Interaction of Glycosylated Human Myelin Basic Protein with Lipid Bilayers†

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Received October 4, 1988; Revised Manuscript Received January 17, 1989

ABSTRACT: Myelin basic protein (MBP), isolated from normal human myelin, was glycosylated with UDP-*N*-acetyl-D-galactosamine and a glycosyltransferase isolated from porcine submaxillary glands. MBP containing 0.85 mol of *N*-acetyl-D-galactosamine per mole of protein was oxidized at carbon 6 by galactose oxidase and complexed with a spin-label, Tempoamine, in order to study its interactions with lipids. When the spin-labeled MBP was reacted with lipid vesicles consisting of DSPG, DPPG, and DMPG, most of the spin-label was motionally restricted in the gel phase, with a correlation time greater than 10^{-8} s. The motion increased with increasing temperature and was sensitive to the lipid phase transition. Interaction with the gel phase of DPPA caused much less motional restriction of the probe. However, melting of the lipid allowed increased interaction and motional restriction of the probe, which was only partially reversed on cooling back to the gel phase. The motional restriction of the probe in these lipids is attributed to its penetration partway into the lipid bilayer in both the gel and liquid-crystalline phases. The fact that the probe bound to the protein can penetrate partway into the bilayer suggests that other hydrophobic side chains and residues of the protein can similarly penetrate into the bilayer. Additional evidence for penetration was provided by digestion of the lipid-bound protein with endoproteinase Lys-C. When nonglycosylated and glycosylated MBP in solution was treated with Lys-C, extensive digestion occurred. A single radioactive peptide which eluted at 25 min was identified as residues 92–105. Less digestion occurred when MBP was combined with DPPG, demonstrating that some Lys-C sites were not as available in the lipid-bound form. The glycosylated MBP in DPPG vesicles was digested even less than the nonglycosylated MBP, suggesting much greater protection of the Lys-C sites. These data demonstrate that glycosylation affects the secondary or tertiary structure of the protein in agreement with recent NMR studies [Persaud, R., Fraser, P., Wood, D. D., & Moscarello, M. A. (1988) *Biochim. Biophys. Acta 966*, 357–361], rendering the Lys-C sites inaccessible to the enzyme.

The myelin sheath is a unique multilayered membrane consisting of approximately 70% lipid and 30% protein. The integrity of this multilayered structure is essential for saltatory conduction of the nerve impulse. Interruption of the structure results in slow conduction velocities along the axon.

Although the lipid composition is complex, consisting of a variety of phospholipids, sphingolipids, and cholesterol, the protein composition is relatively simple since two of the proteins, lipophilin (a proteolipid type) and myelin basic protein (MBP),[1] constitute about 85% of the total protein (Lees & Brostoff, 1984). MBP is primarily an extrinsic membrane protein, although hydrophobic interactions between some amino acid residues of the protein and the bilayer may occur [reviewed in Boggs et al. (1982a)]. Its role in the formation of the multilayer structure appears to be in the compaction process which results in the formation of a "crystalline" multilayer arrangement of the bilayers (Brady et al., 1981). It was shown to have an axial ratio of approximately 1:10 both in solution (Epand et al., 1974) and in lipid vesicles (Brady et al., 1981) by X-ray diffraction. The mechanism by which MBP bridges bilayers is not fully understood at this time.

In studying the mechanism by which this rather elongated molecule interacts with lipid bilayers, we have studied the interaction between selected areas of the protein with lipids by judicious use of reporter groups. In one study, the methyl group of each of the only two methionyl residues in the molecule, located at positions 21 and 167 of the 170 amino acid residue protein, was enriched with [^{13}C]methyl, permitting us to visualize these specific resonances by ^{13}C NMR (Deber et al., 1978). In a more detailed study, a spin-labeled iodoacetamide was coupled to the two methionines (Stollery et al., 1980; Boggs et al., 1980). Different degrees of hydrophobic interaction occurred with different lipids, consistent with results from other studies using a variety of techniques (London & Vossenberg, 1973; Demel et al., 1973; London et al., 1973; Boggs & Moscarello, 1978). Motional restriction of the spin-label bound to the methionines occurred upon interaction with lipid and decreased in the order phosphatidic acid $\simeq$ phosphatidylglycerol $\geq$ phosphatidylserine $\gg$ cerebroside sulfate $>$ phosphatidylethanolamine (Stollery et al., 1980). Temperature studies demonstrated that the protein interacted hydrophobically to a greater extent with liquid-crystalline lipid than with gel phase lipid (Boggs et al., 1980). These studies helped to define the interactions of both ends of the molecule with lipids.

Human MBP can be glycosylated at threonine-95 and at threonine-98 by UDP-*N*-acetyl-D-galactosamine in the presence of a glycosyltransferase from porcine submaxillary gland (Cruz & Moscarello, 1983; Persaud et al., 1988). Thr-95, near the center of the protein (in a region which contains some hydrophobic and apolar amino acids), was glycosylated first.

† This work was supported by a program grant (M.A.M.) and an operating grant (J.M.B.) from the Medical Research Council of Canada.
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[1] Abbreviations: CBS, cerebroside sulfate; DMPE, dimyristoylphosphatidylethanolamine; DMPG, dimyristoylphosphatidylglycerol; DPPA, dipalmitoylphosphatidic acid; DPPE, dipalmitoylphosphatidylethanolamine; DPPG, dipalmitoylphosphatidylglycerol; DSC, differential scanning calorimetry; DSPG, distearoylphosphatidylglycerol; EDTA, ethylenediaminetetraacetic acid; GALNAC, *N*-acetyl-D-galactosamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; Lys-C, endoproteinase Lys-C (EC 3.4.99.30); MBP, myelin basic protein; MBP-SL, spin-labeled, glycosylated MBP; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; Tempoamine, 2,2,6,6-tetramethyl-4-aminopiperidine 1-oxide.

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The second site near the proposed hairpin bend in the molecule which contains the tri-proline sequence was glycosylated more slowly (Persaud et al., 1988). Since the sugar residue was readily oxidized by galactose oxidase, a spin-label reporter group, 2,2,6,6-tetramethyl-4-aminopiperidine 1-oxide (Tempoamine), was coupled to this site. The results of these studies are presented in this paper.

Materials and Methods

Preparation of MBP. Human myelin basic protein was extracted from white matter by a modification of the method of Chou et al. (1977) (Cheifetz & Moscarello, 1985).

Preparation of N-Acetyl-D-galactosaminyl Transferase. An extract of UDP-GALNAC:polypeptide-*N*-acetyl-D-galactosaminyl transferase (EC 2.4.2.41) was prepared from porcine submaxillary glands by the method of Schwyzer and Hill (1977).

Glycosylation of Human MBP. Glycosylation of MBP with UDP-*N*-acetyl-D-galactosamine and the subsequent isolation of the glycosylated MBP by HPLC methods were carried out as described previously (Cruz & Moscarello, 1983). For the preparation of milligram amounts of glycosylated MBP, 50–60 independent incubations were done at the same time, each containing 200 μg of MBP. In this way, we routinely transferred 0.85 mol GALNAC/mol of MBP, most of which was located on Thr-95.

Preparation of Spin-Labeled MBP. Ten milligrams of glycosylated MBP was dissolved in 2 mL of 0.05 M sodium phosphate buffer, pH 7.0. After the solution was divided into four equal aliquots, 10 units of purified galactose oxidase (Avigad, 1978; Hatton & Regoeczi, 1982; Tressel & Kosman, 1982) was added to each aliquot, and the tubes were incubated at 37 °C for 2 h. After the four aliquots were pooled, the solution was made 0.1 M with respect to NaCl by the addition of 200 μL of 1.1 M NaCl. The solution was chilled to 4 °C, and 3.7 mg (40-fold molar excess) of Tempoamine dissolved in 40 μL of ethanol was added with stirring (Nezlin & Sykulev, 1982). The pH was adjusted to 6.0 by the addition of dilute HCl and stirred overnight at 4 °C. The following day, 0.3 mg of $NaCNBH_4$ was added and the solution stirred a further 2 h at 4 °C. The spin-labeled protein (MBP-SL) was dialyzed overnight against several changes of distilled water and then applied to a Sephadex G-25 (medium) column (1.5 × 30 cm) in 0.01 M acetic acid to remove traces of free Tempoamine. The protein which eluted in the void volume was well separated from the residual Tempoamine. After the pH was adjusted to 6.8 by the addition of dilute NaOH, the solution was concentrated in Centricon concentrating tubes (Amicon Corp.) with an exclusion limit of 10000 at 6500 rpm (Beckman JA-20 rotor) at 4 °C.

As a control, unglycosylated MBP was subjected to a parallel spin-labeling and purification procedure. No spin-label was detectable in this material.

Purification of Galactose Oxidase (EC 1.1.3.9). Galactose oxidase (150 units) was purified as described (Avigad, 1978; Hatton & Regoeczi, 1982; Tressel & Kosman, 1982). Contamination of this purified galactose oxidase preparation with proteases was monitored by incubating 10 units with 0.5 mg of MBP in 0.25 mL of phosphate buffer, pH 6.5. Aliquots were removed after 1, 3, 6, and 18 h at 37 °C, combined with sample buffer, and run in a 5–15% gradient SDS gel. Since no evidence of proteolysis was obtained, the galactose oxidase was considered of sufficient purity for use in the Tempoamine labeling of MBP.

Preparation of Lipid Vesicles Containing Protein. A chloroform solution of the particular lipid (Avanti Polar Lipids, Birmingham, AL) to be used was placed in an Eppendorf tube, and the chloroform was removed with a stream of nitrogen. The dried lipids were placed under vacuum for at least 1 h and then dispersed in 300 μL of buffer (0.1 mM EDTA/2 mM HEPES, pH 7.4, with 10 mM sodium chloride) at a temperature above the phase transition temperature of the particular lipid. DMPG vesicles were dispersed in 100 mM sodium chloride instead of 10 mM in the same buffer. A solution of spin-labeled protein in 0.01 M sodium acetate was added to the vesicles below the phase transition temperature of the lipid to give a final protein concentration in the vesicles of 9 wt %, and the mixture was vortexed for 3–5 min. The mixture was centrifuged for 1 min in an Eppendorf centrifuge at 11000 rpm for 15 min at 4 °C, and the pellet was taken up in a 100-μL disposable micropipet for ESR measurements.

Endoproteinase Lys-C (EC 3.4.99.30) Digestion of Glycosylated MBP Incorporated into DPPG Vesicles. To 1 mg of dry DPPG was added 250 μL of chloroform/methanol 2:1 (v/v). The solution was dried in a gentle stream of nitrogen followed by lyophilization for 5 min, producing a shell of lipid on the walls of the tube. The dried lipid was suspended by vortexing in 925 μL of 10 mM HEPES buffer, pH 7.4, containing 100 mM sodium chloride and 0.01% sodium azide. The suspension was sonicated in a bath sonicator at 47 °C (5 °C above the T_c of the lipid) until the suspension was optically clear. The suspension was centrifuged at 40000 rpm in a Beckman Ti 50 rotor for 1 h to pellet the large multilamellar vesicles. The supernatant which contained the single-layered vesicles was removed and divided into 3 × 300 μL aliquots. To each 300-μL aliquot was added 88 μL of ^{14}C-glycosylated MBP containing 2.0×10^4 dpm. The suspension was vortexed for 2 min with occasional warming to 47 °C. After being cooled to 37 °C, the suspension was centrifuged at 12000 rpm in an Eppendorf centrifuge. The pellet contained 96% of the total added radioactivity, demonstrating that most of the added protein was bound to the vesicles. The pellets were suspended in 200 μL of HEPES buffer, pH 7.4, and 0.5 μg of endoproteinase Lys-C (Lys-C) was added, incubated at 37 °C for 0, 2, and 18 h, and then centrifuged at 12000 rpm for 2 min. After 18 h of incubation, 4000 dpm were recovered in the supernatant. Three similar samples were pooled, lyophilized, and resuspended in water and applied to a C:18 μBondapak, reversed-phase column.

Digestion of nonglycosylated and glycosylated MBP was carried out under the same conditions of incubation for 18 h at 37 °C.

ESR Measurements. ESR measurements were done on a Varian E-line Century spectrometer (E-104B) equipped with a Varian temperature-control accessory and a DEC LSI 11 based microcomputer system. The microwave power used was 10 mW. The motional parameter τ_0, which provides information about the motion of the probe bound to the glycosylated basic protein in solution and in the presence of lipids, was determined from the equation:

$$\tau_0 = (6.5 \times 10^{-10})\Delta H_0[(h_0/h_{-1})^{1/2} - 1] \text{ s}$$

where ΔH_0 is the width of the center line and h_0 and h_{-1} are heights of the midfield and high-field lines, respectively.

Results

The spin-label bound to the GALNAC on MBP in aqueous solution has a sharp three-line spectrum (Figure 3F) characteristic of rapid, nearly isotropic motion with a motional parameter, τ_0, of 1.46 ns, at 10 °C, significantly increased from the value of the free Tempoamine in solution, 0.034 ns. It is also considerably greater than that of a spin-label bound to

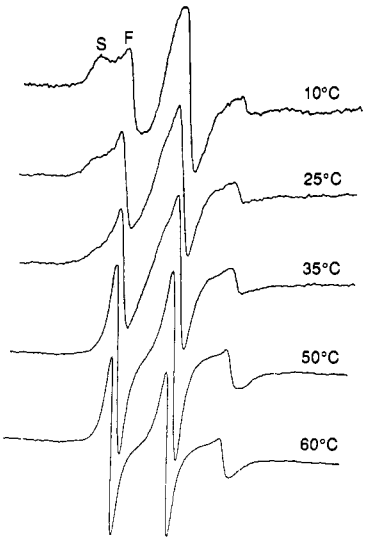

FIGURE 1: ESR spectra of spin-labeled, glycosylated MBP in DPPG vesicles at various temperatures. S indicates the motionally restricted component, and F indicates the more mobile component. The heights of these peaks were measured from the base line for curves shown in Figures 2 and 5.

the methionines of MBP at this temperature, 0.2 ns (Boggs et al., 1980), indicating that the central region of the protein around Thr-95 may have less freedom of motion than regions near the N and C termini.

Interaction of Myelin Basic Protein with Phosphatidylglycerol. In order to study the interaction of the spin-labeled glycosylated MBP (MBP-SL) with dipalmitoylphosphatidylglycerol (DPPG), multilamellar vesicles were prepared containing 9% protein by weight. The spectra, recorded at temperatures from 10 to 60 °C, are shown in Figure 1. At temperatures below the phase transition temperature of the lipid (42 °C), a two-component spectrum was observed. The motionally restricted component, with a hyperfine splitting of 31 G, labeled "S" in the spectrum recorded at 10 °C, disappeared at temperatures above the phase transition; e.g., the spectrum at 50 °C is a sharp three-line spectrum with a hyperfine splitting of 16.36 G.

Changes in the intensity of the midfield line, h_0, with temperature can be used as a measure of the sensitivity of the probe motion to the lipid gel to liquid-crystalline phase transition. As shown for DPPG in Figure 2A, there is a large increase in h_0 at the phase transition. This indicated that at low temperatures, the lipid molecules in the vicinity of the probe are relatively ordered and capable of undergoing a cooperative phase transition, sensed by the probe on the GALNAC. Although a phase transition clearly occurs on the first heating scan, it is broader than that which occurs for the pure lipid [sensed by a fatty acid spin-label (Boggs et al., 1980)], indicating that the protein has some perturbing effect on the lipid in its environment. Changes in the intensity on cooling are more gradual, with two discontinuities in slope at 30–35 and 40–45 °C indicating an additional phase transition at a lower temperature. On reheating, the shape of the curve is in between that observed on the first heating scan and on cooling. This hysteresis between heating and cooling scans, and differences between the first and subsequent heating scans, has also been observed for MBP–DPPG by differential scanning calorimetry (DSC) and from changes in the motion of a spin-label bound to the methionines of MBP (Boggs et al., 1980). After being cooled back to 10 °C, the peak intensity is a little higher than the value found originally in the gel phase.

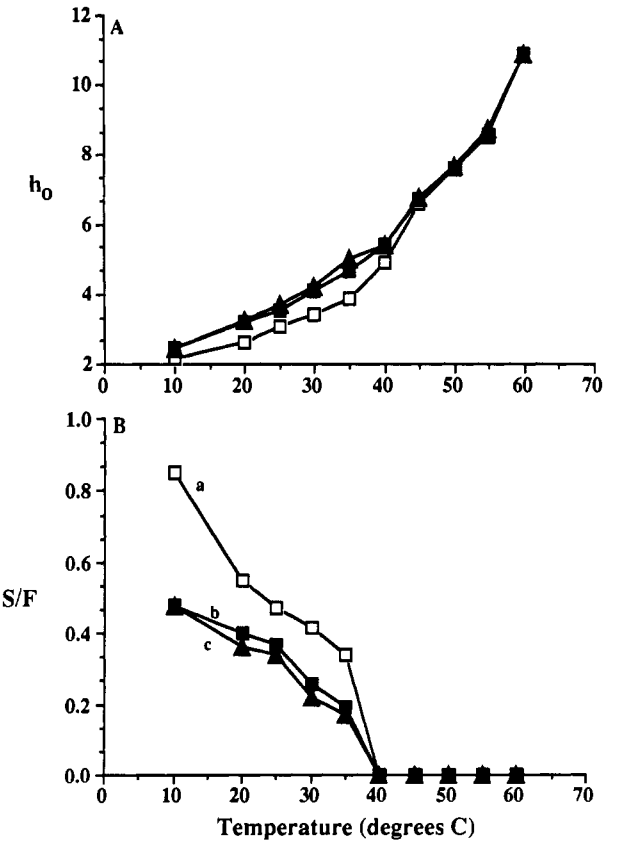

FIGURE 2: Temperature dependence of spectral parameters of spin-labeled glycosylated MBP bound to DPPG. Curve a, first heating scan (□); curve b, reheating scan (■); curve c, cooling scan (▲). (A) Intensity of midfield line, h_0; (B) ratio of heights of motionally restricted and more mobile components, S/F.

Changes in the motion of the probe after cycling through the lipid phase transition can also be detected from changes in the ratio of the motionally restricted and more mobile components in the spectrum. This ratio can be monitored from the heights of the peaks indicated by S and F in Figure 1. It can be measured only up to the lipid phase transition temperature, as only a mobile component occurs in the liquid-crystalline phase. As shown in Figure 2B for DPPG, the S/F ratio decreases sharply with increasing temperature on the first heating scan (curve a), with an increase in slope at 35 °C coinciding with the start of the lipid phase transition. The S/F ratios for the first heating scan are considerably larger than for the cooling (curve c) or reheating scans (curve b). The decrease in phase transition temperature of a portion of the lipid in the vicinity of the probe on cooling and reheating can also be detected from the increase in slope of curves b and c at 25–30 °C.

The appearance of the spectrum on cooling back to 10 °C is shown in Figure 3A and is plotted to the same scale as that of the original spectrum at 10 °C in Figure 3B. It indicates that there is an increase in the amount of a sharp three-line component resembling the spectrum of the MBP-SL in solution. Subtraction of the spectrum of MBP-SL in solution at 10 °C from the spectra in Figure 3A,B gives those in Figure 3C,D. This indicates that an increase in the amount of MBP-SL in solution from 5.5% to 11% after cycling through the phase transition can account for the increase in h_0 at low temperatures shown in Figure 2A, and at least partially accounts for the decrease in the S/F ratio shown in Figure 2B. The decrease in the amount of motionally restricted component in Figure 3C relative to that in Figure 3D may be accounted for by the increase in the amount of MBP-SL in solution.

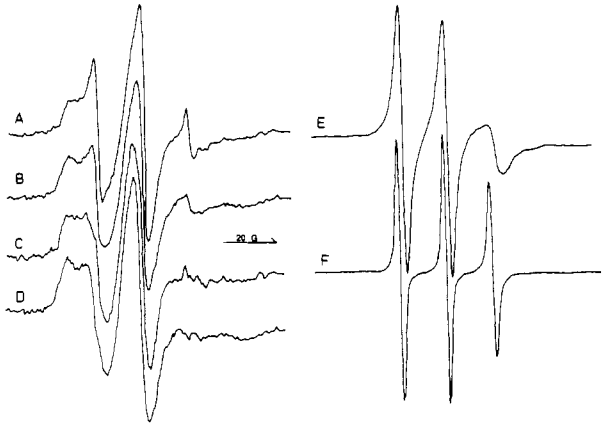

FIGURE 3: ESR spectra of MBP-SL in DPPG vesicles: (A) at 10 °C after cycling through the lipid phase transition temperature; (B) at 10 °C before heating the sample; (C) the result of subtracting the spectrum of MBP-SL in solution at 10 °C from that in (A); (D) the result of subtracting the spectrum of MBP-SL in solution at 10 °C from that in (B); (E) the result of subtracting the spectrum of MBP-SL in solution at 55 °C from that in DPPG at 55 °C; (F) the spectrum of MBP-SL in solution at 55 °C. Spectra in (A) and (B) and in (C) and (D) are plotted on the same scale so their relative heights can be compared.

There may also be some qualitative differences in the motionally restricted components shown in Figure 3C,D, but due to the noisy background, it is difficult to be sure. The hyperfine splitting of 31 G is close to the slow-motion limit, indicating that the correlation time is on the order of 10^{-8} s.

The appearance of the spectra at 50–60 °C shown in Figure 1 suggests that there is a component due to a small amount of MBP-SL in solution at this temperature also. Subtraction of the spectrum of MBP-SL in solution gave the spectrum shown in Figure 3E. It is highly asymmetric with h_{+1} higher than h_0, in contrast to the spectrum of MBP-SL in solution at this temperature (55 °C) (Figure 3F). The τ_0 value for the spectrum in Figure 3E of MBP-SL bound to DPPG is 2.37 ns, 6.4 times greater than that of MBP-SL in solution, indicating interaction with and motional restriction of the probe with the liquid-crystalline phase also. The shape of the spectrum suggests preferential rotation about the x axis may occur which coincides with the N–O bond. This indicates greater restriction of the direction of probe rotation when MBP-SL is added to the lipid bilayer and suggests the spin-label is inserted into the bilayer so that the plane of the ring structure is parallel to the lipid molecules and perpendicular to the bilayer surface. The smaller value of the hyperfine splitting, a_0, of 16.36 G, relative to a value of 16.5 G for MBP-SL in solution, indicates location of the probe in a somewhat less polar environment when added to DPPG. The a_0 value of the free spin-label in water is 16.5 G. It decreases to 14.8 G in hexane. These results suggest that the motional restriction of the probe in the gel and liquid-crystalline phases is due to its penetration partway into the lipid bilayer.

Similar measurements were carried out for DSPG and DMPG. The results showed some differences from DPPG. The phase transition monitored from the peak intensity was more distinct for DSPG, especially for the first heating scan, than for DPPG. In contrast, no distinct phase transition could be detected for DMPG, even on the first heating scan. These results are consistent with an earlier DSC study where the perturbing effect of MBP on the phase transition on heating decreased in the order DMPG > DPPG > DSPG and the hysteresis between heating and cooling scans increased in the order DMPG < DPPG < DSPG (Boggs et al., 1982b). On the first heating scan of DSPG, the motionally restricted component disappeared well before the phase transition while it persisted up to the phase transition for DPPG. For DMPG, however, it persisted to some extent even at temperatures above the phase transition temperature. Thus, motional restriction of the probe decreased in the order DMPG > DPPG > DSPG. These differences may be partially due to the different temperature ranges over which the gel phase exists for these lipids and increased dissociation of the MBP-SL from the lipid as the temperature was increased.

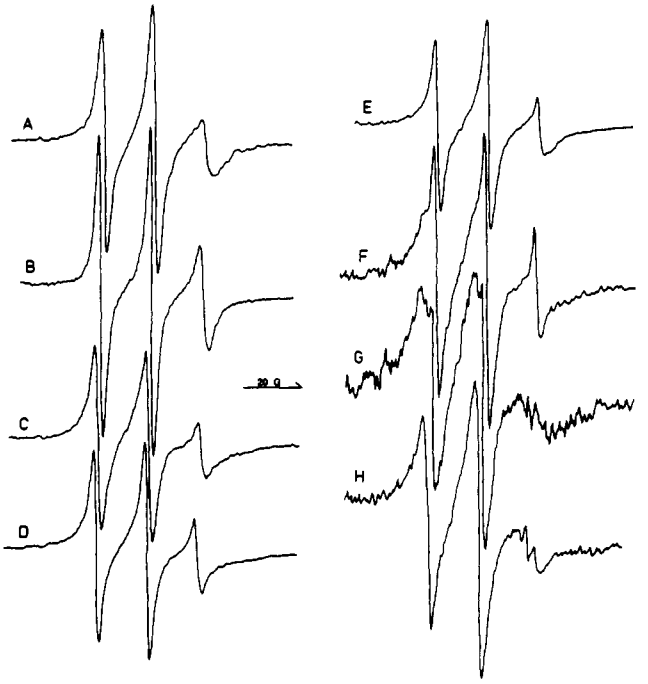

FIGURE 4: ESR spectra of MBP-SL in DPPA vesicles: (A) at 25 °C before heating the sample; (B) at 55 °C before heating the sample above its phase transition temperature; (C) at 65 °C; (D) at 55 °C after heating above the phase transition temperature; (E) at 25 °C after heating above the phase transition temperature; (F) the result of subtracting the spectrum in (B) from that in (D); (G) the result of subtracting the spectrum of MBP-SL in solution at 55 °C from that in (F); (H) the result of subtracting the spectrum of MBP-SL at 25 °C from that in (E). Spectra in (A) and (E) are plotted on the same scale, and spectra in (B) – (D) are plotted on the same scale.

Interaction of Myelin Basic Protein with Phosphatidic Acid. The interaction of the MBP-SL with DPPA was different from phosphatidylglycerol in that no component as motionally restricted as that in DPPG was detected even at temperatures below the lipid phase transition temperature, as shown for the spectrum at 25 °C in Figure 4A. The spectra were characteristic of relatively rapid, nearly isotropic motion at all temperatures. Therefore, changes in the motion of the probe with temperature were monitored from the motional parameter, τ_0, and the midfield line intensity, h_0. The value of τ_0 of the protein-bound probe in DPPA was significantly greater than that of the protein in solution at all temperatures, indicating interaction of the probe with the lipid (Table I and Figure 5B). τ_0 for both MBP in solution and MBP bound to DPPA decreased sharply with increasing temperature up to the transition temperature of the lipid, 60 °C, where it then increased sharply for the lipid-bound protein in contrast to the protein in solution. A corresponding increase in the midfield line intensity (Figure 5A, curve a) occurred up to 55 °C, followed by a sharp decrease at the lipid phase transition temperature. On completion of the phase transition, it continued to rise. Double integration of the spectra showed that the area was the same at all temperatures. Thus, there was no loss of spin-label signal.

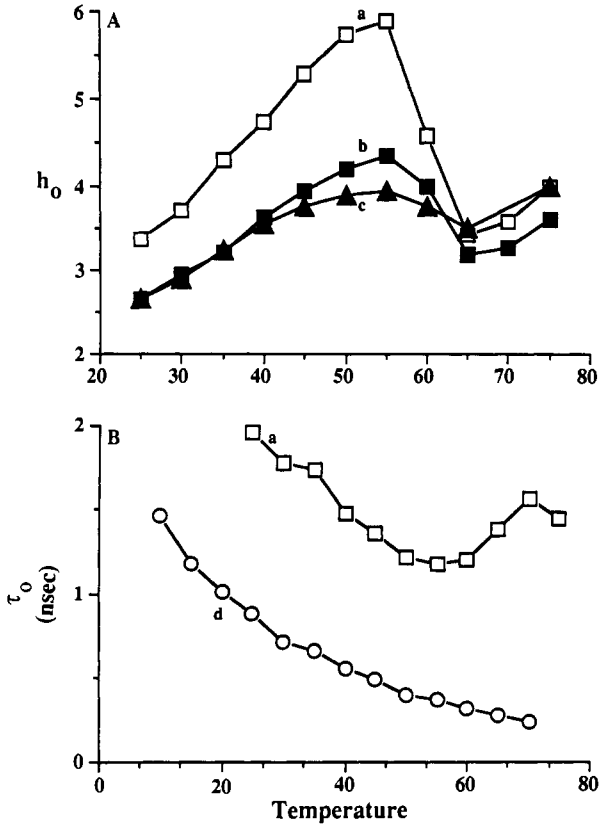

FIGURE 5: Temperature dependence of spectral parameters of spin-labeled glycosylated MBP bound to DPPA. Curve a, first heating scan (□); curve b, reheating scan (■); curve c, cooling scan (▲); curve d, MBP-SL in aqueous solution (○). (A) Intensity of midfield line, h_0; (B) motional parameter, τ_0.

Table I: Motional Parameter, τ_0, of Spin-Labeled Glycosylated MBP in Solution and Bound to Lipids

temp of measurement (°C)	τ_0 (ns)		
	MBP in solution		MBP in lipid
25	0.88	DPPA	1.96[a]
		DPPE	0.88
		myelin	2.12
55	0.37	DPPG	2.37[b]
65	0.27	DPPE	0.37
		CBS	1.18[c]
		myelin	1.26

[a] Before heating above the transition temperature. [b] After subtraction of the spectrum of MBP-SL in solution. [c] CBS, cerebroside sulfate.

The increase in τ_0 and decrease in peak intensity at the phase transition temperature both indicate that the motion of the probe decreased, suggesting its location in a more ordered environment when the lipid melts, i.e., in the lipid bilayer. This is also supported by a decrease in the hyperfine splitting value, a_0, of the spin-label from 16.5 G for MBP in solution or bound to gel phase DPPA to 16.26 G in the liquid-crystalline phase, indicating location of the probe in a more hydrophobic environment when the lipid is in the liquid-crystalline phase.

The changes in the spectra on heating from 55 to 65 °C are shown in spectra B and C, respectively, of Figure 4. In addition to the decrease in height, the shape of the spectrum changes from one in which h_0 is greater than h_{+1} to one in which h_{+1} is greater than h_0, as found for the liquid-crystalline phase of DPPG. This suggests the motion of the probe is more restricted to motion about its x axis when interacting with liquid-crystalline phase lipid. The spectrum in Figure 4C is also broader which accounts for the decrease in height. Another component characteristic of more rapid isotropic motion, which resembles that of MBP-SL in solution, is also present. Therefore, the τ_0 values at temperatures above 60 °C in Figure 5B are not accurate, but changes in the value indicate that a change in the motion has occurred.

On cooling (curve c) and on reheating (curve b) (Figure 5A), the peak intensity follows the same pattern as on the first heating scan, but the value is less at all temperatures. The transition temperature also appears to be decreased and broadened somewhat, particularly on cooling. The lower peak intensity on cooling back to 25 °C indicates motional restriction of a population of the spin-label is still going on in the gel phase.

The spectra on cooling back to 55 and to 25 °C are shown in Figure 4D and E, respectively. They are plotted on the same scale as those at the same temperatures in Figure 4A,B. These show that the more mobile component is retained even at 25 °C. Therefore, τ_0 values could not be calculated for the cooling and reheating scans. The spectrum in Figure 4D also indicates that the broader more motionally restricted component in Figure 4C at 65 °C is retained at 55 °C. However, it is difficult to tell if it is also retained at 25 °C by visual inspection of the spectrum in Figure 4E.

The spectrum on cooling back to 55 °C was resolved into its components by subtracting the spectrum initially obtained at 55 °C in Figure 4B from that in Figure 4D to give the spectrum in Figure 4F. This shows the more motionally restricted component and the more mobile component resulting after cycling through the phase transition. The spectrum of MBP-SL in solution at 55 °C was then subtracted from the spectrum in Figure 4F to give that in Figure 4G. This consists primarily of the more motionally restricted component which exists in Figure 4D at 55 °C. Thus, heating above the lipid phase transition temperature causes a small percentage of the MBP-SL to dissociate from the bilayer as found for DPPG, and also causes some of the MBP-SL bound to the lipid to become more motionally restricted. This motional restriction is retained on cooling back to the gel phase as shown by resolution of the spectrum in Figure 4E at 25 °C by subtraction of the spectrum of MBP-SL in solution at 25 °C. This gave the spectrum shown in Figure 4H. It is not as motionally restricted as found for the gel phase of DPPG, but is considerably more so than that found for DPPA before heating through the phase transition (Figure 4A). These results suggest that the spin-label has penetrated partway into the bilayer in the liquid-crystalline phase and that much of it remains in the bilayer on cooling back to the gel phase. The small increase in height at 55 °C which occurs on cooling after going through the phase transition (Figure 5A, curve c) indicates that partial extrusion of the spin-label occurs when the lipid goes back into the gel phase.

Interaction of MBP with Other Lipids. Interaction of MBP-SL with bovine brain cerebroside sulfate (CBS) also caused motional restriction of the spin-label in the gel phase, similar to that which occurred in phosphatidylglycerol. When the protein was added to myelin vesicles, no motionally restricted component was detected at 25 °C, but the motional parameter of the spin-label increased (Table I), indicating some interaction with the lipid bilayer. The a_0 value was 16.4 G, somewhat smaller than the value in solution. In contrast to the other lipids, interaction of the protein with DPPE had no effect on the motion of the probe in the gel phase and caused only a small decrease in motion in the liquid-crystalline phase. The a_0 value was 16.5 G for both phases, indicating little or no interaction of the probe with this lipid.

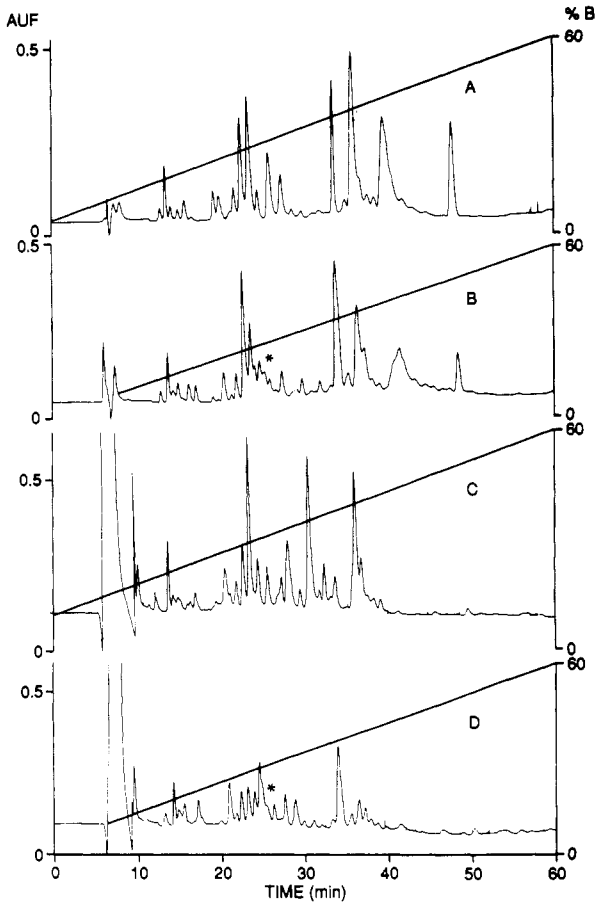

FIGURE 6: Reversed-phase HPLC profiles of proteinase Lys-C digests of (A) native MBP in aqueous solution, (B) glycosylated MBP in solution, (C) nonglycosylated MBP in DPPG vesicles, and (D) glycosylated MBP bound to DPPG vesicles.

Endoproteinase Lys-C Digestion of Nonglycosylated and Glycosylated MBP in Solution and in DPPG Vesicles. (A) MBP in Solution. Endoproteinase Lys-C digests of nonglycosylated and glycosylated MBP were separated by HPLC on a C:18 μBondapak reversed phase column as described under Materials and Methods. The HPLC profiles shown in Figure 6A,B are similar. The peptide containing 80% of the ^{14}C was eluted at about 25 min (marked with a star, Figure 6B). After further purification, it was identified as peptide 92–105, which had been shown previously to contain the major glycosylation site (Cruz & Moscarello, 1983). Hydrolysis of this peptide in 5.7 N HCl for 4 h at 100 °C to liberate sugars, followed by amino acid analysis for amino sugars, demonstrated that all the ^{14}C was recovered as galactosamine and 0.6 mol of galactosamine per mole of peptide 92–105 was recovered. Since acid hydrolysis of sugars results in considerable loss, this yield is reasonable for a peptide which is almost completely glycosylated.

(B) MBP in Lipid Vesicles (DPPG). When endoproteinase Lys-C digestion was performed on nonglycosylated and glycosylated MBP after incorporation into DPPG vesicles, HPLC profiles shown in Figure 6C and Figure 6D, respectively, were obtained. Although considerable digestion was obtained when nonglycosylated MBP (Figure 6C) was incorporated into lipid vesicles, the peptide map was significantly different from that of the nonglycosylated MBP in solution (Figure 6A), showing less extensive digestion. This indicates that some sites were protected from digestion by interaction with the lipid bilayer. The HPLC profile of the Lys-C digest of glycosylated MBP incorporated into DPPG vesicles is shown in Figure 6D. The extent of digestion was even less than for the nonglycosylated MBP in DPPG, demonstrating the even fewer Lys-C digestion sites were available. The virtual absence of hydrophobic peptides eluting after 35 min from the column suggests that these peptides may be buried in the lipid bilayer. The glycosylated peptide 92–105 was recovered at about 25 min. However, less than 10% of the total glycosylated peptide was released from the vesicles, suggesting that the Lys-C sites in the vicinity of the GALNAC were not accessible to the enzyme.

Discussion

Although MBP has been studied extensively for the past 20 years, the detailed interaction of the protein with the lipid bilayer has only been partially elucidated. Earlier studies presented a strong case for penetration of hydrophobic residues or amino acid side chains into the lipid bilayer. London et al. (1973) showed that certain regions of the protein were protected from cleavage by proteolytic enzymes when it was bound to lipid. The results with Lys-C presented in this study confirm this earlier result. The hydrophobic photolabel 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine labeled MBP 2–4 times more when it was incorporated into vesicles than in aqueous solution (Boggs et al., 1988).

To further our understanding of the interaction of localized peptide segments with the bilayer, we took advantage of the ability of MBP to accept carbohydrate. Since GALNAC was localized mainly on Thr-95 at the mole ratios of GALNAC to MBP used here, it provided us with an opportunity to label the protein specifically with only one spin-label per molecule of protein and to study the interactions of the middle portion of the molecule near the tri-proline segment with lipid.

The effect of three synthetic phospholipids, DSPG, DPPG, and DMPG, on the motion of the spin-label was found to be relatively similar; i.e., in all cases, a two-component spectrum was observed, characterized by a motionally restricted and a mobile component, below the phase transition temperature of the respective lipid. A lesser degree of motional restriction also occurred with DPPA once the sample had been cycled through the lipid phase transition. The motional restriction for DPPA was partially but not completely reversible on cooling back to the gel phase.

The motionally restricted component is most likely caused by location of the spin-label in an ordered region of the bilayer. This conclusion is supported by the sensitivity of the probe motion to the lipid phase transition. The probe is probably located close to the bilayer surface, however, since the amino linkage of the spin-label to the sugar is probably protonated. In addition, the sugar to which it is bound is probably too polar to penetrate into the bilayer, unless an unusual change in conformation of the protein occurred. Glycosylation significantly reduced the number of sites of the lipid-bound protein available for proteinase Lys-C digestion and also reduced the amount of peptide 92–105 liberated, so that penetration of this and other segments of the protein into the bilayer is a possible explanation for the results of Lys-C digestion and motional restriction of the spin-label.

Indeed, the fact that glycosylation resulted in even fewer sites available for Lys-C digestion than for the native protein bound to lipid indicated that it affected the interaction of the entire MBP molecule with the lipid bilayer, suggesting significant alteration of the secondary/tertiary structure of the protein. In a recent 500-MHz proton NMR study of glycosylated MBP in solution, not only the specific, glycosylated Thr resonances were shifted but also Val and Ala envelopes were affected, supporting the suggestion of a change in

structure of the entire molecule (Persaud et al., 1988).

Since MBP lacks disulfide bonds, its conformation is relatively unrestricted. Glycosylation might affect the protein conformation by hydrogen bonding (Quiocho, 1986) with sites on the protein, altering its water solubility, or causing steric interference with the putative hairpin bend of the tri-proline segment. In a recent FT-IR study, MBP was shown to adopt considerable β-structure when bound to DPPG vesicles (Surewicz et al., 1987). Although the structure of the glycosylated protein has not yet been studied using this technique, it is possible that the hydrogen-bonding sugar residues could interfere with formation of β-structure. If glycosylation causes a conformational change in the protein, this could protect it against digestion by Lys-C either by significantly increasing its penetration into the bilayer or by changes in the degree of aggregation of the protein, or other unknown effects.

On the other hand, the changes in motion of the spin-label bound to GALNAC in different lipids found in this study have many similarities to the changes found for a spin-label bound to the two methionines of the unglycosylated protein in our earlier study (Boggs et al., 1980). The spin-label was motionally restricted in several lipids in that study also. Motional restriction of the protein-bound spin-label could also be caused by penetration of the spin-label group into the bilayer with the sugar and the peptide backbone in this region of the protein lying on the surface of the bilayer. Hydrophobic side chains and residues elsewhere on the protein could similarly penetrate into the bilayer. Penetration of these groups could perturb the lipid packing and be sufficient to account for the decrease in phase transition temperature and many other effects of MBP on acidic lipids.

The decrease in the motionally restricted component with increase in temperature and its disappearance when the lipid transforms into the liquid-crystalline phase reflect the increased fluidity of the bilayer and possibly also location of the probe in a more fluid region of the bilayer. The hysteresis on cooling suggests a greater disordering effect after the protein has interacted with liquid-crystalline phase lipid. This greater disordering effect may be due to greater interaction (penetration of more side chains or penetration to a greater depth within the bilayer) with the liquid-crystalline phase. The similarity of reheating scans to the cooling scan indicates that these residues continue to penetrate into the bilayer in the gel phase.

It should be noted that the behavior of the protein-bound spin-label in the lipid is markedly different from that of a free spin-label. Even hydrophobic spin-labels such as TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) have very low solubility in gel phase lipid. Their partition coefficient increases greatly during the gel to liquid-crystalline phase transition, and they are squeezed out when the lipid refreezes. The results presented here with the protein-bound spin-label indicate that a hydrophobic group on a protein bound to the bilayer surface is able to partition into the gel phase and to disorder it, in contrast to a small amphipathic molecule which is free to move between the bilayer and the aqueous phase.

The behavior of the spin-labeled protein with DPPA in this study showed similarities to that of MBP spin-labeled at the methionines with the hydrogen-bonding lipid DMPE in our earlier study (Boggs et al., 1980), where there was some interaction with DMPE in the liquid-crystalline phase which was not completely reversed on cooling back. In contrast, in this study the spin-labeled protein showed little or no interaction with DPPE either in the gel or in the liquid-crystalline phase. This is consistent with the lack of perturbing effect of MBP on PE using other techniques (Boggs & Moscarello, 1978; Boggs et al., 1988). The greater interaction of the protein spin-labeled at the methionines with DMPE, found in our earlier study, is probably due to the shorter fatty acid chain length, rather than the different site of labeling. This conclusion is supported by the greater motional restriction of the spin-labeled glycosylated protein with DMPG than with the longer chain PG's in the present study. The motion of the methionine-labeled protein was also restricted more by DMPA in our earlier study than found for the glycosylated protein with DPPA. The shorter fatty acid chain length reduces the cohesive forces between the lipid molecules so that the headgroup interactions can be more easily disrupted when the protein binds.

The similarities in behavior of the spin-label bound to the GALNAC in the center of the protein molecule to that of spin-labels bound to the two methionines near the N and C termini of the protein (Boggs et al., 1980), particularly in the motional restriction and sensitivity to the phase transition, suggest relatively similar interactions with lipid of all regions of this protein.

Registry No. DPPA, 19698-29-4; DPPE, 3026-45-7; DPPG, 4537-77-3; DSPG, 4537-78-4; DMPG, 61361-72-6.

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Interaction of Melittin with Phosphatidylcholine Membranes. Binding Isotherm and Lipid Head-Group Conformation†

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Received October 5, 1988; Revised Manuscript Received January 25, 1989

ABSTRACT: The binding of melittin to nonsonicated bilayer membranes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine was studied with an ultracentrifugation assay and with ^2H and ^{31}P nuclear magnetic resonance. Melittin binding could best be described by a partition equilibrium with $K_p = (2.1 \pm 0.2) \times 10^3$ M^{-1}, measuring the binding isotherm in the concentration range of 0–100 μM melittin and taking into account electrostatic effects by means of the Gouy–Chapman theory. This partition coefficient is smaller than that deduced for small sonicated vesicles and attests to the tighter lipid packing in the nonsonicated bilayers. Deuterium magnetic resonance revealed a conformational change of the phosphocholine head group upon melittin binding. The quadrupole splittings of the α and β segments of the choline head group varied linearly with the amount of bound melittin but in opposite directions; i.e., the α splitting decreased, and the β splitting increased. This conformational change is not specific to melittin but is a response of the phosphocholine head group to positive membrane surface charges in general. Quantitatively, melittin is one of the most efficient head-group modulators, the efficiency per unit charge comparable to that of charged local anesthetics or hydrophobic ions.

Melittin, the main component of bee venom, is a cationic peptide composed of essentially hydrophobic (positions 1–20) and hydrophilic (positions 21–26) amino acids (Haberman & Jentsch, 1967): Gly-Ile-Gly-Ala-Val[5]-Leu-Lys(+)-Val-Leu-Thr[10]-Thr-Gly-Leu-Pro-Ala[15]-Leu-Ile-Ser-Trp-Ile[20]-Lys-(+)-Arg(+)-Lys(+)-Arg(+)-Gln[25]-Gln-$CONH_2$. Because of the uneven distribution of polar and hydrophobic residues, melittin shows amphiphilic properties, interacting strongly with micelles, pure lipid membranes, and biological membranes. Conflicting views about the aggregational state of melittin and about its membrane orientation may be found in the literature, but the data seem to converge to the following picture (Stanislawski & Rüterjans, 1987; Batenburg et al., 1987; Altenbach & Hubbell, 1988): (I) Mellitin is bound in a monomeric form to the lipid membrane. (II) The N-terminus with its 20 mainly hydrophobic amino acids adopts an α-helical conformation in the presence of lipid, with the helix axis more or less parallel to the membrane surface. (III) When added to sonicated phosphatidylcholine vesicles, melittin will not permeate the lipid membrane, but the N- and C-termini will remain on the same side of the membrane.

Melittin induces structural perturbations of the lipid bilayer which have been visualized by a number of different techniques [see Dufourc et al. (1986a,b) and Dempsey and Watts (1987) and references cited therein]. At high concentrations, the peptide disrupts the bilayer, leading to the formation of nonbilayer phases or micelles. In the present study, we were interested mainly in the effect of melittin on the phospholipid head groups, and the concentration of the peptide was kept low enough to leave the bilayer intact.

Phospholipid head groups are sensitive to electric charges at the membrane surface (Seelig et al., 1987). The binding of quite different chemical compounds such as metal ions (Akutsu & Seelig, 1981; Altenbach & Seelig, 1984; Macdonald & Seelig, 1987a,b), hydrophobic ions (Altenbach & Seelig, 1985), or charged local anesthetics (Boulanger et al., 1981; Seelig et al., 1988) induces a unique conformational change of the lipid head group which is quantitatively similar for all positively charged agents and opposite that induced by negatively charged molecules (Seelig et al., 1987; Macdonald & Seelig, 1988). Hence, melittin with its six positive charges should exert a relatively large effect on the phospholipid head groups when bound to the membrane. Deuterium nuclear magnetic resonance (NMR)[1] experiments with head-group-deuteriated 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) have indeed provided first indications of such an effect but have been inconclusive concerning a quantitative interpretation (Dempsey & Watts, 1987).

A reliable and sensitive method for monitoring conformational changes of lipids in membranes is NMR spectroscopy and, in particular, deuterium NMR spectroscopy (Seelig, 1977). Combined with a selective deuteriation of the phospholipid head groups, the measurement of the so-called deuterium quadrupole splitting, $\Delta\nu_Q$, provides a quantitative handle on changes of the head-group conformation and lipid

† Supported by Swiss National Science Foundation Grant 3.521.86.

[1] Abbreviations: NMR, nuclear magnetic resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.

0006-2960/89/0428-4216$01.50/0