polypeptide with membranes. Cardiotoxins

are also basic proteins and are known to penetrate partly into the lipid bilayer. However, infrared spectra of DMPA bilayers in the presence of cardiotoxins show more conformational disorder in the gel phase and less in the fluid phase, and the gel-to-liquid-crystalline phase transition of the cardiotoxin/DMPA complexes is strongly broadened, compared to the effect observed with MBP on PA bilayers (Dufourcq & Faucon, 1978; Vincent et al., 1978; Désormeaux et al., 1992). This comparison shows that MBP has less membrane perturbing effect than cardiotoxins. Comparison with infrared spectra of apocytochrome *c* bound to dimyristoylphosphatidylglycerol bilayers shows that the protein both decreases the frequency of the acyl chain CH_2 stretching band and broadens the phase transition, indicating that apocytochrome *c* induces perturbation of the lipid interchain interactions (Muga et al., 1991). The decrease in frequency resembles the effect of MBP on PG (Surewicz et al., 1987) and may indicate that apocytochrome *c* causes interdigitation of PG. Its effect on DMPA has not been determined.

On the other hand, an increase of the gel-to-liquid-crystalline phase transition induced by a protein or a polypeptide is characteristic of electrostatic interactions. For example, polylysine, which is often used as a model of extrinsic proteins, induces a shift of the phase transition of DMPA bilayers toward higher temperatures, showing that its interactions with lipids are purely electrostatic (Boggs et al., 1981a; Laroche et al., 1988). It is labeled significantly less by a hydrophobic photolabel than MBP (Boggs et al., 1988).

ATR spectra of complexes of MBP with DMPA or EPA give an average angle of the different protein C=O amide groups, over all the protein conformations, in the presence of DMPA or EPA of about 65° with respect to the normal of the bilayer. In order to relate this angle to the overall orientation of the protein with regard to the lipid bilayer, one has to know the content of the secondary structure elements of MBP. Results on the conformation of MBP are contradictory. From circular dichroism measurements, Keniry and Smith (1981) found that MBP bound to various acidic lipids adopts a conformation containing 20−27% α-helices, 10−12% β-sheet, and about 50% random coil. On the other hand, using FTIR spectroscopy, Surewicz et al. (1987) found that the protein bound to DMPG adopted 53% β-sheet and 15% α-helical structure. Using the same technique, we have found that MBP bound to PA contains about 40% α-helix, 35% β-structure, and 20% random coil (A. Nabet, J. M. Boggs and M. Pézolet, unpublished results). Therefore, despite these discrepancies, it appears that the conformation of MBP is rather complex and that the determination of the overall orientation is difficult to make unambiguously.

The significance of the angle of 65° can be interpreted in two different ways. On the one hand, one can assume that the oblique angle is significant for MBP, as has been concluded by Brasseur et al. (1988) for the paramyxovirus F1 fusion peptide and by Ishiguro et al. (1993) for synthetic fusogenic peptides related to a viral fusion peptide, confirming a prediction made by estimating the molecular hydrophobicity potential for different lipid-associating helices (Brasseur, 1991). Since MBP has also been shown to cause fusion (Lampe & Nelsestuen, 1982), some of its helical segments may be expected to interact similarly with the bilayer as viral fusion peptides. On the other hand, one can consider the average angle of 65° determined for the orientation of the C=O amide groups of MBP in the lipid bilayer to be approximate. This angle has been calculated from the order parameter $f(\theta)$ assuming an infinitely narrow orientation distribution of the protein. Determination of the dependence of the order parameter value on the distribution width shows that by using a broader distribution width, a value of −0.2 for the order parameter gives a larger value for the angle than 65° (C.-P. Lafrance, A. Nabet, R. E. Prud'homme, and M. Pézolet, unpublished results). Considering the large size of the protein, it is probable that the distribution of the orientations of all amide C=O groups of the protein is broad. Thus, for an orientation distribution of finite width, the negative $f(\theta)$ value suggests that the average of all the amide C=O groups of the protein may likely be parallel to the lipid bilayer surface, indicating that much of the protein may be spread over the bilayer surface, while some particular hydrophobic residues may penetrate slightly into the membrane.

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