Forsayeth, J., Maddux, B., & Goldfine, I. D. (1986) *Diabetes 35*, 837–846.
Freychet, P., Roth, J., & Neville, D. M. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1833–1837.
Goidl, J. A. (1981) *J. Recept. Res. 2*, 419–433.
Goldfine, I. D. (1981) *Biochem. Actions Horm. 8*, 273–305.
Goldfine, I. D., & Smith, G. J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1427–1431.
Goldfine, I. D., Vigneri, R., Cohen, D., Pliam, N. B., & Kahn, C. R. (1977) *Nature (London) 269*, 698–700.
Harris, J. R., & Agutter, P. S. (1976) in *Biochemical Analysis of Membrames* (Maddy, A. H., Ed.) pp 132–173, Wiley, New York.
Hedo, J. A., & Simpson, I. A. (1984) *J. Biol. Chem. 259*, 11083–11089.
Hedo, J. A., Kahn, C. R., Hayashi, M., Yamada, K. M., & Kasuga, M. (1983) *J. Biol. Chem. 258*, 10020–10026.
Horvat, A. (1978) *J. Cell. Physiol. 97*, 37–47.
Horvat, A., Li, E., & Katsoyannis, P. G. (1975) *Biochim. Biophys. Acta 382*, 609–620.
Jacobs, S., & Cuatrecasas, P. (1981) *Endocr. Rev. 2*, 251–263.
Kahn, C. R. (1976) *J. Cell Biol. 70*, 261–286.
Kasuga, M., Karlsson, F. A., & Kahn, C. R. (1982) *Science (Washington, D.C.) 115*, 185–185.
Monier, S., Brossette, N., Le Marchand-Brustel, Y., & Van Obberghen, E. (1984) *Diabetes 33*, 8.
Pilch, P. F., & Czech, M. P. (1979) *J. Biol. Chem. 254*, 3375–3381.
Ray, T. K. (1970) *Biochim. Biophys. Acta 196*, 1–9.
Ronnett, G. V., Knutson, V. P., Kohanshi, R. A., Simpson, T. L., & Daniel Lane, M. (1984) *J. Biol. Chem. 259*, 4566–4577.
Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., & Ramachandran, J. (1985) *Nature (London) 313*, 756–761.
Vigneri, R., Goldfine, I. D., Wong, K. Y., Smith, G. J., & Pezzino, V. (1978a) *J. Biol. Chem. 253*, 2098–2103.
Vigneri, R., Pliam, N. B., Cohen, D. C., Pezzino, V., Wong, K. Y., & Goldfine, I. D. (1978b) *J. Biol. Chem. 253*, 8192–8197.

Characterization of Dodecylphosphocholine/Myelin Basic Protein Complexes†

George L. Mendz,*,‡ Walter J. Moore,‡ Ian J. Kaplin,§ Bruce A. Cornell,∥ Frances Separovic,∥ David J. Miller,⊥ and Larry R. Brown#

Department of Biochemistry and Electron Microscope Unit, The University of Sydney, Sydney, NSW 2006, Australia, CSIRO Division of Food Research, North Ryde, NSW 2113, Australia, School of Physics, University of New South Wales, Kensington, NSW 2033, Australia, and Research School of Chemistry, Australian National University, Canberra, ACT 2601, Australia

Received May 29, 1987; Revised Manuscript Received July 30, 1987

ABSTRACT: The stoichiometry of myelin basic protein (MBP)/dodecylphosphocholine (DPC) complexes and the location of protein segments in the micelle have been investigated by electron paramagnetic resonance (EPR), ultracentrifugation, photon correlation light scattering, ^{31}P, ^{13}C, and ^{1}H nuclear magnetic resonance (NMR), and electron microscopy. Ultracentrifugation measurements indicate that MBP forms stoichiometrically well-defined complexes consisting of 1 protein molecule and approximately 140 detergent molecules. The spin-labels 5-, 12-, and 16-doxylstearate have been incorporated into DPC/MBP aggregates. EPR spectral parameters and ^{13}C and ^{1}H NMR relaxation times indicate that the addition of MBP does not affect the environment and location of the labels or the organization of the micelles except for a slight increase in size. Previous results indicating that the protein lies primarily near the surface of the micelle have been confirmed by comparing ^{13}C NMR spectra of the detergent with and without protein with spectra of protein/detergent aggregates containing spin-labels. Electron micrographs of the complexes taken by using the freeze-fracture technique confirm the estimated size obtained by light-scattering measurements. Overall, these results indicate that mixtures of MBP and DPC can form highly porous particles with well-defined protein and lipid stoichiometry. The structural integrity of these particles appears to be based on protein–lipid interactions. In addition, electron micrographs of aqueous DPC/MBP suspensions show the formation of a small amount of material consisting of large arrays of detergent micelles, suggesting that MBP is capable of inducing large changes in the overall organization of the detergent.

Understanding the molecular architecture of the myelin sheath requires the characterization of the interactions between its various components, the lipids and proteins. The association of the myelin basic protein (MBP) with lipid components plays an important role in stabilizing the multilamellar structure of myelin. Hence, knowledge of the location, structure, and nature of the binding of the basic protein to lipid systems may provide insights into intermolecular interactions of functional significance in the organization of myelin.

For MBP to contribute to the stability of the myelin double bilayer it must interact with lipids in specific ways. A simple model would envisage the protein acquiring definite conformations in protein–lipid complexes, in the manner of intrinsic structural proteins. The conformational rigidity resulting from the protein–lipid interactions acts as a stabilizing factor on the membrane. Outside this milieu the protein is unable to undergo adequate folding. Another view would consider the

† This work was supported by the National Health and Medical Research Council.
‡ Department of Biochemistry, The University of Sydney.
§ Electron Microscope Unit, The University of Sydney.
∥ CSIRO.
⊥ University of New South Wales.
Australian National University.

0006-2960/88/0427-0379$01.50/0

formation of protein aggregates of well-defined stoichiometry that interact with membrane lipids. The self-associated protein would accommodate the lipid so that the protein–protein interactions become a source of membrane stability.

Smith (1985) found that in water solutions at physiological pH, MBP forms hexamers at concentrations as low as 0.1 mM. Determination of the stability and structure of these protein aggregates when complexed with lipids became an important step in our attempts to understand the molecular architecture of myelin.

A characterization of the complexes formed when myelin basic protein binds to the zwitterionic detergent dodecylphosphocholine (DPC) was obtained in a previous study using circular dichroism and high-resolution nuclear magnetic resonance (NMR) (Mendz et al., 1984). The results suggested (i) the presence of discrete binding sites on the MBP molecules, (ii) changes in protein conformation induced by the interaction, (iii) that the binding occurs with detergent micelles, (iv) that the protein penetrates beyond the dipolar interfacial region of the DPC micelles into the hydrophobic interior, and (v) that the complexes become independent of detergent concentration at a molar ratio of about 200:1. Detailed information regarding the stoichiometry and organization of the detergent/protein aggregates has now been obtained from the application of several analytical methods.

Materials and Methods

Porcine MBP was provided by Dr. Max Marsh of Eli Lilly & Co. Bovine MBP was prepared from white matter of fresh cow brain by the method of Eylar et al. (1969). Purities of the proteins were checked by sodium dodecyl sulfate gel electrophoresis. They displayed an intense band at the position of MBP and a faint band (<3%) at a higher molecular weight. The fatty acid spin-labels 5-doxylstearate [2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy], 12-doxylstearate, and 16-doxylstearate were products of Aldrich Chemical Co. DPC was synthesized by methods previously described (Brown, 1979; Lauterwein et al., 1979).

The proteins were lyophilized twice from 99.5% ^2H_2O and dissolved in 99.96% ^2H_2O (Merck Sharp & Dohme) at a concentration of 0.5–1.0 mM in 5-mm OD precision NMR tubes. The pH was adjusted with ^2HCl or NaO^2H and measured with an Ingold 6030-02 microelectrode, values being reported as meter readings. Increasing amounts of DPC were added to the protein solutions to obtain a series of DPC/MBP molar ratios from 0 to 200.

Samples containing spin-labels were prepared by adding a measured amount of free radical in ethanol to a DPC/MBP aqueous suspension to obtain the final composition desired, lyophilizing the mixture, and dissolving it in ^2H_2O.

Ultracentrifugation. Measurements were carried out in 0.05 M phosphate buffer, pH 7.0, with 14.6×10^{-3} and 73×10^{-3} M dodecylphosphocholine and 7.3×10^{-5} M myelin basic protein. Sedimentation equilibrium experiments were performed in a Beckman Model E analytical ultracentrifuge at 293 K. Samples were spun in a Four-Place An-F rotor with double-sector cells at 20 000 rpm for typically 24 h. After the mixtures reached equilibrium, the MBP concentration was monitored with ultraviolet scanning at 280 and 286 nm for the lower and higher detergent concentrations, respectively. The stoichiometry of the detergent/protein complex was determined by the method of Reynolds and Tanford (1976).

Photon Correlation Laser Light Scattering. The effective hydrodynamic radius of DPC micelles and DPC/MBP complexes was measured at 298 K by photon correlation laser light scattering. The apparatus employs the focused beam of a He–Ne Spectral Physics Type 125, 50-mW laser and detects the 90° scattered light with an E.M.I. Type 9863B/100 photomultiplier. The photocount correlation function measurement was made with a Langley Ford correlator connected to an HP9835A computer with a sample interval of 5–10 μs. Phosphate buffer solutions (0.05 M) at pH 7 were made with deionized ultrafiltered water. Detergent concentrations ranged between 5×10^{-3} and 20×10^{-3} M, and the detergent-to-protein molar ratio was 150:1, to provide sufficient detergent to bind to MBP but to avoid large excesses of detergent micelles without bound protein. Immediately prior to measurements, samples were centrifuged at 5000 rpm for 10 min in a Ti50 Beckman rotor to remove dust particles. Measurements were made within 3–5 minutes after removal of the sample from the centrifuge. The homogeneity in particle size was calculated from the polydispersity index, the normalized variance in the decay rates of the photocount correlation function. The radius and the polydispersity were calculated by cumulants analysis (Koppel, 1972) of the correlation function using a second-order fit.

Electron Paramagnetic Resonance (EPR). EPR measurements were carried out at 298 K with a Varian E4 X-band spectrometer. Rotational correlation times, τ_r, for the spin-labels were calculated from the spectra by (Stone et al., 1965; Cannon et al., 1975)

$$\tau_r = (6.5 \times 10^{-10})\Delta H\left\{\left[\frac{I(0)}{I(+1)}\right]^{1/2} + \left[\frac{I(0)}{I(-1)}\right]^{1/2} - 2\right\}$$

where $I(0)$, $I(+1)$, and $I(-1)$ are the peak-to-peak heights of the 0, +1, and −1 transitions in the first-derivative spectrum and ΔH is the line width (in gauss) of the 0 transition.

Nuclear Magnetic Resonance. Samples were prepared in 0.05 M ^2H_2O phosphate buffer at pH 7. The ^1H NMR spectra at 400 MHz, ^{31}P NMR spectra at 162 MHz, and ^{13}C NMR spectra at 100.5 MHz were obtained on a Bruker WM-400 or a Varian XL-400 spectrometer, operating in the pulsed Fourier transform mode with quadrature detection. Spectra were measured at 298 K over 16K data points. No spectral enhancements were used in the Fourier transformation of free induction decays. Longitudinal relaxation times were measured by the inversion-recovery pulse sequence method and transverse relaxation times by the spin-echo pulse sequence (Carr & Purcell, 1954). The assignments of ^1H and ^{13}C resonances are those of Brown et al. (1981); chemical shifts are given relative to 3-trimethylsilyl [2,2,3,3,-$^2H_4$]propionate at p^2H 7.0.

Electron Microscopy. Samples were negatively stained for electron microscopy by adding 1 drop of detergent or detergent/protein mixture to the appropriate amount of staining solution (1% sodium dodecatungstosilicate) and thoroughly mixing. A drop was placed on a formvar/carbon-coated copper grid, blotted, and allowed to dry. For freeze-fracturing, droplets were frozen in liquid nitrogen cooled Freon 22 before fracturing at 173 K in a Balzer (BAF-300) freeze etch unit. The fracture surface was shadowed at 45° with 2.5 nm Pt/C followed by vertical evaporation of 20 nm of carbon. Replicas were cleaned in Triton X and collected on copper grids for microscopy. Samples were examined in a Philips EM 400 electron microscope operating at 100 kV.

Results

Ultracentrifugation. Ultracentrifugation has been used to determine the stoichiometry of MBP/DPC aggregates (Table I). For comparison Table I also shows data reported previously (Lauterwein et al., 1979) for dodecylphosphocholine

Table I: Stoichiometry and Size of Dodecylphosphocholine/Myelin Basic Protein Complexes and Dodecylphosphocholine Micelles

	exptl method	DPC/MBP	DPC
particle weight	analytical ultracentrifuge	protein: 18 400 ± 2000 detergent: 51 000 ± 7000	19 500 ± 1700[a]
particle diameter	light scattering	20.6 ± 1.0 nm	5.4 ± 0.4 nm
	electron microscopy	25.0 ± 5 nm	4.0 ± 1 nm
stoichiometry	analytical ultracentrifuge	1 ± 0.1 protein molecule 145 ± 20 detergent molecules	56 ± 5 detergent molecules
	EPR	70 ± 5 detergent molecules/micelle	56 ± 5 detergent molecules/micelle

[a] From Lauterwein et al. (1979).

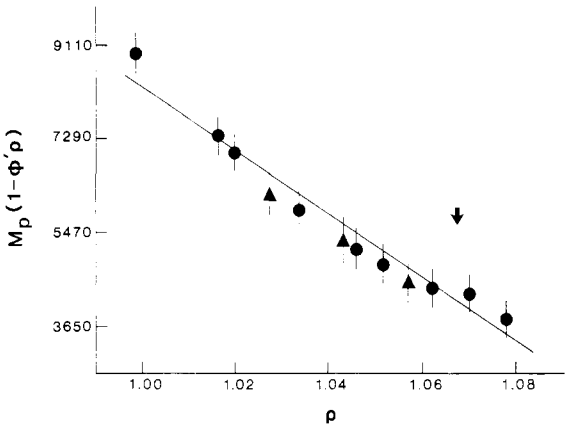


FIGURE 1: Sedimentation equilibrium measurements by the method of Reynolds and Tanford (1976) for a mixture of 14.6×10^{-3} M DPC and 7.3×10^{-5} M MBP in 0.05 M phosphate buffer, pH 7.0 at 20 °C (●). The value $M_p(1 - \phi'\rho)$ is plotted versus the solvent density, ρ, which was altered by mixing H_2O and ^2H_2O in various proportions. M_p is the molecular weight of the protein component of the aggregate, and ϕ' is the volume increment per gram of protein. The arrow indicates the value at which the density of the solvent matches the buoyant density of the detergent, $\rho = 1/\bar{v}$. Measurements for a solution of 73×10^{-3} M DPC and 7.3×10^{-5} M MBP are represented by ▲.

micelles in protein-free solutions. The state of aggregation of MBP bound to dodecylphosphocholine micelles has been determined by equilibrium ultracentrifugation in $H_2O/^2H_2O$ mixtures (Reynolds & Tanford, 1976). At a solvent density that matches the density of the detergent, the apparent molecular weight of the complex is determined by the molecular weight of the protein component. A plot of the apparent value of $M_p(1 - \phi'\rho)$ as a function of solvent density for solutions with a detergent/protein molar ratio of 200:1 is shown in Figure 1. The molecular weight obtained from these data indicates that one molecule of myelin basic protein is incorporated into each aggregate. A relatively inaccurate estimate of the number of detergent molecules associated in the complex can be obtained from the slope of the plot in Figure 1. This yields the value of 145 ± 20 molecules. By comparison, in solutions with detergent only there are 56 ± 5 molecules of dodecylphosphocholine per micelle (Lauterwein et al., 1979). Also shown in Figure 1 are the results of measurements for solutions with a detergent/protein molar ratio of 1000:1. The composition of the aggregates is not significantly altered by the presence of excess lipid.

Light Scattering. The measured effective hydrodynamic radius for detergent micelles was 2.7 ± 0.2 nm. This value compares well with those obtained previously (Brown et al., 1981). The measured radius for the DPC/MBP complex was 10.3 ± 0.5 nm and the polydispersity 0.25. In comparison, a standard sample of monodisperse latex spheres of 42-nm radius yielded a polydispersity of 0.06. Since all samples were treated in a similar way and the rate was above 10 000 counts/s, interference by dust particles in the measurement of the detergent/protein complexes can be ruled out as the source of the calculated polydispersity. The value appears to indicate in this case the presence of particles with different sizes.

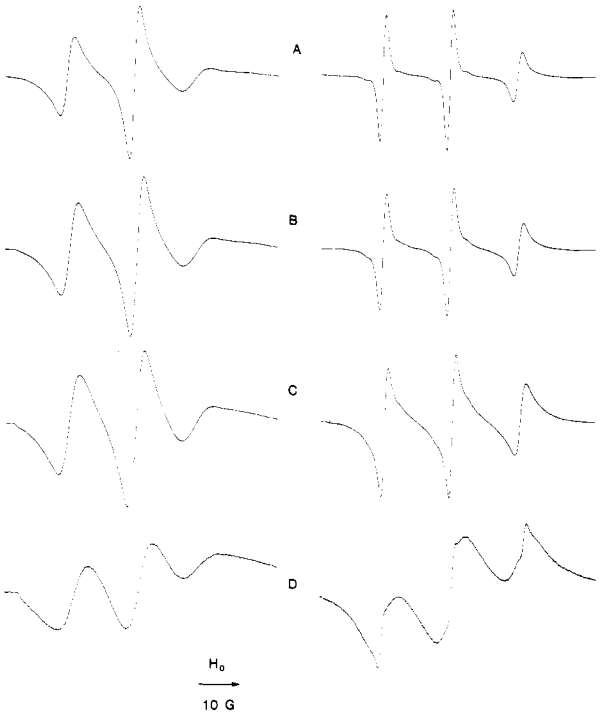


FIGURE 2: X-band EPR spectra for 2×10^{-3} M 5-doxylstearic acid (left) and 16-doxylstearic acid (right) as a function of dodecylphosphocholine/myelin basic protein concentration. Spectra were measured in 0.05 M phosphate buffer at pH 7.0 and 25 °C for DPC concentrations of (A) 0.32, (B) 0.21, (C) 0.05, and (D) 0.02 M. The detergent/protein concentration ratio was 200:1.

EPR Measurements for Spin-Labels Bound to Mixed DPC/MBP Micelles. Figure 2 shows the spectra measured for 2×10^{-3} M 5-doxylstearate (left) and 16-doxylstearate (right) labels as a function of DPC/MBP concentration. The detergent/protein molar ratio is 200:1. At high dodecylphosphocholine concentration (Figure 2A) the spectrum is reasonably sharp. As the detergent concentration is decreased the spectrum remains at first insensitive to the change (Figure 2B). Further decreases in detergent concentration produce a broadening of the spectral lines (Figure 2C,D). The spectra in Figure 2 are interpreted as follows. At low detergent-to-label ratios, micelles are formed that contain more than one spin-label molecule. This leads to broadening of the EPR spectrum through interaction between spin-labels; the degree of broadening depends on the average number of spin-labels per micelle. With increasing detergent concentration the broadening of the spectrum becomes less pronounced. When the detergent concentration is sufficiently high so that, on average, one or less spin-label molecule is bound per micelle, the characteristic spectrum of an isolated spin-label in a milieu provided by the detergent micelle is obtained, and further increases in detergent concentration do not change it (Figure 2A,B).

Table II: Isotropic Hyperfine Splitting Constants (a) and Rotational Correlation Times (τ_r) Measured for Free Spin-Labels Incorporated into Dodecylphosphocholine Micelles and into Dodecylphosphocholine/Myelin Basic Protein Complexes

	1×10^{-4} M spin-label[a]		1×10^{-4} M spin-label, 5×10^{-2} M dodecylPC[a]		2×10^{-3} M spin-label, 3×10^{-1} M dodecylPC, 1.5×10^{-3} M myelin basic protein	
spin-label	a_{14N} (G)	$10^{10}\tau_r$ (s)	a_{14N} (G)	$10^{10}\tau_r$ (s)	a_{14N} (G)	$10^{10}\tau_r$ (s)
5-doxylstearic acid	15.7	1.3	14.8	18	14.9	29.8
12-doxylstearic acid	15.7	1.8	14.8	9.2	14.9	15.0
16-doxylstearic acid	15.7	0.82	14.9	3.5	14.9	6.1

[a] From Brown et al. (1981).

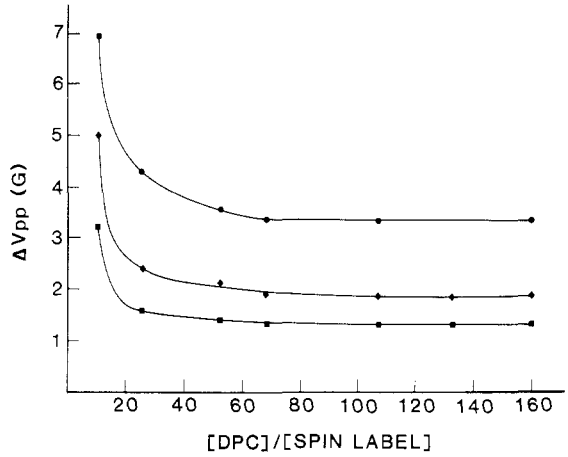


FIGURE 3: Peak-to-peak line width, $\Delta\nu_{pp}$, of the $I = -1$ transition in the X-band spectra of three spin-labels as a function of the ratio of dodecylphosphocholine to spin-label molar concentrations. Spectra were measured in 0.05 M phosphate buffer at pH 7.0 and 25 °C. Titration with a 200:1 mixture of DPC and MBP for 2×10^{-3} M (●) 5-doxylstearate, (◆) 12-doxylstearate, and (■) 16-doxylstearate.

The variation of the peak-to-peak line width of the $I = -1$ transition in the EPR spectra of the three labels as a function of the detergent-to-spin-label ratio in DPC/MBP aggregates is plotted in Figure 3 for detergent/protein mixtures containing approximately 200 detergent molecules per MBP molecule. The high concentrations of detergent (0.02–0.32 M) relative to that of spin-label (2×10^{-3} M) in these experiments ensure that the label is essentially fully bound to the micelles at all detergent concentrations. For each of the three labels the line width of the $I = -1$ transition decreases sharply as the detergent-to-label ratio is increased from 10:1 to approximately 50:1. The line width becomes independent of the detergent-to-spin-label ratio at 65–75 detergent molecules per spin-label, indicating that at this ratio approximately 1 label molecule is bound per micelle. In solutions containing 0.25 M dodecylphosphocholine without protein, the line width becomes independent of the detergent-to-spin-label ratio at approximately 50–60 detergent molecules per spin-label (Brown et al., 1981), supporting and extending previous results which showed that for DPC solutions between 0.02 and 0.1 M there are 50–60 detergent molecules per micelle (Bosch et al., 1980). The present results suggest that the addition of MBP induces only a small change in the size of the micelles. These measurements also provide a direct estimate of the micelle size of the high detergent concentrations used in NMR experiments (see below).

^{14}N hyperfine splittings, a, and rotational correlation times provide further information about the organization of the DPC/MBP complexes. Comparison of the values for free spin-label, DPC micelles, and detergent/protein aggregates shows that there is a decrease of 0.8–0.9 G in the hyperfine splitting when the label is incorporated into DPC micelles (Brown et al., 1981) and of 0.8 G when incorporated into DPC/MBP complexes (Table II). The decrease in the hyperfine coupling constant is indicative of a less polar environment for the nitroxide moiety of the bound spin-label (Griffith & Jost, 1976). The similarity of the splitting in the presence of detergent with or without protein indicates that the local environment of the label is largely unaffected by the presence of MBP. There is a 4–15-fold increase in the rotational correlation time when the spin-label is incorporated into detergent micelles, indicating that the rotational mobility of the nitroxide group is reduced compared to its mobility in the free spin-labels. A 7–23-fold increase in the correlation time is observed when the labels are incorporated into detergent/protein aggregates. The increase in correlation times in detergent micelles in the myelin basic protein/detergent complex indicates a further restriction in the rotational mobility of the label.

Table III: Transverse and Longitudinal Relaxation Times of Protonated Carbon and Phosphorus Nuclei of Dodecylphosphocholine in Solutions with and without Myelin Basic Protein

	transverse relaxation times (T_2) (ms)		longitudinal relaxation times (NT_1) (ms)	
nucleus	DPC	DPC/MBP	DPC	DPC/MBP
C_1	48 ± 5	43 ± 5	7500 ± 280	6800 ± 280
C_2	59 ± 5	57 ± 5	2600 ± 70	2400 ± 70
C_3	43 ± 5	42 ± 5	2030 ± 50	1840 ± 50
C_4–C_9	35–53 ± 4	31–39 ± 4	1120–1260 ± 40	1130–1200 ± 40
C_{10}	42 ± 5	36 ± 5	1060 ± 30	970 ± 30
C_{11}	42 ± 5	30 ± 5	950 ± 30	900 ± 30
$C_{12'14}$	54 ± 5	40 ± 5	1000 ± 20	930 ± 20
C_{13}	64 ± 5	66 ± 5	1000 ± 25	900 ± 25
C_{15}	62 ± 4	62 ± 4	1890 ± 40	1790 ± 40
P	38 ± 1	25 ± 2	3760 ± 50	1120 ± 30

NMR Relaxation Times. Most of the measured spin–lattice relaxation times of the carbon nuclei of dodecylphosphocholine are nearly the same in detergent micelles with and without myelin basic protein. Only small changes are observed in the first three carbon nuclei of the acyl chain (Table III). On the other hand, the formation of detergent/protein aggregates results in the broadening of the group of spectral lines corresponding to carbon nuclei 4–9, 10, 11, and 12 of the acyl chain (Table III, Figure 4).

Both the ^{31}P spin–spin and spin–lattice relaxation times of the phosphate ester group in DPC decrease in detergent/protein complexes relative to those of detergent micelles (Table III).

The locations within the detergent/protein aggregates of the nitroxide moieties of the different spin-labels have been investigated by observing the paramagnetic contributions to the relaxation rates of individually assigned nuclei in the detergent molecules. Figure 5 shows the ^{13}C NMR spectra

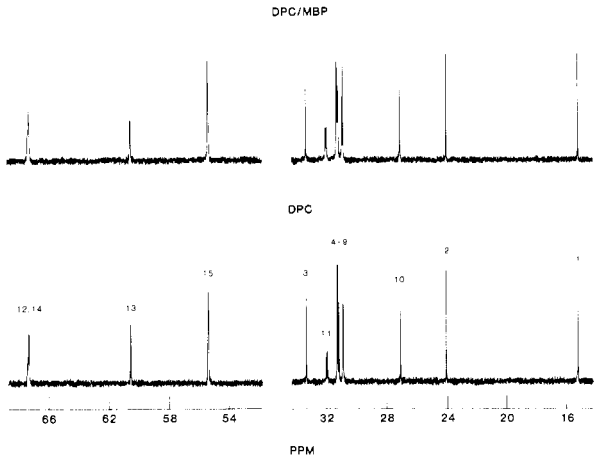


FIGURE 4: 100.5-MHz ^{13}C NMR spectra of 0.075 M dodecylphosphocholine micelles in ^2H_2O 0.05 M phosphate buffer at p^{2}H 7.0 and 30 °C (bottom) and in aggregates with 0.5×10^{-3} M myelin basic protein (top).

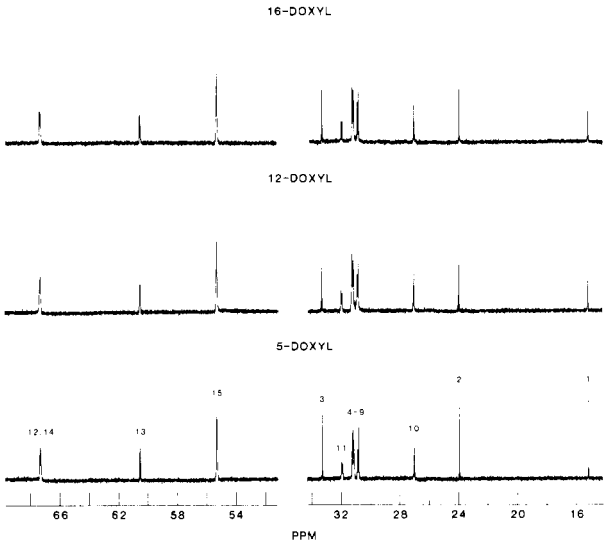


FIGURE 5: Effect of incorporation of spin-labels into dodecylphosphocholine/myelin basic protein complexes. 100.5-MHz ^{13}C NMR spectra of 0.15 M DPC in 150:1 DPC/MBP mixtures in 0.05 M phosphate buffer, p^{2}H 7.0, at 30 °C. Spin-label concentration 0.001 M. From bottom to top (a) 5-doxylstearate, (b) 12-doxylstearate, and (c) 16-doxylstearate.

measured for 0.07 M dodecylphosphocholine in detergent/protein/spin-label mixtures of molar ratios 120:1:0.4. The addition of 12-doxylstearate or 16-doxylstearate results in increased line widths in the detergent ^{13}C spectral lines corresponding to nuclei in positions 1, 2, and 3. The effect is less pronounced in the resonances of successive carbon nuclei in the acyl chain up to position 10. Increased line widths are also observed when 5-doxylstearate is added to the detergent/protein mixture, but in this case the most strongly broadened resonance corresponds to the nucleus in position 10, with noticeable broadening of the lines arising from nuclei in positions 4–9. These results show that the paramagnetic relaxation caused by the different spin-labels is selective for different ^{13}C nuclei of the detergent molecules. The data are qualitatively consistent with localization of the nitroxide labels of 12-doxylstearate and 16-doxylstearate near the center of the micelles and that of the nitroxide label of 5-doxylstearate closer to the phosphate group of the detergent molecule. Comparison of these results with those obtained for DPC micelles (Brown et al., 1981) shows that the presence of myelin basic protein does not appreciably alter the influence of the spin-labels on the detergent ^{13}C resonances and thus suggests

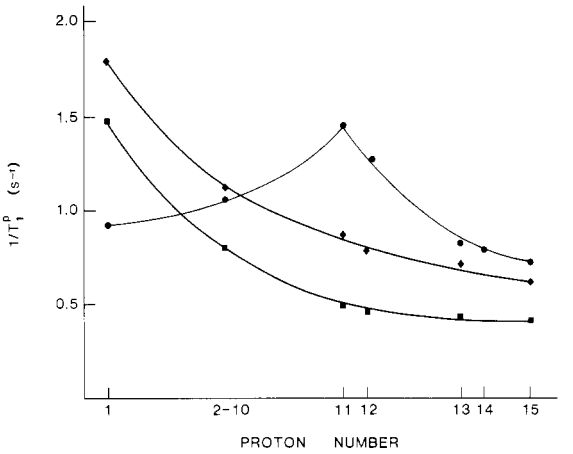


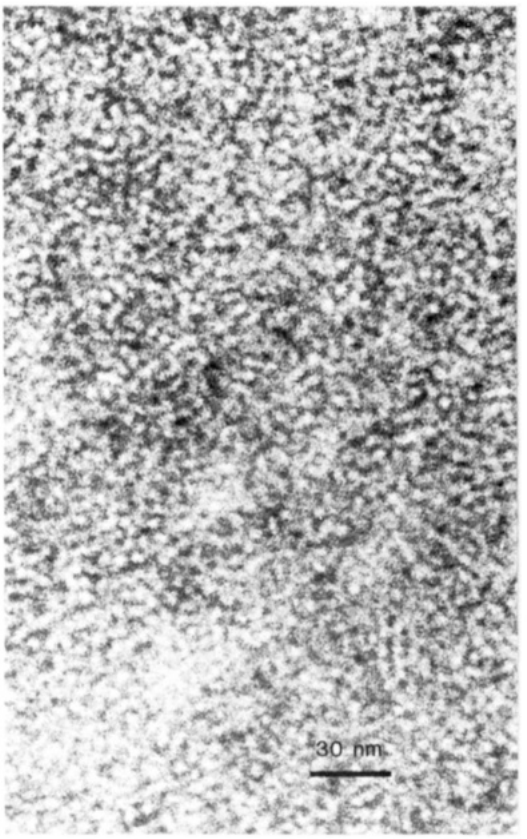
FIGURE 6: Paramagnetic contribution to the ^1H spin–lattice relaxation rates, $1/T_1^P$, for the ^1H resonances of dodecylphosphocholine as a function of nucleus position in the covalent structure of the detergent molecules. Measurements were carried out at 400 MHz in the same mixtures used in Figure 5: (●) 5-doxylstearate; (◆) 12-doxylstearate; and (■) 16-doxylstearate.

that the spatial orientation of the detergent and of the different spin-labels within the micelles is not appreciably changed when MBP is also present in the aggregate.

In the spectrum of dodecylphosphocholine/myelin basic protein complexes the resonances of carbons 12 and 14 of DPC overlap and those of carbons 11–14 show scalar couplings to ^{31}P of several hertz. Consequently, line-width measurements of these resonances are less accurate. To determine more reliably the broadening effects caused by 5-doxylstearate, the selectivity of the paramagnetic effects was also studied by measurement of the ^1H spin–lattice relaxation times. In the absence of spin-label, the proton resonances of DPC have relaxation times between 0.49 and 1.0 s. The selectivity for different protons can be observed in Figure 6, where the paramagnetic contributions to the relaxation rates ($1/T_1$) (the difference in measured relaxation rates with and without the spin-label) are plotted as a function of proton number. For 5-doxylstearate the largest values of $1/T_1^P$ correspond to the methylene groups 11 and 12. This finding is consistent with a location of the nitroxide moiety of this label near the phosphate group of the detergent. Comparison of this result with those given for dodecylphosphocholine micelles shows that the addition of MBP causes little change in the plots of $1/T_1^P$ versus position. This observation provides further evidence that the presence of bound protein causes no major change in the location of the nitroxide label within the micelles.

Analysis of Electron Micrographs. Negatively stained dodecylphosphocholine preparations reveal structures with diameters between 3 and 5 nm that may be interpreted as micelles (Figure 7). Much larger aggregates are observed in electron micrographs of detergent/protein samples prepared by the freeze-fracture technique (Figure 8). The complexes appear in the form of spheroids with diameters estimated between 20 and 30 nm. A characteristic feature observed in negatively stained DPC/MBP samples, and not in samples containing only detergent, is the presence of few very large roughly circular "myelin-like figures" consisting of ordered layers of aggregates (Figure 9) with diameters over 100 nm. The aggregates appear in bright rows alternating with darker regions. The overall pattern is reminiscent of the myelin figures observed in lipid preparations (Sakurai & Kawamura, 1984; Sakurai, 1985).

At a detergent/protein molar ratio of 1000:1 ordered rows of aggregates are also observed, but they do not form the



DPC

FIGURE 7: Electron micrograph of negatively stained dodecylphosphocholine micelles.

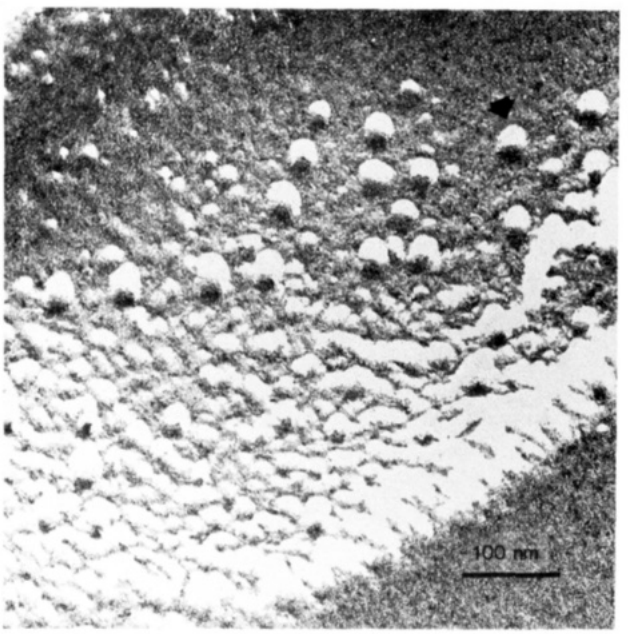


FIGURE 8: Electron micrograph of freeze-fractured sample of a dodecylphosphocholine/myelin basic protein mixture in 0.05 M phosphate buffer at pH 7.0. The arrow indicates a characteristic spheroid of diameter about 30 nm.

roughly circular patterns. These arrays from regions comprising between 5 and 10 rows. The regions lie at angles with respect to one another with no apparent overall order.

Discussion

The previous study of the interaction of myelin basic protein with the detergent dodecylphosphocholine (Mendz et al., 1984)

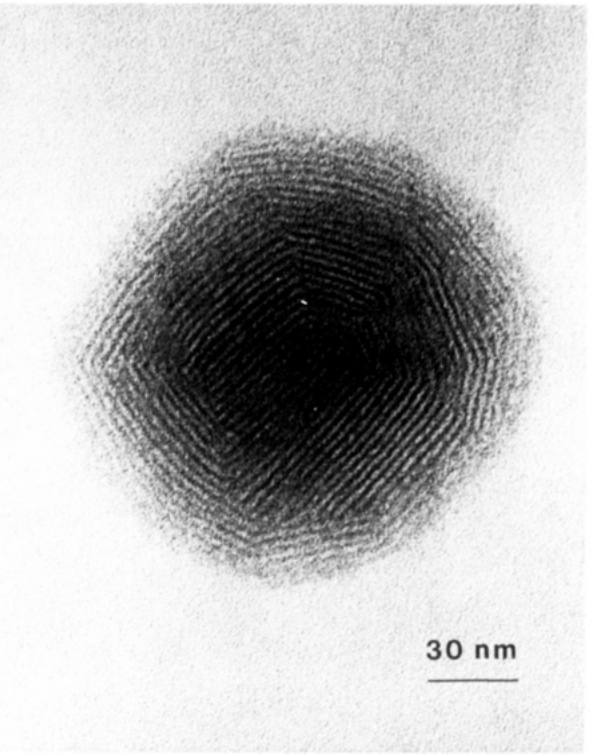


FIGURE 9: Electron micrograph of negatively stained DPC/MBP mixture showing a myelin-like figure. The aggregates appear as bright rows forming regions of parallel arrays.

outlined the effects on the conformation of the protein and established that the interaction occurs at discrete binding sites on the MBP molecule with the micellar form of the detergent. The present study provides direct evidence that the DPC/MBP complex consists of polypeptide chains bound to detergent molecules which retain a micellar organization.

Ultracentrifugation data indicate that myelin basic protein forms a complex of well-defined size and stoichiometry with dodecylphosphocholine micelles. In the presence of MBP, aggregates are formed comprising 1 protein molecule and about 2 detergent micelles, each with a size of 65–75 molecules. Thus, the hexamers observed in aqueous solutions of the protein (Smith, 1985) are no longer present in the association of the protein with the detergent. The data obtained for changes in the ^{1}H NMR spectrum of MBP when DPC is added (Mendz et al., 1984) showed that (i) the changes in chemical shift occur in resonances from residues located at discrete sites in the protein primary structure, (ii) the magnitude of these changes is generally small, and (iii) a number of resonances corresponding to residues at the beginning, middle, and end of the protein sequence remain unchanged. Similarly, the data obtained from circular dichroism measurements indicated the formation of only 19% α-helix. These observations suggest that there is only a limited reorganization in the polypeptide chain structure of the protein to incorporate the detergent.

Ultracentrifugation measurements at a detergent/protein molar ratio of 1000:1 provide information about the way in which the complex is held together. Because the composition of the aggregates is not altered by the presence of excess detergent, it is reasonable to conclude that the stabilizing forces of the complex arise from protein–detergent interactions and that this interaction is very specific.

Light-scattering data show the diameter of these complexes to be about 20 nm. This result is in good agreement with measurements carried out on electron micrographs of de-

tergent/protein mixtures (Figure 8). The complexes appear with good homogeneity in the ultracentrifugation data, but in the light-scattering experiments a measured polydispersity of 0.25 indicates the presence of heterogeneous aggregates. Since heterogeneous size distribution is manifested differently in the two methods, both results can be compatible.

Information about the density of the aggregates is derived from the size of the particle and the amount of material in it. If each protein molecule is considered a prolate ellipsoid with axes 1.5 and 15 nm (Epand et al., 1974), an upper boundary for the volume occupied by a molecule is 26.5 nm^3. The volume of the equivalent sphere calculated from the molecular weight and molar specific volume of MBP is 22.5 nm^3.

An upper limit for the volume occupied by the detergent is obtained by assuming the length of the molecules to be 3.6 nm, corresponding to their fully extended conformation. The volume of 2 DPC micelles is 391 nm^3. On the other hand, the volume of the equivalent sphere calculated from the molecular weight and molar specific volume of DPC gives a lower limit of 79.5 nm^3.

The volume of the equivalent sphere calculated for the DPC/MBP aggregates from the dimensions obtained by light scattering is 4577 nm^3. Consequently, between 3% and 10% of the aggregate particle is occupied by the protein and detergent and the rest by the solvent. The result is a very porous particle indeed. This picture helps one to understand why the line widths of the protein resonances in such a complex remain remarkably sharp and why no part of the protein is protected by the micelles against rapid exchange of its amide groups with the aqueous environment (Mendz et al., 1984).

EPR data indicate that the three spin-labels used are incorporated into the micelles in the complex. NMR (Figures 5 and 6) and EPR data (Table II) establish that the spin-labels cause no appreciable perturbation of the organization of the detergent/protein aggregates. Comparison of these data with measurements on detergent micelles (Brown et al., 1981) indicate that the presence of myelin basic protein has little influence on the environment or location within the micelles of the nitroxide moieties of the micelle-bound spin-labels. Conversely, these results indicate that MBP does not drastically alter the organization of the detergent micelles.

Addition of the protein does not change the ^{13}C longitudinal relaxation times of DPC resonances by more than 10%, a result indicating that the relatively rapid component of the chain motions with correlation times in the order of 10^{-9}–10^{-8} s is not influenced by the protein. Broadening of some ^{13}C NMR lines of the detergent spectrum after the addition of protein (Figure 4) suggests the incorporation of at least segments of the polypeptide into the micelles. The resonances most affected are those corresponding to carbon nuclei in positions 4–12 in the acyl chain covalent structure. The selective decrease of ^{13}C transverse relaxation times suggests that the detergent–protein interaction introduces a new slow motion of the DPC molecules which is capable of modulating the dipolar interactions at certain regions of the chain sufficiently to influence T_2 but which is too slow to affect T_1. The addition of the 5-doxylstearic acid spin-label to detergent solutions (Brown et al., 1981) and to detergent/protein mixtures produces increased broadening of the resonances of carbon nuclei 4–10 (Figure 5) with maximal effect on the protein nuclei at positions 11 and 12 (Figure 6). These results together with the selective broadening of only a few ^1H NMR resonances of the protein spectrum (Mendz et al., 1984) suggest that the polypeptide backbone is mainly outside the micellar surface, with only specific regions of the protein molecule penetrating the micelles. Different segments could be immersed at various depths but mostly in the region just below the interface.

Sixl and co-workers (1984) found that myelin basic protein induces large effects on the ^{31}P and the ^2H NMR spectra of bilayers of dimyristoylphosphatidylglycerol (DMPG) but does not perturb any part of the head groups in dimyristoylphosphatidylcholine (DMPC) bilayers. They ascribed this result to the preferential binding of MBP to the acidic lipids to form protein/lipid complexes as had been previously reported (Boggs & Moscarello, 1978a,b; Boggs et al., 1982). The decrease in longitudinal and transverse relaxation times induced in the phosphorus nucleus of DPC may arise from substantial electrostatic interactions of the protein with the zwitterionic detergent. However, considering the results with DMPC and since we observe no significant changes in the relaxation times of the ^{13}C nuclei in the choline head group in the presence of MBP, the possibility that the perturbation of the phosphorus nucleus relaxation times is due to changes in the shape and/or size of the micelles or the rate of diffusion of detergent molecules within a micelle cannot be ruled out.

In membrane studies several hypothesis have been advanced to explain the nature of the slow motions of the acyl chains induced by lipid–protein interactions: (i) exchange between boundary and bulk lipid sites; (ii) rotation of the protein or relative motion of structured segments; (iii) reorientational motion of lipid molecules while bound to the protein surface; and (iv) change in the average size and/or shape of the vesicles due to the incorporation of proteins (Bloom & Smith, 1975). In the present studies EPR and NMR data provide evidence supporting a model in which the incorporation of the protein induces only a small change in the micelles. The process could be either the formation of different micelles at discrete binding sites in the protein or the attachment of the micelles already present in solution after they have been suitably enlarged and deformed to contour specific regions of the protein.

Electron micrographs of negatively stained DPC/MBP solutions reveal the presence of a few myelin-like figures, i.e., very large arrays of detergent micelles (Figure 9), which are not observed in solutions containing only DPC. These arrays were not present in sufficient numbers, typically one or less per sample examined, to have an effect on the ultracentrifugation or light-scattering measurements if they are present in suspensions, but their frequency probably indicates that they are not just artifacts produced by uneven drying of the plates. These were observed like isolated patches (blobs) in which aggregates did not show any particular organization. Myelin figures have been previously reported as an effect of phospholipid organization (Sakurai, 1985). In the present case these large arrays probably suggest the ability of MBP to induce supraggregate structures, but whether they occur in the suspensions or during drying of the plates cannot be defined.

The well-defined stoichiometry of MBP/DPC complexes even in the presence of excess detergent suggests that these complexes are based on quite specific protein–detergent interactions. This is consistent with previous results (Mendz et al., 1984) indicating that only limited and quite distinct regions of the MBP amino acid sequence interact with lipids and with the present evidence that distinct micelles are retained within the MBP/DPC complex.

ACKNOWLEDGMENTS

We are grateful to Dr. G. C. Fletcher for the use of the light-scattering apparatus at the School of Mathematics and Physics, Macquarie University, and to Dr. G. B. Ralston,

Department of Biochemistry, University of Sydney, for the ultracentrifuge runs.

Registry No. DPC, 29557-51-5.

REFERENCES

Bloom, M., & Smith, I. C. P. (1985) in *Progress in Protein–Lipid Interactions* (Watts, A., & De Pont, R., Eds.) Chapter 2, Elsevier, Amsterdam.

Boggs, J. M., & Moscarello, M. A. (1978a) *J. Membr. Biol. 39*, 75–96.

Boggs, J. M., & Moscarello, M. A. (1978b) *Biochim. Biophys. Acta 515*, 1–21.

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

Bösch, C., Brown, L. R., & Wüthrich, K. (1980) *Biochim. Biophys. Acta 603*, 298–312.

Brown, L. R. (1979) *Biochim. Biophys. Acta 557*, 135–148.

Brown, L. R., Bösch, C., & Wüthrich, K. (1981) *Biochim. Biophys. Acta 642*, 296–312.

Cannon, B., Polnaszek, C. F., Butler, K. W., Eriksson, L. E. G., & Smith, I. C. P. (1975) *Arch. Biochem. Biophys. 267*, 505–518.

Carr, H. Y., & Purcell, E. M. (1954) *Phys. Rev. 94*, 630.

Epand, R. M., Moscarello, M. A., Ziereuberg, B., & Vail, W. J. (1974) *Biochemistry 13*, 1264–1267.

Eylar, E. H., Salk, J., Beveridge, G. L., & Brown, L. V. (1969) *Arch. Biochem. Biophys. 132*, 34–48.

Griffith, O. H., & Jost, P. C. (1976) in *Spin Labelling—Theory and Applications* (Berliner, L. J., Ed.) Chapter 12, Academic, New York.

Koppel, D. E. (1972) *J. Chem. Phys. 57*, 4814–4820.

Lauterwein, J., Bösch, C., Brown, L. R., & Wüthrich, K. (1979) *Biochim. Biophys. Acta 556*, 244–264.

Mendz, G. L., Moore, W. J., Brown, L. R., & Martenson, R. E. (1984) *Biochemistry 23*, 6041–6046.

Reynolds, J. A., & Tanford, C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4467–4470.

Sakurai, I. (1985) *Biochim. Biophys. Acta 815*, 149–152.

Sakurai, I., & Kawamura, Y. (1984) *Biochim. Biophys. Acta 777*, 347–351.

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

Smith, R. (1985) *Febs Lett. 183*, 331–334.

Stone, R. J., Buckman, T., Nordio, P. L., & McConnell, H. M. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 1010–1017.

Fluorescent Probes of Electrostatic Potential 1 nm from the Membrane Surface†

Anthony P. Winiski,‡ Moises Eisenberg,§ Marek Langner,∥ and Stuart McLaughlin*,∥

Departments of Biochemistry, Pharmacological Sciences, and Physiology and Biophysics, State University of New York, Stony Brook, New York 11794

Received May 20, 1987; Revised Manuscript Received August 18, 1987

ABSTRACT: We measured the electrostatic potential 1 nm from the surface of charged phospholipid bilayer membranes to test the predictions of the Gouy–Chapman theory. Fluorescent probes (anthraniloyl, 5-(dimethylamino)naphthalene-1-sulfonyl, Lucifer yellow) were attached covalently to the sialic acid residue of the ganglioside galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide (G_{M1}). These fluorescent gangliosides were incorporated into neutral [phosphatidylcholine (PC)] or charged [phosphatidylserine (PS)] phospholipid bilayers, and the fluorescence was quenched with the cations thallium and 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (tempamine). We calculated the electrostatic potential at the chromophore from the quenching ratio using the Boltzmann relation: the average potential was −30 mV for PS bilayers in 0.1 M $NaNO_3$. We assume the chromophore is 1 nm from the surface because X-ray diffraction measurements demonstrate that the sialic acid residue of G_{M1} is 1 nm from the surface of a PC/G_{M1} bilayer [McDaniel, R. V., & McIntosh, T. J. (1986) *Biophys. J. 49*, 94–96]. We also used thallium and tempamine to quench the fluorescence of chromophores located at the surface of the PS membranes; in 0.1 M $NaNO_3$ the average surface potential was −80 mV, which agrees with other measurements. The Gouy–Chapman theory predicts that the potential 1 nm from a membrane with a surface potential of −80 mV is −24 mV; this prediction agrees qualitatively with the experimental results obtained with fluorescent gangliosides.

The Gouy–Chapman theory describes how the electrostatic potential in the aqueous phase at the surface of a bilayer membrane, ψ_0, should depend on the surface charge density, σ, and monovalent salt concentration, C. When ψ_0 is small

$$\psi_0 = \sigma/(\epsilon_r \epsilon_0 \kappa) \quad (1)$$

where ϵ_r is the dielectric constant of the aqueous solution, ϵ_0 is the permittivity of free space, and $1/\kappa$ is the Debye length, which is proportional to $C^{-1/2}$ and equal to 1 nm for $C = 0.1$ M. Several different experimental techniques demonstrate the Gouy–Chapman theory describes adequately the dependence of ψ_0 on σ and C (Hartley & Roe, 1940; Davies, 1951; McLaughlin, 1977; Eisenberg et al., 1979; Cafiso & Hubbell, 1981; Hartsel & Cafiso, 1986; Winiski et al., 1986).

† This work was supported by National Science Foundation Grant BNS 85-01456, National Institutes of Health Grant GM 24971, and Anna Fuller Foundation Fellowship 667.

‡ Department of Biochemistry.

§ Department of Pharmacological Sciences.

∥ Department of Physiology and Biophysics.

0006-2960/88/0427-0386$01.50/0