Physicochemical Characterization of Dodecylphosphocholine/Palmitoyllysophosphatidic Acid/Myelin Basic Protein Complexes†

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ABSTRACT: The stoichiometry of dodecylphosphocholine/palmitoyllysophosphatidic acid/myelin basic protein complexes and the location of the protein in the micelles have been investigated by electron paramagnetic resonance, ultracentrifugation, small-angle X-ray scattering, ^{31}P, ^{13}C, and ^{1}H nuclear magnetic resonance spectroscopy, and electron microscopy. Ultracentrifugation measurements indicated that well-defined complexes are formed by association of one protein molecule with approximately 133 detergent molecules. The spin-labels 5-, 12-, and 16-doxylstearate have been incorporated into detergent/protein aggregates. Electron paramagnetic resonance spectral parameters and ^{13}C and ^{1}H nuclear magnetic resonance relaxation times showed that the addition of myelin basic protein does not affect the environment and location of the labels or the organization of the micelles. Previous results suggesting that the protein lies primarily near the surface of the micelles have been confirmed by comparing ^{13}C spectra of the detergents with and without protein with spectra of detergent/protein aggregates containing the spin labels. Electron micrographs of the complexes taken by using the freeze–fracture technique revealed the presence of particles with an estimated radius about three times the radius of the micelles measured by small-angle X-ray scattering. The structural integrity of the complexes appears to be based on intramolecular protein interactions as well as protein–detergent interactions.

The severe myelin deficiency in the central nervous system of the mutant shiverer mouse that accompanies the low level of myelin basic protein (MBP) expression (Dupouey et al., 1979) showed that investigations of the conformation and location of MBP bound to lipid bilayers would be relevant to the understanding of the molecular architecture of myelin. The ratio of neutral to negatively charged lipids in the myelin membrane of the central nervous system is approximately 4:1 (Kirschner et al., 1984). This relative abundance of charged lipids together with the basic character of MBP suggested the importance of studying the association of the protein with mixtures of neutral and anionic lipids. The effects of myelin basic protein on the aggregation (Fraser et al., 1986), fusion (Lampe & Nelsesteuen, 1982; Lampe et al., 1983), and permeabilization (Cheifetz et al., 1985) of mixed lipid vesicles have been studied, but practically no new information has been obtained about the conformation of the bound protein and its location relative to the bilayer (Deber et al., 1978; Sixl et al., 1984), other than the clarification of the possible role of the charge heterogeneity of MBP (Moscarello et al., 1986).

The structure of biological membranes is not modeled by detergent micelles as closely as by lipid vesicles, but, for proteins that bind at hydrophobic–hydrophilic interfaces such as MBP, micelles may provide a suitable system to study the conformations adopted by the protein when it binds neutral and charged amphiphiles, the type of forces involved in these interactions, and the location of the protein relative to mixed detergent–water interfaces.

An investigation on the interactions of myelin basic protein and peptides derived from it with dodecylphosphocholine/palmitoyllysophosphatidic acid micelles helped to outline (i) the conformational changes induced in the protein by association with the mixed detergent micelles, (ii) the presence of discrete binding sites in the polypeptide chain that are at least partially different for neutral and charged detergents, and (iii) the role of hydrophobic and ionic forces in the interactions (Mendz et al., 1990). The present work has provided a more complete understanding of the physicochemical properties of the mixed detergent/protein complexes by yielding detailed information about their stoichiometry, size, and organization.

Materials and Methods

Dodecylphosphocholine (DPC) was synthesized by the method described by Brown (1979). Palmitoyllysophosphatidic acid (LPAP) was obtained from Serdary Research Laboratories Inc. (London, Ontario). Rabbit MBP was prepared according to the method of Law et al. (1984). Porcine MBP was kindly provided by Dr. Max Marsh of Eli Lilly & Co. The fatty acid spin labels 5-doxylstearate [2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinyloxy], 12-doxylstearate, and 16-doxylstearate were purchased from Aldrich Chemical Co. (Milwaukee, WI).

The proteins were lyophilized twice from 99.5% $^{2}H_2O$ (Australian Institute for Nuclear Science and Engineering, Lucas Heights, NSW) and dissovled in 99.6% $^{2}H_2O$ (Merck,

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Sharp & Dohme) at concentrations of 0.5–1.0 mM in 5-mm o.d. precision NMR tubes (Wilmad, Buena, NJ). 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/LPAP mixtures were added to the protein solutions to obtain a series of detergent/protein molar ratios from 0 to 250.

Samples containing spin labels were prepared by adding a measured amount of free radical in ethanol to DPC/LPAP/MBP aqueous suspensions 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 12.5×10^{-3} M DPC/LPAP detergent mixtures at a molar ratio of 4:1, and 5×10^{-5} M rabbit myelin basic protein. Mixtures of detergents in buffer similar to the ones in the sample compartments were placed in the reference compartments of the cells. 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 20000 rpm for typically 24 h. After the mixtures reached equilibrium, the MBP concentration was monitored with ultraviolet light at 280 nm. The stoichiometry of the detergent/protein complex was determined by the method of Reynolds and Tanford (1976).

Electron Paramagnetic Resonance (EPR). Samples containing 2×10^{-3} M spin labels and 4:1 DPC/LPAP detergent mixtures at concentrations ranging from 5 to 180×10^{-3} M, with or without bovine MBP at a detergent/protein molar ratio of 180:1, were prepared in 0.05 M ^2H_2O phosphate buffer, pH 7.0, and placed in capillary tubes. 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)

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

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.

Small-Angle X-ray Scattering (SAXS). DPC/LPAP detergent mixtures at concentrations of 160:40 ($\times 10^{-3}$) M were prepared in 0.05 M phosphate buffer, pH 7.0. Suspensions including the protein were prepared by adding 1×10^{-3} M porcine MBP. The samples were allowed to equilibrate for over 24 h. Measurements were carried out with the high-resolution SAXS camera at the Research School of Chemistry, The Australian National University (Aldissi et al., 1988). The instrument employs 0.154-nm X-rays and has a 1-m focal length; wavelength selection and focussing of X-rays are achieved by a bent quartz crystal. Owing to the very low contrast between the detergent micelles and the background water, 0.03×10^{11} cm^{-2}, the desired higher intensities of the beam were obtained by not employing focussing in the vertical direction (i.e., not using a full Huxley–Holmes geometry), which was not needed for these experiments. The small-angle scattering patterns were recorded on a one-dimensional position-sensitive detector (Gabriel, 1977). Data were processed by using the ORL-ANU program suite, and all the scattering patterns were recorded on an absolute intensity scale calibrated with a 1-mm water cell. Scattering length densities were calculated in the usual way (Jacrot, 1976). Spectra are plotted as different functions of the momentum transfer Q, with

$$Q = (4\pi \sin \theta)/\lambda \quad (2)$$

where θ is the half-scattering angle and λ the wavelength of the X-rays.

Nuclear Magnetic Resonance (NMR). Samples were prepared in 0.05 M ^2H_2O phosphate buffer, pH 7.0. The ^1H NMR spectra at 400 MHz, ^{31}P NMR spectra at 162 MHz, and ^{13}C NMR spectra at 100.5 MHz were measured on a Bruker WM-400 or a Varian XL-400 spectrometer, operating in the pulsed Fourier transform mode with quadrature detection. ^1H NMR spectra of the detergents were measured at 298 K over 16K data points, acquiring 16 transients with radio-frequency pulses of 90° (7–15 μs), a spectral width of 2053 Hz, and a repetition time of 17 s. No spectral enhancements were employed in the Fourier transformation of these free induction decays. ^{13}C NMR spectra were acquired at 300 K over 16K data points. Spectra of the mixtures of detergents and spin labels, with or without the protein, were acquired by accumulating 12000–18000 transients and using a radio-frequency pulse of 75° (17 μs), with a spectral width of 6920 Hz and a repetition time of 10 s. Relaxation measurements of the ^{13}C resonances of DPC in detergent mixtures, with or without MBP, were performed at 300 K by averaging 256 transients over 16K data points per spectrum, with spectral width of 7485 Hz, radio-frequency pulses of 90° (22 μs), and a repetition time of 15 s. Free induction decays were transformed by employing a line broadening of 3 Hz. ^{31}P NMR spectra of DPC/LPAP mixtures, with or without MBP, were acquired at 300 K, by averaging 56 transients over 16K data points. A radio-frequency pulse of 90° (31.2 μs) was used, with a spectral width of 3065 Hz and a repetition times of 22 s. Free induction decays were transformed with a line broadening of 1 Hz. Longitudinal relaxation times (T_1) were measured by the inversion–recovery pulse sequence (Vold et al., 1968), with delay times of 0.001–15 s for ^{13}C resonances and 0.001–20 s for ^{31}P resonances. Transverse relaxation times (T_2) were measured with the spin-echo pulse sequence (Carr & Purcell, 1954), with delay times of 0.004–0.72 s for ^{13}C resonances and 0.004–0.068 s for ^{31}P resonances. In the relaxation experiments, arrays of 18 spectra were collected with interleaved acquisitions for each experiment. T_1 and T_2 values were calculated by nonlinear regression analysis (Miller, 1981). 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 prepared by freezing droplets in liquid nitrogen cooled Freon 22 and fractured 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 400 kV.

Results

Ultracentrifugation. Ultracentrifugation was used to determine the stoichiometry of DPC/LPAP/MBP aggregates (Table I) and to obtain information on their homogeneity. For comparison, Table I also shows data reported previously for DPC micelles in protein-free solutions (Lauterwein et al., 1979; Mendz et al., 1988). The state of aggregation of MBP bound to DPC/LPAP mixed micelles was determined by equilibrium centrifugation in $H_2O/^2H_2O$ mixtures (Reynolds & Tanford, 1976). At a solvent density that matches the density of the detergent mixture, the apparent molecuar 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

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

	exptl method	DPC/LPAP/MBP	DPC
particle weight	analytical ultracentrifuge	protein, 18 400 ± 2000	
		detergent, 49 300 ● 5500	detergent, 19 500 ● 1700[a]
particle diameter	SAXS	7.4 ± 1 nm	
	light scattering		5.4 ± 0.4 nm[b]
	electron microscopy	25.0 ± 5 nm	4.0 ± 1 nm[b]
stoichiometry	analytical ultracentrifuge	1 ± 0.1 protein molecule 133 ± 20 detergent molecules	56 ± 5 detergent molecules[a]
	EPR	70 ± 5 detergent molecules/micelle	56 ± 5 detergent molecules/micelle[a]

[a] From Lauterwein et al. (1979). [b] From Mendz et al. (1988).

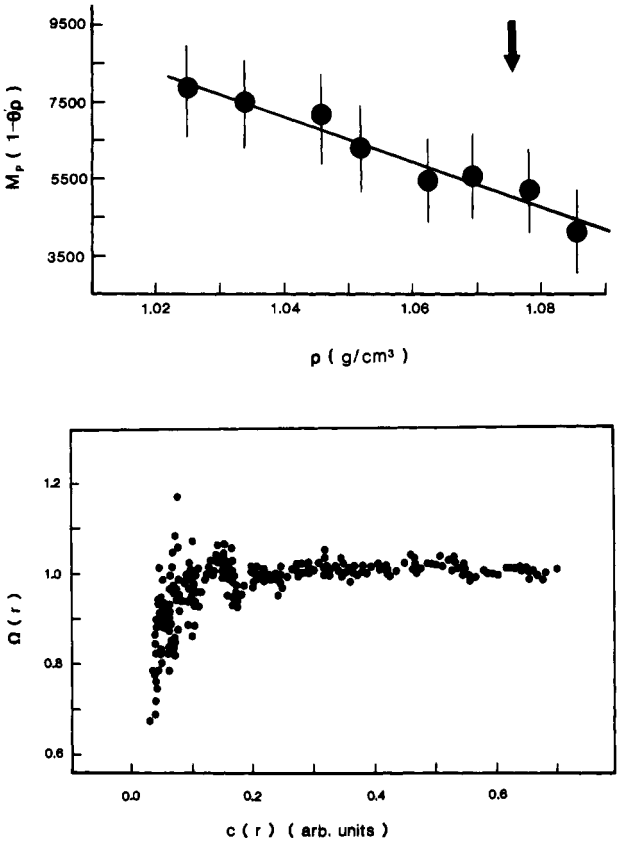

FIGURE 1: (Top) Sedimentation equilibrium measurements by the method of Reynolds and Tanford (1976) for a mixture of 12.5×10^{-3} M DPC/LPAP (4:1 molar ratio) and 5×10^{-5} M rabbit MBP in 0.05 M phosphate buffer, pH 7.0 at 293 K. The value $M_p(1 - \phi'\rho)$ is plotted versus the solvent density ρ, 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 apparent weight of the aggregate corresponds to that of the protein. (Bottom) Plot of experimentally determined values of the dimensionless function $\Omega(r)$ versus the total protein concentration in the cell.

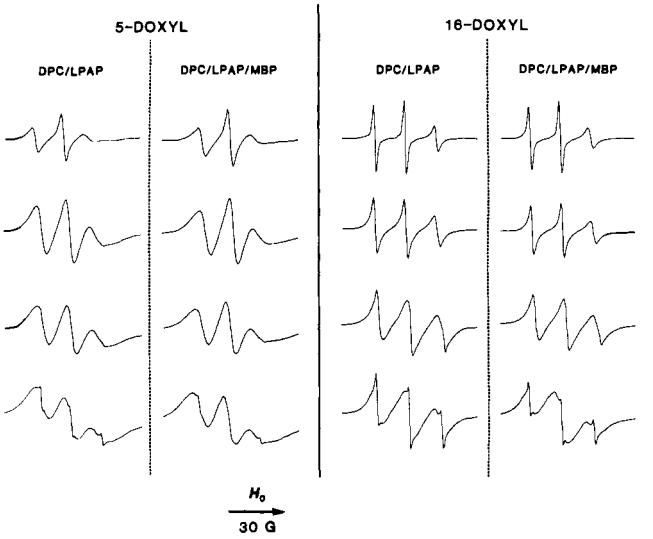

FIGURE 2: X-band EPR spectra for 2×10^{-3} M 5-doxylstearic acid (left) and 16-doxylstearic acid (right) as a function of DPC/LPAP and DPC/LPAL/MBP concentrations. Spectra were measured in 0.05 M phosphate buffer at pH 7.0 and 293 K for DPC/LPAP concentrations of 160:40, 40:10, 20:5, and 8:2 ($\times 10^{-3}$) M from top to bottom, respectively, and DPC/LPAP/MBP concentrations of 160:40:1, 40:10:0.25, 20:5:0.125, and 8:2:0.005 ($\times 10^{-3}$) M from top to bottom, respectively.

a function of solvent density for solutions with a DPC/LPAP molar ratio of 4:1 and a detergent/protein molar ratio of 250:1 is shown in Figure 1 (top). Since the density of the detergent mixture employed has not been established, the stoichiometry of the complex cannot be calculated by direct application of the method. However, the measured values of the apparent molecular weight of the complex in different solvent mixtures shows that, for molecules of MBP with a mass of 18.4 kDa, only the value of the protein monomer would fit the data. The arrow in Figure 1 indicates the solvent density, 1.07586 g·cm^{-3}, at which the apparent molecular weight of the complex is that of the MBP monomer. The corresponding value of the specific volume of the detergent mixture is 0.929 cm^3·g^{-1}, which is in the range of values obtained for detergent micelles. Assuming that the molar ratio of DPC and LPAP in the aggregates is that of the detergent mixture, the slope of the plot in Figure 1 (top) yields a relatively inaccurate estimate of 106 ± 16 DPC and 27 ± 4 LPAP molecules in the complex. By comparison, in DPC/MBP suspensions there are 145 ± 20 detergent molecules per complex (Mendz et al., 1988), and in solutions of only DPC there are 56 ± 5 molecules per micelle (Lauterwein et al., 1979). Since these calculations assumed the homogeneity of the DPC/LPAP/MBP aggregates, it became necessary to establish the validity of this hypothesis. Milthorpe and co-workers (1975) introduced a function $\Omega(r)$ of the activity of the monomer at different positions of the radial distance r in the ultracentrifugation cell, as a means of fitting reaction models to sedimentation equilibrium data obtained with self-associating solutes. The $\Omega(r)$ function provides a very sensitive test to determine the homogeneity of a sample. Figure 1 (bottom) shows a typical plot of experimentally determined values of Ω as a function of the protein concentration in the cell, $c(r)$, for one of the ultracentrifugation runs. The value 1 for $\Omega(r)$ indicated a homogeneous distribution of the protein in the sample.

EPR Measurements for Spin-Labels Bound to Mixed DPC/LPAP/MBP Micelles. Spectra of 2×10^{-3} M 5-doxylstearate and 16-doxylstearate spin labels measured in detergent and detergent–protein suspensions are shown in Figure 2. Three lines were recorded in the first-derivative spectra corresponding to the $I = +1$, 0, and -1 electronic

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

	1×10^{-4} M spin label[a]		2×10^{-3} M spin label; 18×10^{-2} M detergent		2×10^{-3} M spin label; 18×10^{-2} M detergent; 1×10^{-3} M protein	
	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	13.0	24.7	13.0	28.7
12-doxylstearic acid	15.7	1.8	13.0	14.9	13.0	21.3
16-doxylstearic acid	15.7	0.82	13.0	5.8	13.0	8.1

[a] From Brown et al. (1981)

transitions. The detergent/spin label molar ratios in the samples decreased from the top row to the bottom row of spectra in Figure 2. At high relative detergent concentrations (top row), the spectra arising from label molecules in the micelles are reasonably sharp, indicating that one or less spin label molecule is bound per micelle. As the detergent concentration was decreased relative to the label concentration, micelles were formed that contained more that one spin label molecule; the interactions between them resulted in broadening of the spectral lines. At very low detergent/spin label molar ratios, superpositions of broad and narrow spectra were observed, corresponding to label molecules in the micelles and free in solution, respectively. The variation of the peak-to-peak line width of the $I = -1$ transition in the EPR spectra of the 5-, 12-, and 16-doxylstearate labels as a function of the detergent/spin/label ratio in DPC/LPAP solutions and DPC/LPAL/MBP suspensions are plotted in Figure 3. For the bound labels, the line widths of this transition decreased sharply as the detergent/label ratio was increased from 5:1 to approximately 50:1. For the three labels, the line widths became independent of the detergent/spin label ratio at approximately 65 detergent molecules per spin label in DPC/LPAP solutions and DPC/LPAP/MBP suspensions, indicating that at this ratio approximately 1 label molecule is bound per micelle. The results suggested that the addition of MBP did not induce any significant change in the size of the DPC/LPAP micelles. These measurements also provided a direct estimate of the micelle size at the high detergent concentrations used in the SAXS and NMR experiments.

^{14}N hyperfine splittings, a, and rotational correlation times, τ_r, provided further information about the organization of the DPC/LPAP/MBP complexes. Comparison of the α_{14N} values for free spin label, for DPC/LPAP micelles, and for DPC/LPAP/MBP aggregates showed a decrease of 2.7 G in the hyperfine splitting when the labels were incorparated into DPC/LPAP micelles or DPC/LPAP/MBP complexes (Table II). The decrease in a_{14N} is indicative of a less polar environment for the nitroxide moiety of the bound spin label (Griffith & Jost, 1976). The comparable values of the splitting in the presence of detergent with or without protein indicated that the local environment of the label is largely unaffected by the presence of MBP. These results were similar to those found for DPC micelles and DPC/MBP complexes (Mendz et al., 1988). The rotational correlation time increased 7–9-fold when the spin labels were 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 9–22-fold increase in the correlation time was observed when the labels were incorporated into the DPC/LPAP/MBP aggregates. This increase in the correlation times in the detergent/protein complexes indicated a further restriction in the rotational mobility of the label.

The picture suggested by the combined results of the ultracentrifugation and EPR measurements is that detergent/protein aggregates are formed by attachment of two DPC/LPAP micelles to MBP molecules, similarly to the situation found for DPC/MBP complexes (Mendz et al., 1988).

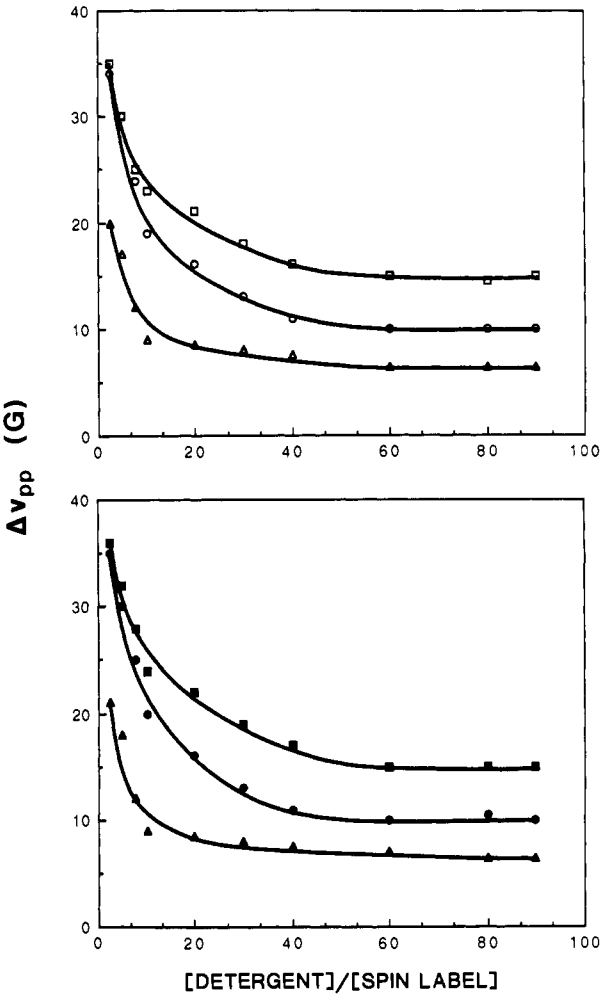

FIGURE 3: Peak-to-peak linewidth, $\Delta\nu_{pp}$, of the $I = -1$ transition in the X-band spectra of three spin labels as a function of the ratio of detergent to spin label molar concentrations. Spectra were measured in 0.05 M phosphate buffer at pH 7 and 293 K. (Top) Titration with a 160:40 mixture of DPC/LPAP for 2×10^{-3} M (□) 5-doxylstearate, (○) 12-doxylstearate, and (△) 16-doxylstearate. (Bottom) Titration with a 160:40:1 mixture of DPC/LPAP/MBP for 2×10^{-3} M (■) 5-doxylstearate, (●) 12-doxylstearate, and (▲) 16-doxylstearate.

Small-Angle X-ray Scattering. After subtraction of the water scattering spectrum, the scattering functions of the detergent mixtures with or without MBP are very similar. There is a broad peak at $Q = 0.015$ nm^{-1}, which in all probability arises from diffraction, since there is a second broad, weak feature at twice this Q value. The results indicated that at these concentrations, the aggregates are close enough to give an interference pattern, suggesting a certain degree of ordering in the suspensions or the presence of larger aggregates. The

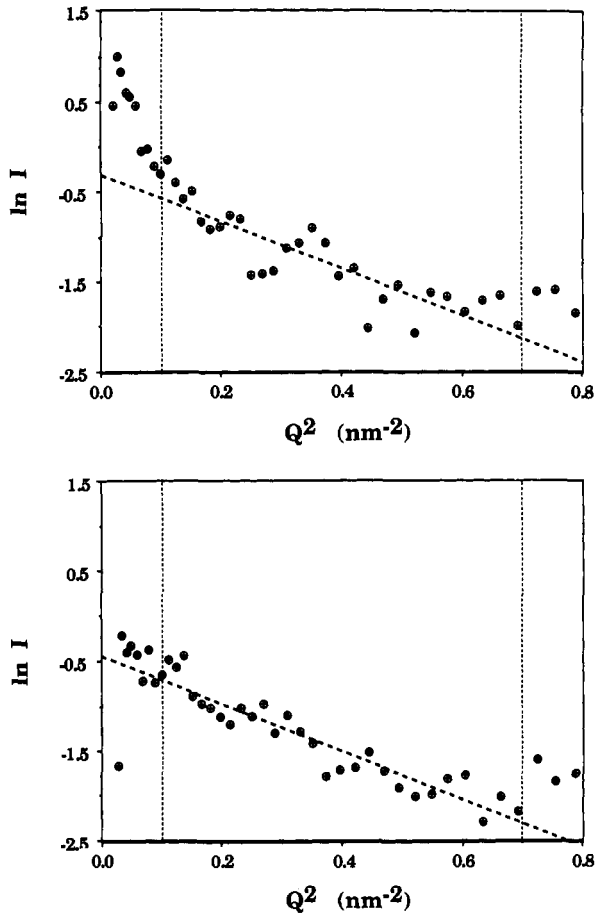

FIGURE 4: Guinier plots, showing the logarithm of the intensity I as a function of the square of the momentum transfer Q, for a 160:40 ($\times 10^{-3}$ M) DPC/LPAP detergent mixture (top) and for a similar mixture of detergents with 1×10^{-3} M porcine MBP added (bottom) prepared in 0.05 M phosphate buffer at pH 7.0 and 293 K. The plots show the limits of the scattering data employed for the Guinier approximation and the regression lines obtained in these analyses. The QR_g values were 0.882 and 0.855 for the top and bottom scattering plots, respectively.

calculated lattice parameter was 4.2 nm, which corresponds to a 4.8-nm center-to-center distance, assuming hexagonal close packing. In ln I versus Q^2 plots, the scattering showed at low Q values a reasonably linear region for detergent/protein suspensions and a less good correlation for the detergent suspensions (Figure 4). The data in these regions were analyzed by using the Guinier approximation (Porod, 1982), where QR_g values were less than 1.0. The aggregates in the suspension containing MBP appeared to have a well-defined size with a radius of gyration (R_g) of approximately 2.8 nm. The size of the micelles in the solution containing only the detergents was more varied, with a range of R_g values centered at about 2.7 nm.

The results of EPR measurements suggested that the detergent micelles are made up of approximately 65–75 molecules, with a molecular mass of 24.6 kDa. Assuming the micelles to be spherical, a radius of gyration of 2.7 nm corresponds to a sphere with a radius of 3.5 nm, giving an upper limit of 180 nm^3 for its volume. A lower limit calculated from the molecular weights of DPC and LPAP and from the specific volume obtained from the ultracentrifugation measurements yields a value of 38.1 nm^3. The combined results of the ultracentrifugation and EPR measurements suggested that the aggregates are composed by attachment of two DPC/LPAP micelles to an MBP molecule. Considering the protein as a prolate ellipsoid of axes 1.5 and 15 nm (Epand et al., 1974),

Table III: ^{13}C and ^{31}P Transverse and Longitudinal Relaxation Times of Protonated Carbon and Phosphorus Nuclei of Dodecylphosphocholine/Palmitoyllysophosphatidic Acid Suspensions with and without Myelin Basic Protein

	transverse relaxation time (T_2) (ms)		longitudinal relaxation time (T_1) (ms)	
nucleus	DPC/LPAP	DPC/LPAP/MBP	DPC/LPAP	DPC/LPAP/MBP
C_1	22 ± 3	20 ± 4	2650 ± 800	2642 ± 1200
C_2	29 ± 4	30 ± 3	1427 ± 300	1316 ± 500
C_3	31 ± 5	56 ± 7	824 ± 90	995 ± 100
C_4–C_8	39–41 ± 5	35–49 ± 6	596–643 ± 40	594–621 ± 40
C_9	40 ± 5	41 ± 5	643 ± 45	621 ± 30
C_{10}	33 ± 2	32 ± 3	520 ± 25	520 ± 25
C_{11}	32 ± 6	44 ± 7	502 ± 40	464 ± 30
C_{12}	16 ± 3	21 ± 3	482 ± 15	503 ± 15
C_{13}	21 ± 2	23 ± 2	503 ± 20	490 ± 20
C_{14}	16 ± 3	21 ± 3	482 ± 15	503 ± 15
C_{15}	22 ● 1	21 ± 2	637 ± 30	612 ± 30
P	19 ± 3	5 ± 1	1449 ± 100	550 ± 90

an upper boundary for the volume occupied by a molecule is 26.5 nm^3. A lower limit for the volume of the protein is 22.5 nm^3, the value corresponding to the volume of the equivalent sphere calculated from the molecular weight and molar specific volume of MBP. An upper limit for the volume of two mixed detergent micelles would be 360 nm^3 and a lower limit 76.2 nm^3. Thus, the volume of the aggregates would be between 98.6 and 387 nm^3. The observed radius of gyration of DPC/LPAP/MBP complexes was 2.8 nm, with a radius of 3.7 nm for a spherical micelle, but if the complex of molecular mass 67.7 kDa were compact and spherical it ought to have a radius of 4.6 nm. Hence, it is reasonable to conclude that the value of 3.7 nm corresponds to the mixed detergent micelles in the complexes and not to the overall radius of the aggregates.

NMR Relaxation Times. The values measured for the spin–spin and spin–lattice relaxation times of the carbon nuclei of DPC in mixed DPC/LPAP detergent micelles, with or without myelin basic protein, were nearly the same. Small differences in these values were observed only for carbon nuclei in positions 2 and 3 of the acyl chain (Table III), with position 1 corresponding to the methyl group of the chain. The formation of detergent/protein aggregates resulted in an apparent narrowing of the spectral line corresponding to carbon nuclei 3 of the acyl chain of DPC (Table III), but since this resonance closely overlaps a resonance arising from LPAP, the increase measured in the transverse relaxation time may simply reflect a small change in the relative chemical shift of boths peaks induced by the presence of MBP.

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

The locations within the mixed detergent micelles and the mixed detergent/protein aggregates of the nitroxide moieties of the three spin-labels were investigated by observing the paramagnetic contributions to the relaxation rates of individually assigned nuclei in the DPC detergent molecules. Figure 5 shows the ^{13}C NMR spectra of 10×10^{-3} M dodecylphosphocholine in DPC/LPAP/spin label and DPC/LPAP/MBP/spin label mixtures at a molar ratio of 100:25:0.4 and 100:25:1:04, respectively. Addition of 12-doxylstearate and 16-doxylstearate to detergent mixtures with or without the protein, resulted in increased line widths in the DPC ^{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 of the acyl chain up to position 10. The presence

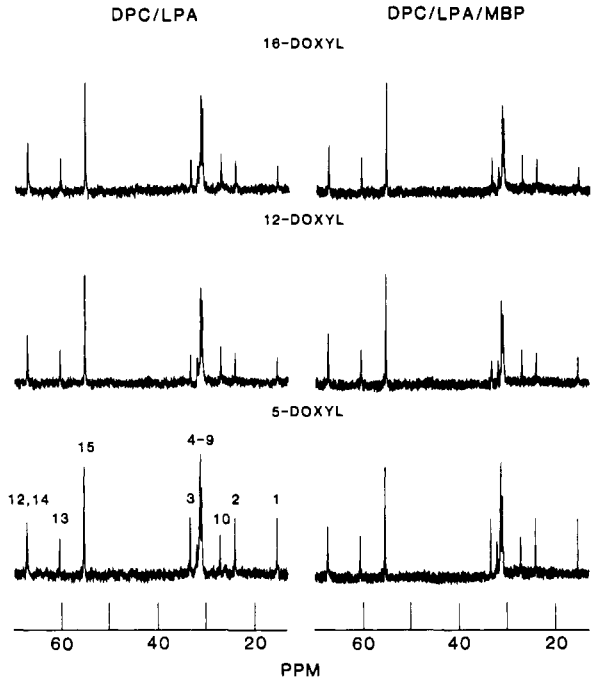

FIGURE 5: Effect of incorporation of spin labels into DPC/LPAP micelles (left) and DPC/LPAP/MBP complexes (right). 100.5-MHz ^{13}C NMR spectra of 10×10^{-3} M dodecylphosphocholine in 100:25 detergent and 100:25:1 detergent/protein molar ratios, respectively, in 0.05 M phosphate buffer at pH 7 and 300 K. The spin label concentration was 0.04×10^{-3} M. Shown from bottom to top are 5-doxylstearate, 12-doxylstearate, and 16-doxylstearate.

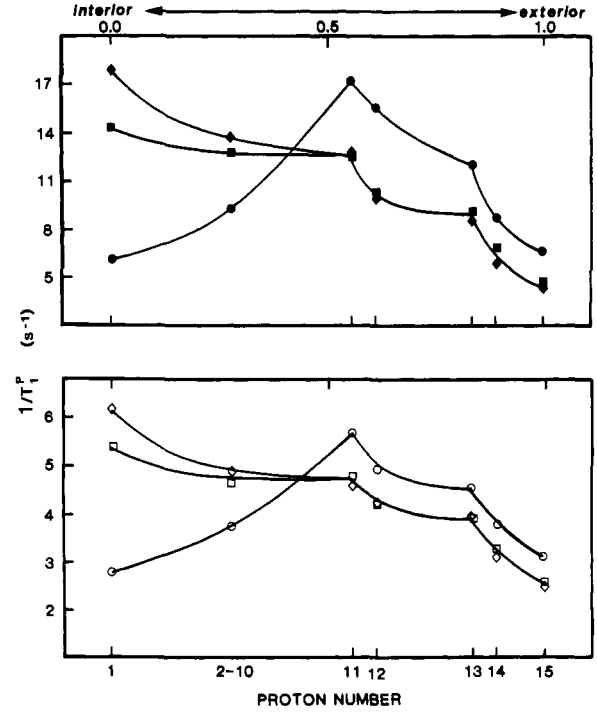

FIGURE 6: Paramagnetic contributions in the ^1H spin–lattice relaxation rates, $1/T_1^P$, for the ^1H resonances of dodecylphosphocholine as a function of nucleus position in the covalent structure of the detergent molecules. Measurements were carried out at 400 MHz in the same mixtures used in Figure 5, with the data for the detergents without protein at the bottom and that with protein at the top: (●,○) 5-doxylstearate, (■,□) 12-doxylstearate, and (◆,◇) 16-doxylstearate.

of 5-doxylstearate in mixed detergent suspensions with or without MBP increased the line widths of resonances arising from carbon nuclei 4–9, and the strongest broadening was observed for the resonance of the nucleus in position 10. The results showed that the presence of the spin labels caused a selective paramagnetic relaxation in the different ^{13}C nuclei of DPC molecules. The data are qualitatively consistent with localization of the nitroxide labels of 12- and 16-doxylstearate near the center of the mixed detergent micelles and that of the 5-doxylstearate closer to the phosphate group of DPC. The presence of myelin basic protein did not alter significantly the effects of the three spin labels on the ^{13}C resonances of DPC and thus suggested that the spatial orientation of this detergent and of the different spin labels within the mixed detergent micelles was not appreciably changed when MBP is also present in the aggregates.

In the spectrum of DPC in mixed detergent micelles, the resonances of carbons 12 and 14 overlap and those of carbons 11–14 show scalar couplings to the phosphorus nucleus of several hertz. As a consequence, measurements of the line widths of these resonances are less accurate. The broadening effects of the 5-doxylstearate label were determined more reliably by studying the selectivity of the paramagnetic effects on the ^1H spin–lattice relaxation times of the DPC resonances. In the absence of spin label, the proton resonances of DPC have relaxation times between 0.51 and 1.28 s in mixed DPC/LPAP micelles and in mixed detergent/protein complexes. The selectivity for different protons can be observed in Figure 6, where the paramagnetic contributions to the relaxation rates ($1/T_1^P$) (the difference in measured relaxation rates with and without the spin label) are plotted as a function of proton number. In suspensions of mixed detergent micelles and mixed detergent/protein complexes, the largest values of the paramagnetic contribution for the 5-doxylstearate label corresponded to the methylene groups at positions 11 and 12, whereas for the 12- and 16-doxylstearate labels the largest values of $1/T_1^P$ corresponded to the methyl protons at position 1. These findings are consistent with a location of the nitroxide moiety of the 5-doxylstearate label near the phosphate group of the detergent and of the nitroxide group of the 12- and 16-labels near the end of the acyl chains of DPC. The addition of MPB caused little changes in the shapes of the plots of $1/T_1^P$ versus proton position. These observations provided further evidence that the presence of bound protein causes no major change in the location of the nitroxide label within the micelles. However, the values of the paramagnetic contributions in the detergent/protein complexes were significantly larger than those in the mixed detergent micelles, suggesting that the motions of the labels in the complexes were more restricted than in the mixed detergent micelles.

Analysis of Electron Micrographs. Large aggregates were observed in electron micrographs of detergent/protein samples prepared by the freeze–fracture technique (Figure 7). These appeared in the form of spheroids with diameters of 20–30 nm, similar to those observed for DPC/MBP complexes (Mendz et al., 1988).

Discussion

A previous study of the interaction of myelin basic protein with mixed DPC/LPAP detergent micelles (Mendz et at., 1990) outlined the effects on the conformation of the protein and established that the interaction occurs at discrete binding sites on MBP and that a balance between hydrophobic and ionic forces is achieved in the interactions of the protein with the detergents. The present study provides direct evidence that the DPC/LPAP/MBP complexes consist of polypeptide chains bound to detergent molecules that retain a micellar organization.

Ultracentrifugation data indicated that myelin basic protein forms a complex of well-defined size and stoichiometry with

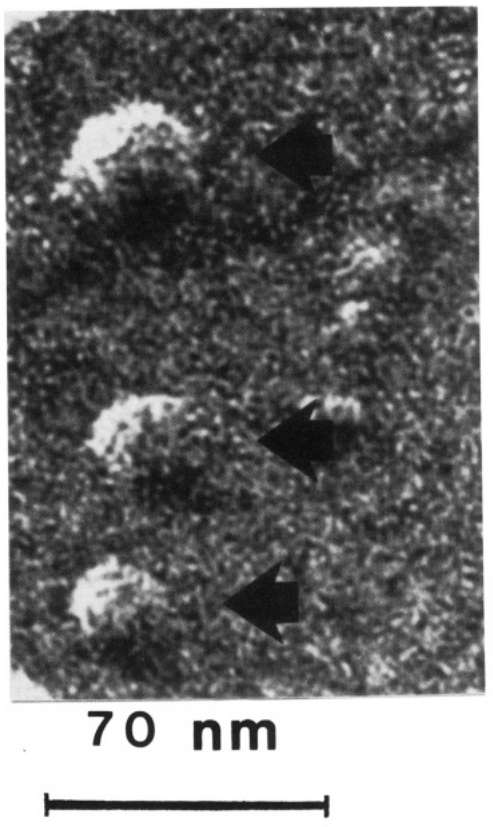

FIGURE 7: Electron micrograph of freeze-fractured sample of a DPC/LPAP/MBP suspension in 0.05 M phosphate buffer at pH 7.0. The arrows indicate characteristic spheroids of diameters 20–30 nm.

mixed dodecylphosphocholine/palmitoyllysophosphatidic acid micelles. The aggregates formed in the presence of MBP consisted of 1 protein molecule and approximately two mixed detergent micelles, each with an average size of 65–75 molecules. The stoichiometry of these aggregates is similar to the complexes formed when myelin basic protein binds to dodecylphosphocholine micelles (Mendz et al., 1988). The changes observed in the ^{1}H NMR spectrum of the protein in DPC/LPAP suspensions (Mendz et al., 1990) showed that (i) the changes in chemical shift occur in resonances from residues located at discrete sites in the polypeptide chain and that these changes are at least partially different for the zwitterionic and anionic detergents, (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 remained unchanged. The data obtained from circular dichroism measurements indicated that at detergent/protein molar ratios above 100:1, approximately 20% of α-helix is induced in the protein, and this amount of helix is independent of the composition of the DPC/LPAP mixture for a range of molar ratios. Since it was also shown that the formation of helices in segments of the intact protein is limited by intramolecular interactions, these observations suggest that to accomodate the detergents there is only a limited reorganization in the polypeptide chain structure that originates both from specific protein intramolecular interactions and protein–detergent interactions.

EPR data indicated that the three spin labels were incorporated into the micelles in the detergent/protein complexes. NMR (Figures 5 and 6) and EPR data (Table II) established that the spin-labels caused no appreciable perturbation of the organization of the detergent/protein aggregates. Comparison of the data on mixed detergent micelles and mixed detergent/protein aggregates indicated 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 indicated that MBP does not drastically alter the organization of the detergent micelles.

The addition of the 5-doxylstearic acid spin-label to mixed detergent solutions and to mixed detergent/protein suspensions produced increased broadening of the resonances of carbon nuclei 4–10 (Figure 5) with maximal effect on the proton nuclei at positions 11 and 12 (Figure 6). The effect of the 12- and 16-doxylstearate spin labels broadened the resonances arising from carbon nuclei at positions 1, 2, and 3 (Figure 5) and had a maximal effect on the proton at position 1. These results together with the selective broadening of only a few ^{1}H NMR resonances of the protein spectrum by each of the spin labels (Mendz et al., 1990) suggested that the polypeptide backbone is mainly outside the micellar surface, with only specific regions of the protein molecule penetrating into the micelles. Different segments could be immersed at various depths but mostly in the region just below the interface. The larger paramagnetic contributions observed in suspensions containing MBP could be explained by "crowding" of the interior of the mixed detergent micelles by segments of the protein, which would result in a diminished rate of diffusion of the spin labels within the micelles.

Addition of MBP did not produce significant changes in the longitudinal and transverse relaxation times of the carbon nuclei of the acyl chain and headgroup of DPC, with the exception of the values measured for the carbon nucleus at position 3 (Table III), indicating that the perturbation produced in the mixed detergent micelles by binding the protein did not affect the overall motion of the DPC molecules. This result is in contrast with the observations made for DPC/MBP complexes (Mendz et al., 1988) in which the presence of the protein induced a slow motion on the detergent molecules capable of modulating the dipolar interactions at certain regions of the acyl chain. An explanation for this difference may be that the interior of the mixed DPC/LPAP micelles can accommodate small perturbations more readily by rearranging the acyl chains of DPC and LPAP, which have different lengths.

Myelin basic protein induces large effects on the ^{31}P NMR spectra of bilayers of dimyristoylphosphatidylglycerol but does not perturb any part of the headgroups in dimyristoylphosphatidylcholine bilayers (Sixl et al., 1984). The effect was ascribed 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 resonance of DPC may arise from electrostatic interactions of the protein with LPAP, which would result in the restriction of the motion of the phosphate ester group of the neutral detergent. However, considering that no significant changes in the ^{13}C relaxation times of the resonances of the choline headgroup of DPC were observed in the presence of MBP and that a decrease of the relaxation times of the phosphorus resonance was also observed in the interactions of the basic protein with DPC micelles (Mendz et al., 1988), the possibility that the perturbation of the motion of the phosphorus nucleus may be due to changes in the shape and/or size of the micelles cannot be ruled out.

In a study of interactions of MBP and DPC, the size of particles present in electron micrographs of DPC/MBP samples prepared by freeze–fracture corresponded well with the size determined by light-scattering measurements (Mendz et

al., 1988). Particles with diameters of 20–30 nm were observed in DPC/LPAP/MBP samples prepared by the freeze–fracture technique, but the SAXS data provided no evidence to support their presence. Several explanations are possible. In the first place the particles revealed by the micrographs of frozen DPC/LPAP/MBP samples may just be artifacts created during the preparation of the samples. This is not likely since they appear in all the samples and are similar to those observed in DPC/MBP samples. Another possibility is that the particles are not present in sufficient numbers in the DPC/LPAP/MBP suspensions to have an effect on the SAXS measurements. In this case they would not correspond to the detergent/protein aggregates formed by attachment of two DPC/LPAP micelles to an MBP molecule. The supramolecular structures revealed by electron microscopy might then be formed by association of a number DPC/LPAP/MBP aggregates. An alternative explanation is that the detergent/protein aggregates constitute very porous particles mostly occupied by the solvent, similar to the DPC/MBP complexes (Mendz et al., 1988). In this case, considering that there was already an extremely low contrast between the detergent micelles and the solvent, the particles may become invisible to X-ray scattering measurements owing to the lack of contrast between the solvent in the aggregates and in the bulk of the suspensions. Indeed, this could also explain why only the micelles were detected in DPC/LPAP/MBP suspensions.

The EPR and NMR data provided evidence supporting a model in which the association of the protein with two detergent micelles induced 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 micelles already present in solution after they have been suitably modified to contour specific regions of the protein. The former process appears more probably considering that although the binding sites for DPC and LPAP in the MBP molecule are at least partially different, the association with mixed detergent micelles is characterized by a balance between ionic interactions with LPAP and hydrophobic interactions with DPC resulting in a well-defined complex, and the changes induced in the conformation of the protein molecule are independent of the DPC/LPAP ratio, for at least the range of values 3:1 to 9:1, at high detergent/protein molar ratios (Mendz et al., 1990). Moreover, at low detergent/protein ratios the amount of α-helix induced in suspensions of MBP titrated with both detergents depended only of the relative concentrations of DPC and LPAP and was independent of the order in which they were added; similarly, the ^{1}H NMR spectra of the protein depended only of the DPC/LPAP ratio (Mendz et al., 1990). Overall it appears that while LPAP facilitates the binding of MBP to micelles, the final organization of the DPC/MBP and DPC/LPAP/MBP aggregates is rather similar.

Registry No. LPAP, 22002-85-3; DPC, 29557-51-5.

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