Interaction of Two Complementary Fragments of the Bovine Spinal Cord Myelin Basic Protein with Phosphatidylglycerol Bilayers, Studied by ^2H and ^{31}P NMR Spectroscopy†

Manajit Hayer-Hartl,‡,§,⊥ Peter J. Brophy,‖ Derek Marsh,‖ and Anthony Watts*,‡

Department of Biochemistry, University of Oxford, Oxford OX1 3QU, U.K., Department of Biological Sciences, Stirling University, Stirling FK9 4LA, U.K., and Abteilung Spektroskopie, Max-Planck-Institut für Biophysikalische Chemie, D-3400 Göttingen, FRG

Received March 17, 1993; Revised Manuscript Received June 2, 1993

ABSTRACT: The interaction of two complementary fragments of myelin basic protein from bovine spinal cord with bilayers of dimyristoylphosphatidylglycerol has been studied by broad line ^2H and ^{31}P NMR. The fragments, produced by cleavage at the single tryptophan, consist of an N-terminal portion of molecular mass 12.6 kDa and a C-terminal portion of molecular mass 5.8 kDa. The phosphatidylglycerol lipid was deuterated at all three segments of the glycerol headgroup. The approximately linear dependence of the ^2H quadrupole splittings and ^{31}P chemical shift anisotropy on protein/lipid ratio in the complexes indicates that the lipids interacting with the protein fragments were in fast exchange on the NMR time scale ($\approx 10^{-4}$–10^{-5} s). The relative gradients of the dependence on protein/lipid ratio of both these parameters decrease with the size of the protein fragment and correlate reasonably well with both the net charge on the protein and the lipid binding stoichiometries in the absence of salt. The results are therefore consistent with a model in which the perturbation of the quadrupole splittings either is determined by the net surface potential or is constant for the different protein fragments. Either possibility is consistent with the reduced activity of the fragments relative to the whole protein.

The ^2H NMR spectra of phospholipids specifically deuterated in their polar headgroups are sensitive to the interactions, principally electrostatic, within the bilayer surface between different phospholipid types (Sixl & Watts, 1982, 1983; Watts, 1987; Watts & van Gorkom, 1991). There is now convincing evidence that, in many cases, such interactions are controlled by the electrostatic field arising from the charges on the headgroups of amphiphiles incorporated within the bilayer (Scherer & Seelig, 1989, and references therein). Additionally, membrane proteins, especially charged peptides and extrinsic proteins, are found to induce similar structural changes in the phospholipid headgroups on binding to charged lipid surfaces (Roux et al., 1988; Kuchinka & Seelig, 1989; Sixl et al., 1984; Dempsey & Watts, 1987; Sixl & Watts, 1985). It is again likely that these changes are induced by the electrostatic fields arising from the charged residues on the protein (Roux et al., 1989). Such interactions could well be important in determining the specificity, mode of interaction, and activity of peripherally associated membrane proteins, as well as potentially being implicated in transmembrane signaling by surface-associated ligands.

The myelin basic protein (MBP)[1] is a peripheral protein which comprises approximately 30 wt % of the total protein in central nervous system myelin. As well as its role in the compaction of myelin, it is also the factor responsible for the induction of experimental allergic encephalomyelitis (Eylar et al., 1970). Positively charged residues are roughly evenly distributed throughout the amino acid sequence of the protein from bovine CNS myelin. The whole MBP contains 31 positively charges residues per 18.4-kDa protein, at physiological pH. BNPS–skatole cleaves the bovine protein at the single tryptophan residue, producing a large N-terminal fragment (residues 1–116), F1, of molecular mass 12.6 kDa, and a relatively small C-terminal fragment (residues 117–170), F2, of molecular mass 5.8 kDa (Martenson et al., 1975). The F1 fragment bears 20 of the total complement of positively charged residues, and the F2 fragment bears the other 11 positively charged residues (cf. Figure 1). It has been suggested that the dynamic specificity of lipid association with MBP may be essential in stabilizing the molecular arrangement and integrity of the myelin sheath [see e.g., Boggs et al. (1982)].

In the present work we have compared the relative effects of the F1 and F2 fragments of the MBP on the ^2H quadrupole splittings and ^{31}P chemical shift anisotropy of aqueous dispersions of dimyristoylphosphatidylglycerol perdeuterated in the headgroup, using broad line NMR. Particular attention is given to the contributions of charge effects and relative binding stoichiometries on the lipid headgroup interaction, as compared with that of the whole protein. This current NMR study, and an earlier NMR study on intact MBP (Sixl et al., 1984), on the interaction with the lipid headgroups, is complementary to other ones that have investigated the effects

† This work has been supported in part by the European Community CODEST programme, Contract No. ST2J-0368-C(EDB) and Grant No. MS-13 from the Multiple Sclerosis Society of Great Britain and Ireland.

* To whom correspondence should be addressed.
‡ University of Oxford.
§ Stirling University.
‖ Max-Planck-Institut.
⊥ Present address: Program of Cellular Biochemistry and Biophysics, Rockefeller Research Laboratories, Sloan-Kettering Institute, 1275 York Ave., New York, NY 10021.

[1] Abbreviations: MBP, myelin basic protein; F1, the N-terminal 12.6-kDa fragment of myelin basic protein; F2, the C-terminal 5.8-kDa fragment of the myelin basic protein; CNS, central nervous system; BNPS-skatole, 2-[(2-nitrophenyl)sulfenyl]-3-methyl-3-bromoindolenine; DMPG-d_5, 1,2-dimyristoyl-*sn*-glycero-3-phospho[$^2H_5$]glycerol; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethenesulfonic acid; NMR, nuclear magnetic resonance; csa, chemical shift anisotropy; ESR, electron spin resonance; SDS–PAGE, polyacrylamide gel electrophoresis in sodium dodecylsulfate; HPLC, high-pressure liquid chromatography.

0006-2960/93/0432-9709$04.00/0

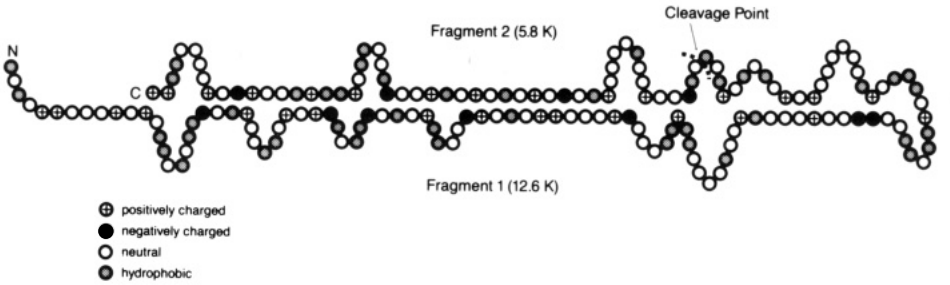

FIGURE 1: Schematic diagram of the disposition of positively charged residues (Lys and Arg) throughout the sequence of bovine myelin basic protein and the fragments F1 and F2 produced by cleavage with BNPS–skatole at the single Trp. Negatively charged (Asp and Glu) and hydrophobic (Leu, Ile, Trp, Phe, Thr, and Ala) residues are also indicated, as well as intervening nonpolar segments (loops) between the charged residues.

on the lipid chain mobility by using spin label ESR (Sankaram et al., 1989; Boggs et al., 1981). Therefore, they provide further information on the role that the basic protein may play in the assembly of compact myelin and hence on its encephalitogenic activity.

MATERIALS AND METHODS

Dimyristoylphosphatidylglycerol perdeuterated in the glycerol headgroup (DMPG-d_5) was synthesized by the method of Harlos and Eibl (1980) and Sixl and Watts (1983). The lipid was purified by HPLC on silica gel, using a chloroform–methanol–water solvent system.

Myelin basic protein was extracted and purified from bovine spinal cord, as described by Deibler et al. (1972). The protein was further purified by chromatography on a Sephadex G-75 Superfine column equilibrated with 10 mM HCl. The basic protein was cleaved by BNPS–skatole at the single tryptophan residue (Martenson et al., 1975). The methionone residues were regenerated as described by Jones and Rumsby (1977) and the fragments purified by chromatography on Sephadex G-75 Superfine. Protein purity was checked by SDS–PAGE; protein and fragments migrated as single bands, according to their molecular weights.

The buffer used throughout for sample preparation was 2 mM Hepes, 1 mM EDTA, and 0.1 M NaCl, pH 7.8, made with deuterium-depleted water (Aldrich Chemical Co.). Lipid–protein complexes were formed by adding the protein fragments to DMPG-d_5 lipid dispersions in buffer at up to 40–45 wt % (with respect to lipid + protein) and incubated at room temperature.

For the binding experiments, a fixed amount (10 mg) of DMPG was dispersed in buffer (1 mL), and various amounts of protein were added to the dispersion. After incubation (1–2 h) at room temperature, the protein–lipid complex was centrifuged (30000g, 10 min). The residual protein in the supernatant and the protein in the complexes was determined by the method of Lowry et al. (1951). Lipid determinations were made using the method of Eibl and Lands (1969). The amount of lipid recovered in the complexes was always >95% of the original DMPG added initially.

Deuterium NMR spectra were recorded at 46.1 MHz on a Bruker WH300 spectrometer, employing either quad echo detection with a pulse spacing of 30 μs and a 90° pulse width of 4 μs or single 90° pulses of duration 29 μs. Proton-dipolar decoupled ^{31}P NMR spectra were recorded at 121.5 MHz on the same spectrometer, with single 90° pulses of 18 μs and gated broad band decoupling.

RESULTS

Binding Studies. The binding curves of the whole myelin basic protein and the F1 and F2 fragments to DMPG bilayers were determined chemically under conditions similar to those used for preparation of the samples for NMR. These results are given in Figure 2. The binding is very tight for all the peptides and saturates at stoichiometries of approximately 30–34 lipids per peptide in each case. These values are different from the stoichiometries determined at low ionic strength (Sankaram et al., 1989), where it was found that the number of lipids associated at saturation binding was proportional to the molecular weight of the peptide.

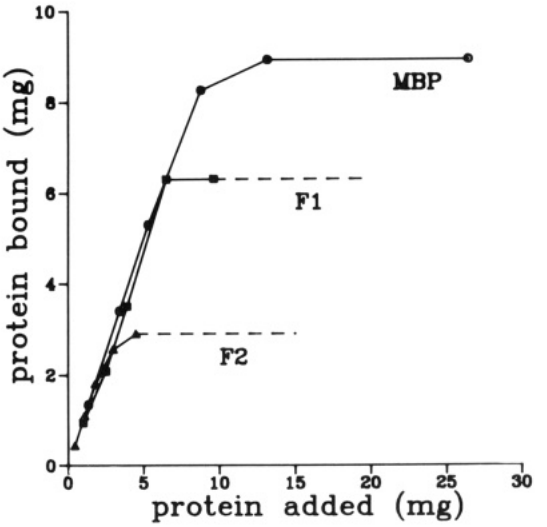

FIGURE 2: Dependence of the binding (expressed as mg/10 mg of DMPG) of MBP (○), the F1 fragment (□), and the F2 fragment (△), to DMPG dispersions, on the total weight of protein added. Buffer: 2 mM Hepes, 1 mM EDTA, and 0.1 M NaCl, pH 7.8. A fixed quantity of lipid (10 mg) was used in each case; see the text for details.

^{2}H NMR Spectra. The broad-line ^{2}H NMR spectra of aqueous dispersions of DMPG-d_5 in the presence of increasing amounts of the fragment F1 are given in Figure 3A. The spectra are a superposition of "Pake doublets" arising from the powder patterns of the deuterated α-, β-, and γ-segments of the DMPG headgroup. In the absence of protein, the spectral component from the α-segment (which has the largest quadrupole splitting) is split into four resonances, arising from inequivalence of the geminal deuterons in both the D- and L-stereoisomers of the glycerol headgroup (Wohlgemuth et al., 1980; Sixl & Watts, 1983). With increasing protein content, the quadrupole splittings of all three deuterated headgroup segments are seen to decrease progressively. This was also found to be the case for the F2 fragment of the protein (spectra not shown). Qualitatively similar effects of protein binding on the DMPG-d_5 ^{2}H NMR spectra have been observed previously with the whole MBP protein (Sixl et al., 1984). The quadrupole splittings of the α-C^{2}H$_2$ segment of

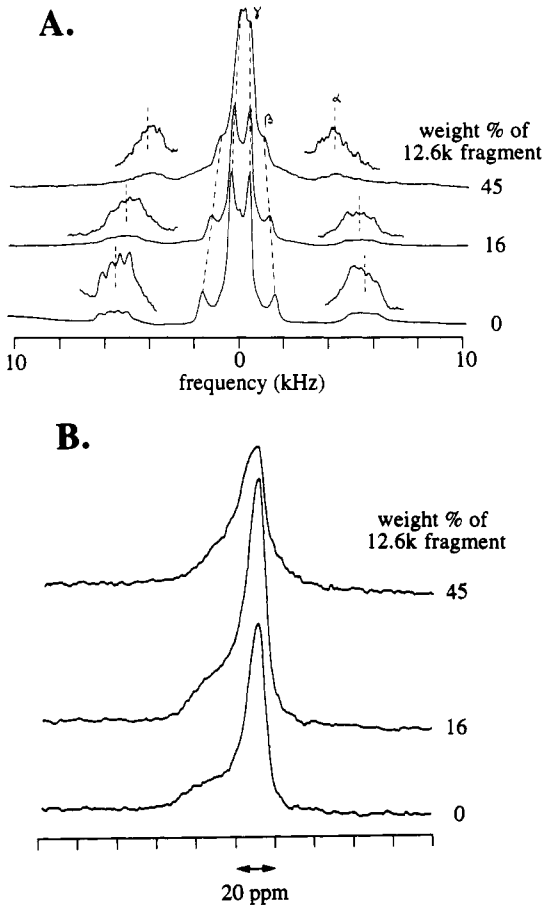

FIGURE 3: (A) 46.1-MHz ^2H NMR spectra of DMPG-d_5 with the indicated wt % of fragment F1 added. (B) 121.5-MHz proton-dipolar decoupled ^{31}P NMR spectra of DMPG-d_5 with the indicated wt % of fragment F1 added. Spectra were recorded at 35 °C.

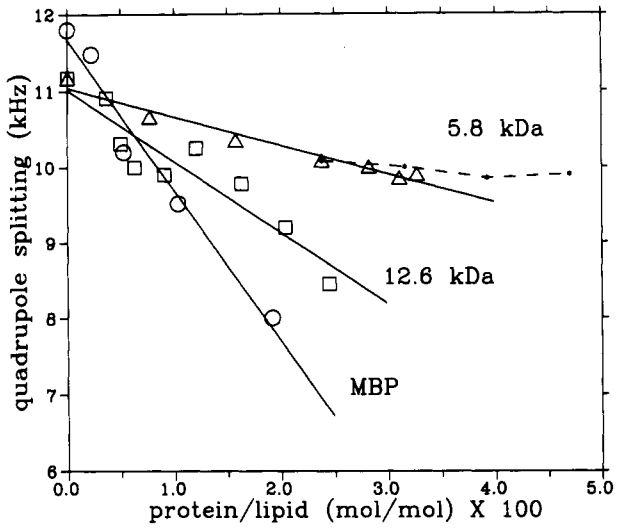

FIGURE 4: Dependence of the α-C^2H_2 quadrupole splitting of DMPG-d_5 on protein/lipid ratio in complexes with MBP (○) [data from Sixl et al. (1984), T = 35 °C] and the fragments F1 (□) and F2 (△). The abscissa corresponds to protein bound, as assayed in Figure 2; the solid lines are linear regressions. Asterisks and the dashed line correspond to the total added protein for the F2 fragment, before correction for the fraction unbound.

the headgroup are plotted as a function of the amount of protein bound in Figure 4, for the whole MBP and for the F1 and F2 fragments. All three sets of data display an approximately linear dependence on the protein/lipid mole ratio when correction has been made for the proportion of protein unbound. This result is consistent with a rapid exchange on the ^2H NMR time scale of the lipids interacting with the protein with those free in the bilayer regions to which no protein is bound. [It will be noted that the characteristic frequencies of the ^2H quadrupole splittings are considerably lower than the rate of translational diffusion of lipid molecules in fluid bilayers; see Knowles and Marsh (1991) for a review.] It is seen from Figure 4 that the extent of decrease in quadrupolar splitting with protein/lipid mole ratio increases with increasing size of the protein fragment in the order MBP > F1 > F2. The gradients of the dependence of the quadrupolar splittings on protein/lipid molar ratio obtained from the linear regressions in Figure 4 are given in Table I.

Table I: Gradients of the α-C^2H_2 Quadrupole Splittings (kHz) Deduced from ^2H NMR and the Chemical Shift Anisotropies (ppm) Deduced from ^{31}P NMR, with Respect to Protein/Lipid Molar Ratio in Complexes of MBP, Fragment F1, and Fragment F2 with DMPG-d_5 at 35 °C

	MBP[a]	F1	F2
^2H [kHz/(mol/mol) × 10^{-2}]	2.0 ± 0.2	0.94 ± 0.14	0.38 ± 0.05
^{31}P [ppm/(mol/mol) × 10^{-2}]	0.3	0.2	0.1

[a] Data taken from Sixl et al. (1984); see text.

The quadrupole splittings for protein-free bilayers (at a protein to lipid mole ratio of 0; Figure 4) are not identical, being 11.2 kHz for the titration of the two fragments of MBP and 11.8 kHz for the intact protein experiment. However, these quadrupole splittings are within experimental error, and the results for the intact protein were obtained in earlier experiments (Sixl et al., 1984), and some small variability in lipid, protein, spectral measurement and temperature control may have occurred; it is the slopes rather than intercepts of the results which are analyzed further (see below).

^{31}P NMR Spectra. The broad-line, proton-dipolar decoupled ^{31}P NMR spectra of aqueous dispersions of DMPG-d_5 in the presence of increasing amounts of the fragment F1 are given in Figure 3B. The powder patterns are characteristic of a lamellar liquid-crystalline arrangement of the lipid molecules, both in the presence and in the absence of the protein fragments. Similar to the results with ^2H NMR, the ^{31}P chemical shift anisotropy is found to decrease systematically with increasing protein content. Analogous results were obtained for the F2 fragment (spectra not shown) and previously for the whole MBP (Sixl et al., 1984). The csa was found to have an approximately linear dependence on protein/lipid mole ratio for both the whole MBP and the F1 and F2 fragments, again consistent with rapid exchange on the timescale characteristic of the ^{31}P chemical shift anisotropy. The gradients of the dependence on protein/lipid molar ratio are given in Table I and, as for the ^2H quadrupole splittings, show a systematic dependence on the molecular weight of the protein fragment in the order MBP > F1 > F2.

DISCUSSION

The results from protein binding assays and NMR studies of the lipid headgroups indicate that both the whole MBP protein and its complementary fragments, associate in a similar manner (predominantly electrostatic) with the negatively charged lipid bilayers. This is essentially in agreement with other previous studies on the MBP fragments (Sankaram et al., 1989; Boggs et al., 1981; London et al., 1973) and other peptides and proteins (Sixl & Watts, 1985; Dempsey & Watts, 1987; Watts, 1987; Watts & Van Gorkom, 1992). The most pronounced quantitative differences in the interaction with the various peptides are manifest by a progressive increase in the degree of perturbation of the NMR spectrum from the

lipid headgroups with increasing molecular weight of the protein fragment. It is these differences that it is attempted to interpret here.

The NMR spectra of Figures 3 and 4, after correction for the degree of protein binding, show that the lipids are in fast exchange on the NMR time scale between sites associated with, and remote from, the protein. The dependence of the ^2H quadrupole splitting, $\Delta\nu_Q$ (or ^{31}P NMR csa, $\Delta\sigma_{eff}$), on lipid/protein molar ratio, n_t, is therefore given by (Sixl et al., 1984)

$$\Delta\nu_Q = (\Delta\nu_Q^p - \Delta\nu_Q^f)n_p/n_t + \Delta\nu_Q^f \quad (1)$$

where $\Delta\nu_Q^p$ is the quadrupole splitting of a lipid directly associated with the protein, n_p is the number of lipid association sites on the protein, and $\Delta\nu_Q^f$ is the quadrupole splitting for the lipids remote from the protein, which is taken to be that of the free lipid bilayers. The linear dependence of the quadrupole splitting on protein/lipid molar ratio for the different fragments, determined by the gradient $(\Delta\nu_Q^p - \Delta\nu_Q^f)n_p$, potentially may be correlated either with the stoichiometry of binding or with the net charge stoichiometry of the lipid/protein complexes.

Seelig and co-workers (Seelig et al., 1987; Scherer & Seelig, 1989) have demonstrated that the quadrupole splitting of lipids ^2H-labeled in the headgroup are sensitive to the electrostatic field at the lipid polar group–water interface [cf. also Roux et al. (1989)]. In the low potential approximation of electrostatic double layer theory, which should be applicable at moderately high degrees of protein binding, the electrostatic surface potential is directly proportional to the surface charge density [see, e.g., Cevc and Marsh (1987)]. The same is also true for the electrostatic field at distances close to the bilayer surface. In the absence of the protein, the net charge density per lipid is $-e$, where e is the elementary electronic charge. In the presence of the protein, the net charge per lipid in the immediate region of the protein is $(Z - n_p)e/n_p$, where $+Ze$ is the net charge on the protein. Therefore the gradient of the protein/lipid ratio dependence can be approximated by

$$(\Delta\nu_Q^p - \Delta\nu_Q^f)n_p \propto Ze \quad (2)$$

Hence in this model, the gradients of the protein/lipid ratio dependence of the quadrupole splitting (or csa) should be determined simply by the net charges, Ze, on the different protein fragments. These are $Z = +20$, $+13$, and $+7$ for MBP, fragment F1, and fragment F2, respectively (cf. Figure 1), i.e., they are in the ratio 1.0:0.65:0.35. These values are seen to be in moderately good agreement with the ratios of the gradients of the quadrupole splittings (1.0:0.47:0.19) and csa's (1.0:0.67:0.30) given in Table I.

It will be noted that partial neutralization of the negative surface charge on DMPG by addition of a positively charged amphiphile has been found also to give rise to a progressive decrease in the quadrupole splittings of the α-C^2H_2 group and in the ^{31}P csa, but a slight increase was found in the quadrupole splittings of the β-C^2H group (Marassi & MacDonald, 1991). In the presence of MBP or its fragments, on the other hand, the quadrupole splittings of both α- and β-deuterated groups are found to be decreases, although to a lesser extent for the β-C^2H group. This might tend to suggest that interactions other than simply electrostatic are contributing to the change in lipid headgroup conformation on binding the MBP and its fragments. However, the extent to which this might be true also for admixture of the positively charged amphiphile is not entirely clear, since higher mole fractions of the amphiphile were found to give rise to nonbilayer phases.

In addition, it is found that the binding of MBP and its fragments produces a *differential* response in the change of quadrupole splittings for each headgroup segment which is characteristic of electrostatic interactions between bilayers and protein rather than a simple structural perturbation of the whole headgroup region. If the latter were the case, then a constant, or generalized, reduction in measured parameter by a similar factor for each segment, including the phosphate group, would be observed. However, the fractional gradients in the variation of quadrupole splittings and csa with MBP binding are different for each individual segment (Sixl et al., 1984). In contrast, for the case of the fusogenic peptide melittin, increasing concentrations of the peptide bilayers reduces the quadrupole splitting for α- and β-methylenes of deuterated dimyristoylphosphatidylcholine bilayers by a similar amount for each segment, implying a structural, in this case, rather than electrostatic interaction of the peptide with zwitterionic bilayers (Dempsey & Watts, 1987).

An alternative interpretation is to assume that the protein-induced perturbation of the quadrupole splitting, $(\Delta\nu_Q^p - \Delta\nu_Q^f)$, is the same for all three fragments, and therefore that the gradients of the quadrupole splitting should be in the ratio of the binding stoichiometries. For the binding determined at low ionic strength (which is the appropriate limiting value, rather than those determined in 0.1 M NaCl in Figure 2), the stoichiometries are n_p = 36, 24, and 11 mol/mol for MBP, fragment F1, and fragment F2, respectively (Sankaram et al., 1989), i.e., they are in the ratio 1.0:0.67:0.30. These ratios are rather similar to those determined from the charge stoichiometries and again are in moderately good accord with the ratios of the gradients of the ^2H quadrupole splittings and ^{31}P csa's given in Table I.

It is of interest to compare the present results on the interactions with the lipid headgroups with the previous study (Sankaram et al., 1989) of the effects on the lipid chain mobility. In the latter work it was found that the degree of restriction of the lipid chain motion becomes progressively greater as the size of the protein fragment increases. If these results can be extrapolated to those on the lipid headgroups, they would favor the first of the two above interpretations, namely, that the various protein fragments have a differential effect on the headgroup conformation and chain packing density, related to their different charges. The spin label ESR spectra (Sankaram et al., 1989) also contained a second spectral component that was interpreted as corresponding to spin-labeled chains whose motion was restricted by direct interaction with (penetrant) sections of the protein (cf. the nonpolar loops in Figure 1). The extent of this component was also found to be dependent on the size of the protein fragment. It is unclear whether such interactions would be sensed directly by the lipid headgroups, but the slower characteristic timescale of ^2H and ^{31}P NMR (Watts, 1988) would ensure that this component would be in fast exchange, giving rise to single-component NMR spectra as is observed.

In summary, the different fragments cause different degrees of perturbation of the lipid headgroup conformations in a manner that is consistent with their different net charges and the different lipid binding stoichiometries observed at low ionic strength. This illustrates the way in which peripheral proteins can modulate cooperatively the surface structure of negatively charged lipid bilayers to which they are bound. In the case of the MBP such effects may contribute to the stability of compact myelin. The relative sizes of the perturbations caused by the fragments, in comparison with the whole protein,

also correlates with their reduced encephalitogenic activity (Martenson et al., 1975).

REFERENCES

Boggs, J. M., Wood, D. D., & Moscarello, M. A. (1981) *Biochemistry 20*, 1065–1073.

Boggs, J. M., Moscarello, M. A., & Paphadjopoulos, D. (1982) in *Lipid–Protein Interactions* (Jost, P. C., & Griffith, O. H., Eds.) Vol. 2, pp 1–51, Wiley-Interscience, New York.

Cevc, G., & Marsh, D. (1987) *Phospholipid Bilayers. Physical Principles and Models*, p 442, Academic Press, New York.

Deibler, G. E., Martenson, R. E., & Kies, W. (1972) *Prog. Biophys. Biochem. 2*, 139–165.

Dempsey, C. E., & Watts, A. (1987) *Biochemistry 26*, 5803–5811.

Eylar, E. H., Caccam, J., Jackson, J., Westall, F., & Robinson, A. B. (1970) *Science 168*, 1220.

Harlos, K., & Eibl, H. (1980) *Biochemistry 19*, 895–899.

Knowles, P. F., & Marsh, D. (1991) *Biochem. J. 274*, 625–641.

Kuchinka, E., & Seelig, J. (1989) *Biochemistry 28*, 4216–4221.

London, Y., Demel, R. A., Geurts van Kessel, W. S. M., Vossenberg, F. G. A., & Van Deenen, L. L. M. (1973) *Biochim. Biophys. Acta 311*, 520–530.

Marassi, F. M., & MacDonald, P. M. (1991) *Biochemistry 30*, 10558–10566.

Martenson, R. E., Deibler, G. E., Kramer, A. J., & Levine, S. (1975) *J. Neurochem. 24*, 173–182.

Roux, M., Neumann, J.-M., Bloom, M., & Devaux, P. F. (1988) *Eur. Biophys. J. 16*, 267–273.

Roux, M., Neumann, J.-M., Hodges, R. S., Devaux, P. F., & Bloom, M. (1989) *Biochemistry 28*, 2313–2321.

Sankaram, M. B., Brophy, P. J., & Marsh, D. (1989) *Biochemistry 28*, 9692–9698.

Scherer, P. G., & Seelig, J. (1989) *Biochemistry 28*, 7720–7728.

Seelig, J., MacDonald, P. M., & Scherer, P. G. (1987) *Biochemistry 26*, 7535–7541.

Sixl, F., & Watts, A. (1982) *Biochemistry 21*, 6446–6452.

Sixl, F., & Watts, A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1613–1615.

Sixl, F., & Watts, A. (1985) *Biochemistry 24*, 7906–7910.

Sixl, F., Brophy, P. J., & Watts, A. (1984) *Biochemistry 23*, 2032–2039.

Watts, A. (1987) *J. Bioenerg. Biomembr. 19*, 625–653.

Watts, A. (1988) in *Dynamic Properties of Biomolecular Assemblies* (Harding, S., & Rowe, A. J., Eds) pp 320–347, Royal Chemical Society, London.

Watts, A., & Van Gorkom, L. C. M. (1991) in *The Structure of Biological Membranes* (Yeagle, P. L., Ed.) pp 307–377, CRC Press Inc., Cleveland, OH.

Wohlgemuth, R., Waespe-Sarcevic, N., & Seelig, J. (1980) *Biochemistry 19*, 3315–3321.