Membrane Proteins in Reverse Micelles: Myelin Basic Protein in a Membrane-Mimetic Environment†

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ABSTRACT: The solubility, reactivity, and conformational dynamics of myelin basic protein (MBP) from bovine brain were studied in reverse micelles of sodium bis(2-ethylhexyl) sulfosuccinate (AOT)–isooctane and water. Such a membrane-mimetic system resembles the aqueous spaces of native myelin sheath in terms of physicochemical properties as reflected in the high affinity of MBP for interfacial bound water. This is marked by the unusual profile of the solubility curve of the protein in reverse micelles, which shows optimal solubility at a much lower molar ratio of water to surfactant ($[H_2O]/[AOT] = w_0$) than that reported for other water-soluble proteins. The role of counterions and/or charged polar head groups in the solubilization process is revealed by comparison of the solubility of MBP in nonionic surfactant micellar solutions. Whereas MBP is unfolded in aqueous solutions, insertion into reverse micelles generates a more folded structure, characterized by the presence of 20% α-helix. This conformation is unaffected by variations in the water content of the system (in the 2.0–22.4w_0 range). The reactivity of ϵ-amino groups of lysine residues with aqueous solutions of *o*-phthalaldehyde demonstrates that segments of the peptide chain are accessible to water. Similar results were obtained with the sequence involved in heme binding. In contrast, the sole tryptophan residue, Trp-117, is shielded from the aqueous solvent, as indicated by lack of reaction with *N*-bromosuccinimide. The invariance of the wavelength maximum emission in the fluorescence spectra as a function of w_0 is consistent with this result. The internal dynamics of the protein in the vicinity of the tryptophan, as investigated by time-resolved fluorescence anisotropy techniques, indicates that the average distribution angle of the fluorophore absorption dipole remains constant with increasing w_0, reflecting hindrance to its motion, whereas the rotational correlation time decreases, indicating a more relaxed local conformation around the chromophore, although the overall protein structure remains largely unaffected. The results reported here demonstrate that MBP, inserted in reverse micelles, displays distinctive properties that mirror its behavior as a water-soluble perimembrane protein in myelin, where it interacts with both interfacial water and charged membrane surfaces.

Of the many components that make up the multilamellar myelin sheath of the central nervous system, the Folch-Pi proteolipid and the myelin basic protein (MBP)[1] constitute about 80% of the total protein. Both proteins, located in different domains of the myelin lamellae, have been completely sequenced: the former recently by Stoffel et al. (1983) and Lees et al. (1983) and the latter by Eylar (1970). Although the function of these proteins is still poorly understood, they are probably involved in myelin compaction and in maintenance of its integrity.

The conformation of myelin proteins has been studied by a variety of physical and chemical techniques, after solubilization in homogeneous organic or aqueous solvent mixtures (Boggs et al., 1982). From sequence and proteolytic studies, Stoffel et al. (1984) and Laursen et al. (1984) have presented models for the molecular arrangement of the Folch-Pi proteolipid in the myelin of central nervous system. Conformational measurements of the proteolipid, as recently determined by Delahodde et al. (1984) in a membrane mimetic environment, are compatible with such models.

MBP, located between the cytoplasmic apposition of surfaces of the oligodendroglial membrane, is an extrinsic water-soluble protein (Golds & Braun, 1976). In the latter solvent, the protein behaves as an extended, flexible, polyelectrolyte with little periodic secondary structure (Martenson, 1978). It is generally believed, however, that in the native membrane MBP exhibits a more folded, compact conformation (Martenson, 1981). In situ, MBP is localized in the aqueous interstitial space sandwiched between two lipids bilayers. If one considers only those lipid layers adjacent to the aqueous space, this is equivalent to its location in an inverted bilayer environment with the polar heads of the lipids in contact with the interfacial water and hydrophobic tails inside the hydrocarbon bilayers (Figure 1).

These observations prompted us to investigate the behavior of MBP in reverse micelles, which are water-containing organized assemblies of surfactants in organic solvents. Bound water entrapped within these micelles, which may be considered as rigid, monodisperse spheres, determines their size and exhibits peculiar physical and chemical properties. While different from bulk water [for a review, see Fendler (1982)], such interfacial water (Kuntz & Kauzmann, 1974) is comparable in many respects to water close to biological membranes and proteins. Recently, interest in these membrane

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[1] Abbreviations: MBP, myelin basic protein; AOT, sodium bis(2-ethylhexyl) sulfosuccinate; NATA, *N*-acetyltryptophanamide; ACTH, adrenocorticotropin hormone.

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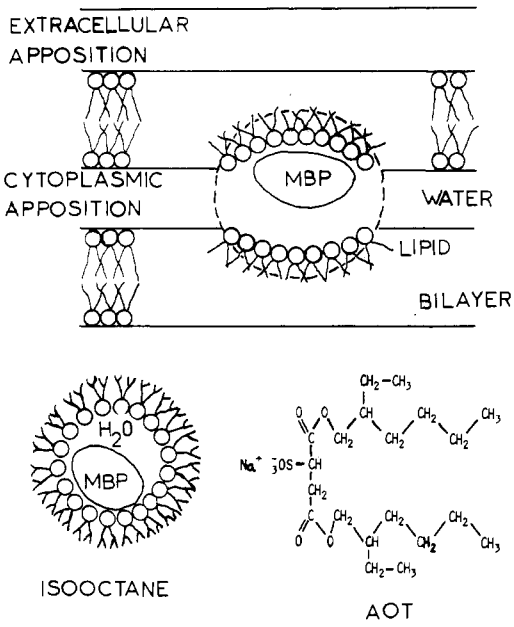

FIGURE 1: Schematic representation of MBP in myelin and in a reverse micelle. (Upper part) The dotted circle underlines an inverted bilayer environment of MBP at the cytoplasmic apposition. (Lower part) Left-hand side shows the scheme of MBP in an AOT reverse micelle. Surfactant-charged head groups (designated as circles) are in contact with the water pool, while the hydrocarbon chains are directed toward the apolar solvent. Right-hand side shows the structure of the surfactant AOT.

mimetic agents has been extended to the guest molecules, which can be solubilized in the aqueous core of reverse micelles, including peptides (Thompson & Gierasch, 1984), enzymes (Luisi et al., 1977), and integral membrane proteins (Delahodde et al., 1984), as well as nucleic acids (Imre & Luisi, 1982).

Since many biological phenomena occur at surfaces rather than in homogeneous solvent mixtures, the primary purpose of this investigation is to describe the properties of MBP at an interfacial environment, where it should possess a lower degree of motional freedom than in aqueous solution, and to provide new insights regarding its structure, reactivity, and dynamics in this environment. Because reverse micelles are optically transparent, they can be studied by a large range of spectroscopic techniques. In this work absorption spectroscopy was utilized to study the solubility of MBP in reverse micelles; circular dichroism was carried out in order to characterize the significant conformational change observed in micellar solutions. In addition, MBP possesses a single tryptophan serving as an intrinsic probe of the protein motion; fluorescence techniques including emission spectra, excited-state lifetimes, and anisotropy in steady-state and time-resolved modes were employed to investigate the dynamics of the protein embedded within this membrane-mimetic system.

From these measurements, a picture of MBP emerges with increased structural order and/or higher conformational rigidity, which is consistent with what is known of the organization of native myelin (Braun, 1984). Such structural features can be crucial for the myelin arrangement, for the immunological properties, and for the physiological role of MBP, possibly leading to a different regulation of myelin metabolism in demyelinating diseases (Ong & Yu, 1984).

Materials and Methods

Sodium bis(2-ethylhexyl) sulfosuccinate (AOT), a generous gift from the Cyanamid Co. (France), was purified according to Wong et al. (1976) and carefully dried in vacuo. The purity of each batch was checked as recommended by Luisi et al. (1984). The absorption of a 50 mM AOT solution in isooctane did not exceed 0.03 at 280 nm, and titration by 0.1 N NaOH did not detect more than 0.03% residual acidity. Tetraethylene glycol mono-*n*-dodecyl ether (Nikkol BL-4SA) was used without further purification as purchased from Nikko Chemicals Co. (Tokyo, Japan). Isooctane (Uvasol grade) was from Merck (Darmstadt, FRG); *N*-acetyltryptophanamide (NATA) was from Sigma. *N*-Bromosuccinimide, 99% pure, was purchased from Koch Light (Colnbrook, Bucks, England). *o*-Phthalaldehyde (analytical grade), L-leucine, and *N*-acetylglycyl-L-lysine methyl ester acetate (research grade) were purchased from Serva (Heidelberg). Myelin basic protein was extracted from bovine brain, purified by the method of Deibler et al. (1972) and lyophilized.

Preparation of Reverse Micelles. The micellar solutions were prepared by addition of measured volumes of isooctane to dry, preweighed amounts of AOT. The desired volumes of MilliQ (Millipore) purified water (v/v) were added with Hamilton syringes and the samples shaken until optically clear. Dried, preweighed quantities of MBP were added and left for several hours at room temperature. Dissolution was achieved by gentle shaking followed by 2 min of sonication in a Branson-type sonicator when necessary. A final centrifugation at 5000 rpm removed the undissolved protein.

For solubilization studies, because of possible problems associated with a large excess of solid protein (i.e., uptake of water and AOT), experiments were always carried out with the minimal amount of undissolved protein necessary to achieve saturation. This was determined previously by trial and error.

The absorption spectra were measured on a Cary Model 118 spectrophotometer. The base line was always run with micellar solutions at the same water to surfactant molar ratio (w_0) as used for the protein. The molar extinction coefficient of MBP in reverse micelles was found to be identical with that of aqueous solutions and taken as 1.07×10^4 (M_r 18 500) at 278 nm.

Titration of Tryptophan and ϵ-Amino Groups of Lysine Residues by Water-Soluble Reagents. The reaction of *N*-bromosuccinimide with tryptophan or with the model compound NATA was carried out as described by Spande & Witkop (1967). For the reaction in reverse micelles, micellar solutions of MBP were prepared as above except that water was replaced by 0.01 M acetic acid. Micellar solutions of the reagent were obtained by injecting into a 50 mM AOT/isooctane solution the required volume of 0.1 M *N*-bromosuccinimide in 0.01 M acetic acid. Both solutions were mixed and shaken in a stoppered cell before being read at 280 nm.

The reaction of ϵ-amino groups of lysine residues was performed according to Benson & Hare (1975). Micellar solutions of *o*-phthalaldehyde were obtained by injecting the desired volume of an 11 mM aqueous solution of the reagent (0.2 M in borate, pH 9.5, containing 0.06 mM 2-mercaptoethanol) into a 100 mM AOT/isooctane solution (2% v/v, corresponding to $w_0 = 11.2$). A total of 0.1 mL of a micellar solution of MBP (1–3 nmol) was added to 1 mL of the reagent in a stoppered cuvette; at the same time, micellar solutions of leucine and *N*-acetylglycyllysine methyl ester (2–20 nmol) were prepared and used as standards. Upon excitation at 340 nm, maximal emission fluorescence intensity was observed at 455 nm, after 15 min, on a Jobin et Yvon type 3 spectrofluorometer (excitation bandwidth 0.2 nm, emission bandwidth 10 nm). In the range of studied concentrations for MBP and standards, a good linearity of the emitted fluorescence was obtained.

Circular Dichroism. Circular dichroism measurements were performed on a Cary Model 60 spectropolarimeter equipped with a 6001 attachment. Some measurements were done on a Jobin et Yvon Mark V spectropolarimeter. The wavelength range scanned was 250–190 nm. The instrument was calibrated with (+)-*d*-camphorsulfonic acid at 290 nm. The calibration at low wavelengths was checked with hemoglobin. Blank runs of AOT solutions in isooctane, at appropriate w_0 values, were subtracted from the corresponding sample spectra. The spectra reported are the average of at least three scans of several different preparations. Measurements were made at 20 °C, in cylindrical quartz cells of 1.0-, 0.5-, and 0.2-mm cell path. Ellipticity values [θ] are expressed on a mean residue basis in units of deg·cm^2·dmol^{-1}. The mean residue weight for MBP, calculated from the amino acid composition, was taken as 110.

Fluorescence Measurements. Corrected fluorescence emission spectra were recorded with a SLM 8000 spectrofluorometer coupled to a Hewlett-Packard 9815 A minicomputer. "Magic angle" configuration (excitation polarizer in the vertical position and emission polarizer orientated 55° from the vertical) was used for collecting the emitted fluorescence light in order to eliminate polarization effects (Spencer & Weber, 1970). Excitation wavelength of 295 nm was used. Bandwidths were 2 and 4 nm for the excitation and emission, respectively (single-grating monochromators). Quartz cells of 1 × 1 cm were used. Thermoregulation of the sample was achieved with a circulating water thermostat Hüber HS 40, and the temperature was monitored with a thermistor probe coupled to a Digitec 5810 digital thermometer.

Steady-state anisotropy measurements were performed on the same apparatus with the T-format configuration. The excitation wavelength was selected at 295 nm (2-nm bandwidth), and the emission light was collected through 1 M $CuSO_4$ cut-off filters (1-cm optical path). The T-format configuration allows the measurement of $R_{vert} = I_{vv}/I_{vh}$ and of $R_{horz} = I_{hv}/I_{hh}$, where the first subscript refers to the excitation light and the second to the emission. The anisotropy was calculated as

$$r = \frac{R_{vert} - R_{horz}}{R_{vert} + 2R_{horz}} \quad (1)$$

Nanosecond anisotropy decay measurements were performed on the same time-correlated single photon counting instruments as already described (Vincent & Gallay, 1984) equipped with the nanosecond excitation source from Edinburgh Instruments (Birch & Imhoff, 1981). The flash lamp was run in 1 atm of nitrogen, 50 kHz, and 7 kV with an electrode gap of ~0.6 mm. Excitation wavelength was selected at 295 nm (bandwidth 5 nm). Emission was collected through a 1 M $CuSO_4$ cut-off filter (1-cm optical path). $I_{vv}(t)$ and $I_{vh}(t)$ were collected successively after one of two runs of 400-s cumulation. In order to avoid the color effect of the phototube, the apparatus response function was obtained after cumulation during the same time of the fluorescence decay of a short lifetime standard (*p*-terphenyl in cyclohexane, τ = 0.95 ns; Berlman, 1971) according to Wahl et al. (1974). Analysis of the data was performed according to previously described methods (Vincent et al., 1982; Vincent & Gallay, 1984).

Results

Solubilization of MBP in Reverse Micelles. The anionic surfactant sodium bis(2-ethylhexyl) sulfosuccinate can solubilize variable amounts of water in apolar solvents, the concentration of which is generally expressed as $w_0 = [H_2O]/[AOT]$, the molar ratio of water to surfactant. Comparison of the ultraviolet spectra of MBP in aqueous and in micellar solutions at various w_0 values shows little difference, if any, while lack of scattering of the micellar solutions in the 350-nm region indicates excellent solubilization. The amount of solubilized protein can be calculated from the absorption maximum at 276–278 nm.

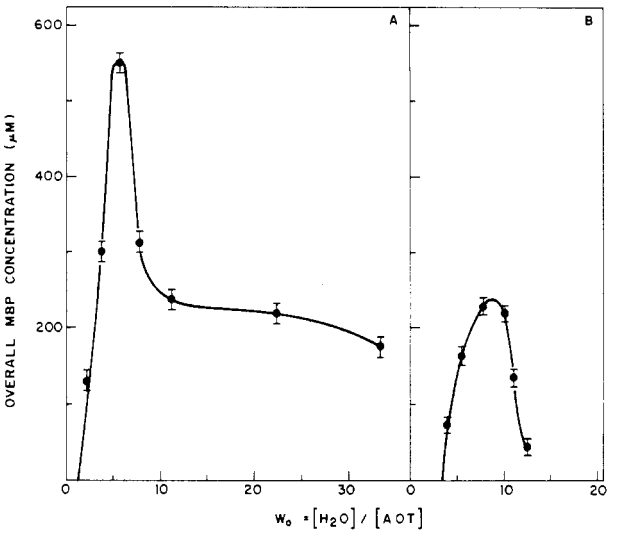

FIGURE 2: Solubilization curves of MBP in surfactant/isooctane/water reverse micelles. The overall concentration is calculated from absorbance measurements at 278 nm and plotted vs. w_0, the molar $[H_2O]/[surfactant]$ ratio. Each point represents an average of several determinations carried out independently. Each determination represents a separate experiment, at a given w_0 value. Bars represent experimental errors. In curve A, the surfactant is 50 mM sodium bis(2-ethylhexyl) sulfosuccinate (AOT) and in curve B is 100 mM tetraethylene glycol mono-*n*-dodecyl ether (Nikkol).

The solubilization of MBP in reverse micelles has been studied as a function of the water content of the system, at a constant concentration of AOT (50 mM). From Figure 2A it is obvious that the protein is totally insoluble in the absence of water. The solubility curve, starting around a w_0 value of 2.0, increases sharply with increasing amounts of water up to a maximum value for w_0 = 5.6 and then decreases rapidly until a plateau is reached at a value of 11.2. It is interesting to point that optimal solubilization of other water-soluble proteins, so far reported, occurs at much higher water to surfactant ratios (Luisi & Wolf, 1982) at similar AOT concentrations. In the very narrow range of maximal solubilization, where the mean aggregation number (i.e., the number of molecules of surfactant per micelle) is in the vicinity of 100 (Grandi et al., 1981), the saturating overall concentration of MBP reaches a value of about 0.55 mM; however, in the water pool of reverse micelles, at 0.5% v/v water content, this represents a protein concentration 200-fold higher. At this point, the calculated ratio of the number of protein molecules to the number of micelles initially present in the system is equal to 1.

The solubilization of MBP in reverse micelles was also investigated with a nonionic surfactant, tetraethylene glycol mono-*n*-dodecyl ether (Nikkol BL 4S4). Comparison of the two curves, obtained by plotting the overall protein concentration solubilized at optimal surfactant concentration against w_0 (Figure 2B), shows major dissimilarities. At values of w_0 lower than 3, the solubilization is nil; above 3, it increases up to a maximum located between 8 and 10 and becomes very low for w_0 = 12.6. Moreover, the maximal overall concentration of solubilized MBP (0.23 mM) in micelles made of 100 mM Nikkol, water, and isooctane reaches about half the value

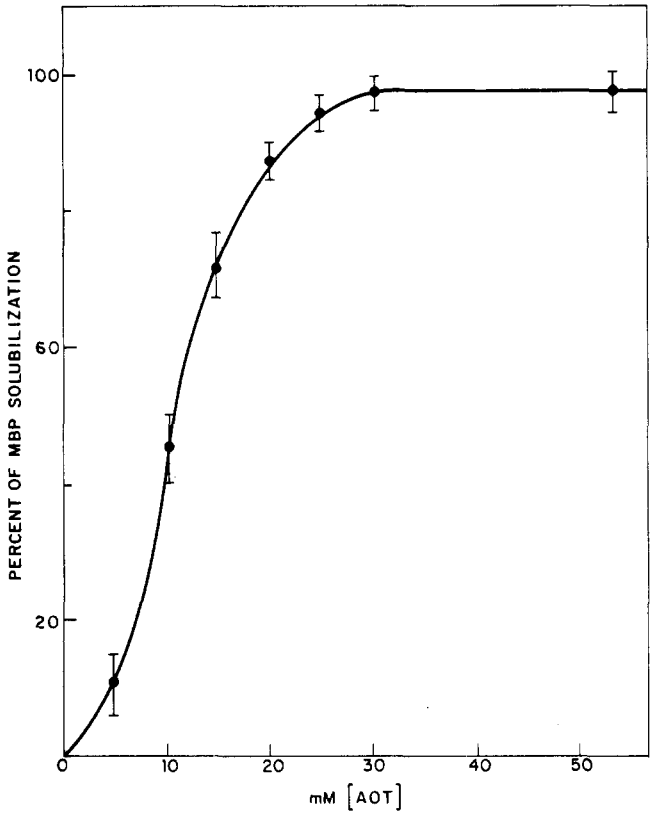

FIGURE 3: Solubilization curve of MBP as a function of AOT concentration at optimal w_0 value of 5.6. The percent of solubilization was taken as the ratio of weighed to solubilized protein (2 mg of MBP in 1 mL of micellar solution). Other experimental conditions are as in Figure 1.

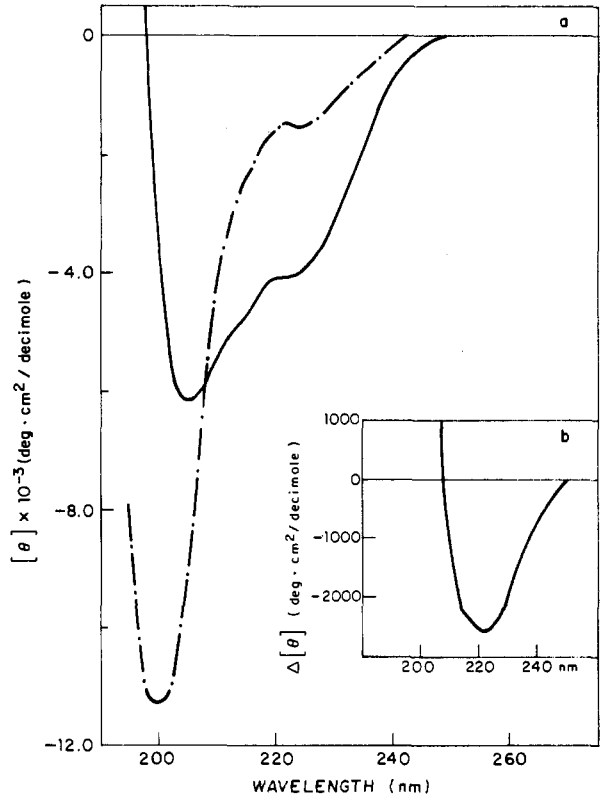

FIGURE 4: Circular dichroism spectra of MBP. (a) Far-ultraviolet circular dichroism spectrum of MBP in aqueous (-·-) and micellar (—) solutions of 50 mM AOT/isooctane/water, at w_0 = 5.6. Each curve represents an average of five measurements. (b) Calculated difference spectrum between aqueous and micellar solutions.

obtained with AOT micelles under optimal experimental conditions.

The percent of solubilization, taken as the ratio of solubilized to weighed protein, was also studied as a function of AOT concentration, for a constant optimal w_0 value of 5.6. Figure 3 shows the solubilization of 2 mg of dry MBP in 1 mL of micellar solution. At an AOT concentration of 10 mM the solubilization is around 50%, reaching 100% for 30 mM AOT. In contrast to the variation of solubility with varying w_0 seen in Figure 2A, the variation of solubility with AOT, at optimal w_0, resembles that reported for several water-soluble enzymes (Grandi et al., 1981).

Circular Dichroism. The circular dichroism spectrum of MBP in the far-ultraviolet (250–190 nm) was measured in aqueous and in micellar solutions. Figure 4a illustrates the conformational change of MBP occurring in reverse micelles of 50 mM AOT, at w_0 = 5.6. The spectrum exhibits two negative extrema at 205 and 222 nm, with ellipticities of −6100 and −4000 deg·cm²·dmol⁻¹. When compared to the ellipticities measured in aqueous solutions (−11 700° at 198 nm and −1500° at 222 nm), these values reveal a shift from 198 to 205 nm in reverse micelles, in addition to the striking differences in ellipticities at the negative extrema. Figure 4b shows the calculated difference circular dichroism spectrum between aqueous and micellar solutions of MBP. The spectrum is characterized by a negative extremum at 222 nm, indicating that the most probable structure generated consists of α helices. However, since the difference spectrum lacks a distinctive second maximum near 208 nm, the presence of some nontypical β sheet and β turns (Chang et al., 1978) cannot be completely ruled out.

The conformation of MBP was investigated as a function of AOT concentration. In the 25–100 mM range, the observed spectra were unaffected by surfactant concentration. Measurements were also carried out at increasing water to surfactant ratios, i.e., for w_0 values ranging from 2.0 to 22.4. The range of ellipticities was −3900 ± 100 deg·cm²·dmol⁻¹ at 222 nm and −6100 ± 200 deg·cm²·dmol⁻¹ at 205 nm. It was concluded that the conformation of MBP was not dependent on the water content of the micellar system.

When measured in reverse micelles of Nikkol, the nonionic surfactant, at w_0 = 5.6, the circular dichroism of MBP was changed relative to aqueous solution, but less so than in AOT. The value at 222 nm is −2500 and that at 205 nm is −7000. Calculations of the secondary structure according to Chen et al. (1974) yield a value of approximately 22% α helix for MBP in AOT and 10% in Nikkol reverse micelles.

Reactivity of MBP Amino Acid Side Chains in Reverse Micelles. The reactivity of ϵ-amino groups of lysine residues in micellar MBP solutions was studied with a fluorogenic water-soluble reagent, *o*-phthalaldehyde (Benson & Hare, 1975). In order to increase the overall concentration of the water-soluble reagents, the water to isooctane ratio (v/v) was raised to 2% by taking an AOT concentration of 100 mM and a w_0 value of 11.2. Within the above concentration limits, it has been shown in the preceding section that the conformation of MBP is not measurably affected.

Under these experimental conditions, the relative fluorescence intensities per nanomole, expressed in arbitrary units, were 228 ± 10 for MBP and 18 ± 1 for the standard lysine peptide (*N*-acetylglycyllysine methyl ester). It is worthwhile to note that the value obtained for an α-amino acid (leucine) was 25 ± 1. Since the α-amino groups of MBP and of the standard lysine peptide are both acetylated, all the measured fluorescence arises from the ϵ-amino groups. Therefore, the ratio of the fluorescence intensities of the lysine residues of

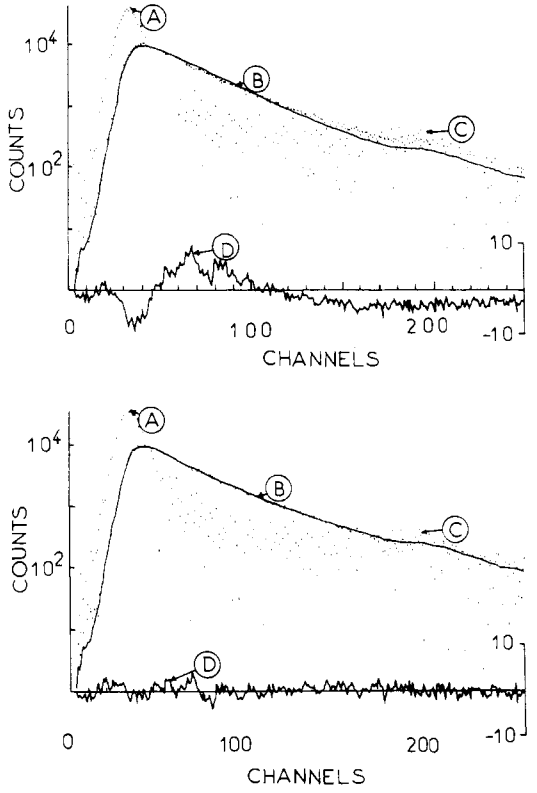

FIGURE 5: Example of fluorescence total intensity decay of MBP in water, at 20 °C. Excitation wavelength 295 nm. Emission was collected with a cut-off 1 M $CuSO_4$ filter (1-cm optical path) through "magic angle" configuration as described under Materials and Methods. (A) Apparatus response function decay according to Wahl et al. (1974). (B) Solid line is lamp-convolved theoretical parameter curve. (C) Dotted line is experimental decay curve. (D) Deviation function. Time calibration was 0.099 ns/channel. (Upper panel) Monoexponential model, τ = 2.7 ns, χ^2 = 13.8; (lower panel) biexponential model, τ_1 = 1.7 ns, τ_2 = 3.9 ns, a_1 = 0.69; a_2 = 0.31, χ^2 = 1.82.

MBP to that of the sole lysine of the standard peptide can be used as an estimate of the number of residues accessible to aqueous solutions of *o*-phthalaldehyde. The value obtained, 12.7 ± 1.5, seems to indicate that most of the 13 lysine residues of MBP are accessible.

Tryptophan residues were titrated with *N*-bromosuccinimide in aqueous and micellar MBP solutions, as well as in micellar solutions of the model compound *N*-acetyltryptophanamide (NATA) at w_0 = 11.2. *N*-Bromosuccinimide titrates the single tryptophan residue in aqueous solutions of MBP and in micellar solutions of NATA. In micellar solutions of MBP only 0.35 equiv/mol was titrated. Additional information about this reaction is provided by the molar ratio of reagent to tryptophan used during the titration. For the titration of the micellar solution of NATA and for the aqueous solution of MBP, 2.0 and 4.5 equiv of bromosuccinimide were consumed respectively, whereas, in micellar solutions of MBP, 8.5 equiv was used. As noted by Spande & Witkop (1967), an increased consumption of *N*-bromosuccinimide indicates a side reaction, mainly involving tyrosine residues in the presence of tryptophan that is refractory to oxidation.

Fluorescence Studies. The fluorescence emission spectrum of the single Trp residue in MBP was recorded in aqueous and in micellar solutions and compared to the spectrum of NATA under the same experimental conditions. The results are summarized in Table I. In water, the emission maxima were 357 and 350 nm for NATA and MBP, respectively. Upon incorporation of MBP and NATA into micellar solutions, a

Table I: Maximum Fluorescence Emission Wavelength of NATA and MBP in Micellar and Aqueous Solutions

w_0	NATA (nm)[a]	MBP (nm)[a]
1.4	336	
2.0	337	334
2.8	340	
5.6	342	335, 344[b]
11.2	343	
22.4	345	334
water	357	350

[a] Solubilized in 50 mM AOT, at 20 °C. [b] Solubilized in 100 mM Nikkol, at 20 °C.

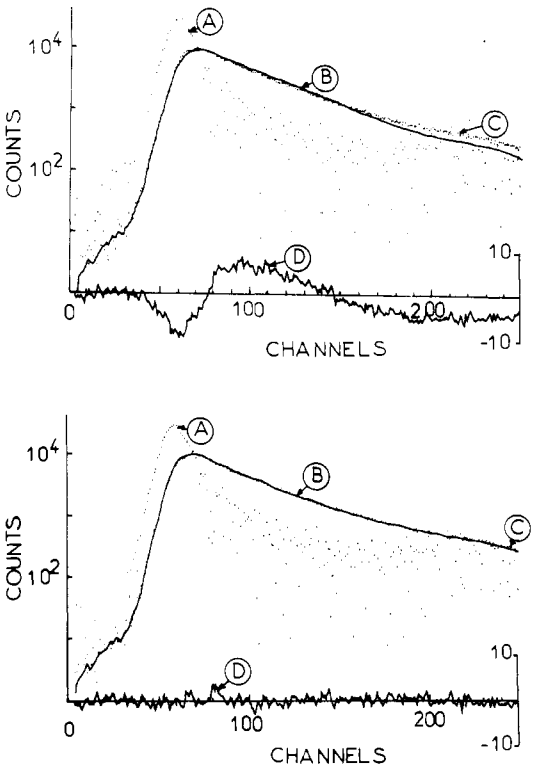

FIGURE 6: Example of fluorescence total intensity decay of MBP in reverse micelles, w_0 = 2.8, at 20 °C. Same experimental conditions as in Figure 5. Monoexponential model, τ = 3.3 ns, χ^2 = 22.5; biexponential model, τ_1 = 4.5 ns, τ_2 = 1.5 ns, a_1 = 0.41, a_2 = 0.59, χ^2 = 1.18.

blue shift of the emission maxima was observed. However, differences between the behavior of NATA and the protein were detectable as a function of water present in the system. For NATA, the emission maximum was increasingly blue-shifted with decreasing values of w_0, whereas for MBP the blue shift remained constant at all w_0 values studied.

The total fluorescence intensity emission decay of MBP in micellar solutions and in water were multiexponential (Figures 5 and 6). The calculated kinetic parameters are summarized in Table II. Upon increasing w_0, the mean excited-state lifetime $\langle\tau\rangle$ decreased by ≃30%. Moreover, at high water content (w_0 = 22.4), its value dropped to a value even lower than that in pure water.

Steady-state anisotropy measurements of the Trp emission of MBP were also performed in aqueous solution and in reverse micelles. In the first solvent, a very low value of the steady-state anisotropy was observed (r = 0.045) in agreement with the very fast rotational relaxation of the Trp-117 in MBP measured by time-resolved technique (Munro et al., 1979). Incorporation of MBP into micellar solutions evoked a large increase of the steady-state anisotropy values with the highest value r = 0.167 for w_0 = 2.8 (Table III).

Table II: Total Emission Kinetics of the Single Trp Residue of MBP in Aqueous and Micellar[a] Solutions

w_0	a_1	τ_1 (ns)	a_2	τ_2 (ns)	$\langle\tau\rangle$ (ns)[b]
2.8	0.43 ± 0.02	4.4 ± 0.1	0.57 ± 0.02	1.3 ± 0.1	3.49 ± 0.04
5.6	0.32 ± 0.02	4.1 ± 0.2	0.68 ± 0.02	1.2 ± 0.2	2.97 ± 0.09
11.2	0.25 ± 0.05	4.0 ± 0.4	0.75 ± 0.05	1.4 ± 0.3	2.62 ± 0.08
22.4	0.22 ± 0.02	3.7 ± 0.2	0.78 ± 0.02	1.2 ± 0.2	2.38 ± 0.04
water	0.32 ± 0.01	3.9 ± 0.1	0.68 ± 0.01	1.7 ± 0.1	2.82 ± 0.04

[a] Measurements carried out in 50 mM AOT, at 20 °C. The samples had an absorbance at 295 nm of ≃0.2. The results are the mean of two to five measurements. [b] The mean excited-state lifetime $\langle\tau\rangle$ was calculated as $\langle\tau\rangle = \sum_i a_i\tau_i^2/\sum_i a_i\tau_i$, where a_i and τ_i are the respective amplitude and lifetime of the ith component (Chen et al., 1977).

Table III: Steady-State Anisotropy Values and Nanosecond Anisotropy Decay Parameters of MBP in Micellar Solutions[a]

w_0	r	A_1	ϕ_1 (ns)	A_2	$r_{0\,\mathrm{eff}}$
2.8	0.167 ± 0.004	0.133 ± 0.018	7.3 ± 2.5	0.093 ± 0.007	0.226 ± 0.012
5.6	0.161 ± 0.001	0.111 ± 0.005	4.6 ± 1.0	0.090 ± 0.007	0.201 ± 0.004
11.2	0.144 ± 0.001	0.123 ± 0.011	2.0 ± 0.7	0.088 ± 0.011	0.211 ± 0.022
22.4	0.142 ± 0.001	0.133 ± 0.018	1.4 ± 0.7	0.103 ± 0.008	0.236 ± 0.026

[a] Measurements carried out in 50 mM AOT, at 20 °C. The samples had an absorbance at 295 nm of ≃0.2. The results are the mean over two to five determinations.

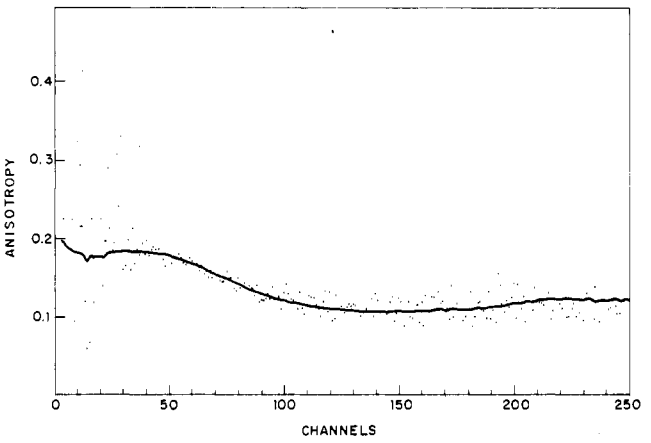

FIGURE 7: Fluorescence anisotropy decay of MBP in reverse micelles of AOT/isooctane/water (w_0 = 11.2) at 20 °C: ϕ_1 = 2.7 ns, A_1 = 0.115, A_2 = 0.079, $r_{0\,\mathrm{eff}}$ = 0.194. Experimental decay (···); theoretical curve for the decay of emission anisotropy (—). Time calibration was 0.099 ns/channel.

The emission anisotropy kinetics of the Trp-117 was monitored in micellar solutions. As an example, Figure 7 illustrates the anisotropy decay of MBP emission for w_0 = 11.2, at 20 °C. As shown, the Trp chromophore exhibits two kinds of rotational motions. The first is moderately fast with a correlation time of ≃2 ns, and the second has a much slower rotation, which on our instrument appears as an infinite time component compared to the excited-state lifetime. This result was observed for every w_0 value listed. The anisotropy decay curves were fitted to a nonexponential function of the form

$$r_t = A_1 e^{-t/\phi_1} + A_2 \qquad (2)$$

where $A_1 = r_{0\,\mathrm{eff}} - A_2$ ($r_{0\,\mathrm{eff}}$ being the anisotropy at zero time and A_2 the residual anisotropy at infinite time). ϕ_1 is an apparent correlation time that reflects the rotational motion of the fluorophore in a restricted volume.

By use of this model, the anisotropy decay parameters obtained for different w_0 values are presented in Table III. It can be observed that decreasing w_0 results in an increase of the correlation time ϕ_1 and in an unchanged value of the infinite component A_2, within the experimental error. The values of the r_0 effective at zero time of the decay curve agree with the limiting anisotropy value of tryptophan measured at the excitation wavelength of 295 nm, in vitrified propylene glycol at −58 °C (Valeur & Weber, 1977).

From the constant term A_2 of the anisotropy decay an average angle θ for the distribution of the Trp absorption dipole can be calculated according to the formalism of Lipari & Szabo (1980), i.e.

$$\frac{A_2}{r_{0\,\mathrm{eff}}} = \left\langle \frac{3\cos^2\theta - 1}{2} \right\rangle^2$$

For MPB, a constant value of about 29° for θ was found for any water to surfactant molar ratio checked.

Discussion

Reverse micelles made of AOT/water/isooctane provide a unique system for the study of MBP solubilized and incorporated into the aqueous core (water pool) of these assemblies. Comparison with the myelin cytoplasmic spaces reveals a number of similar features. In both structures the affinity of surface groups for water is the dominant force opposing close apposition of membranes surfaces (Kirschner et al., 1984; Maitra, 1984). The presence of salt in the aqueous spaces of native myelin corresponds to the presence of sodium counterions in the water pool of AOT reverse micellar systems (Wong et al., 1976; Thompson & Gierasch, 1984). Thus, the existence of physicochemical forces responsible for maintaining the packing of the myelin membrane may also explain the unusual solubility properties of MBP in the water core of reverse micelles. Moreover, the dimension of the aqueous spaces containing MBP in myelin and in reverse micelles is of a comparable order of magnitude (30–50 Å) (Kirschner et al., 1984; Chatenay et al., 1985).

Solubility of MBP in Reverse Micelles. One salient feature of this work is the very sharp profile of the solubility curve of MBP at low water to surfactant molar ratios, around w_0 = 6.0 (Figure 2A). Optimal solubilization of other water-soluble proteins reported by Luisi & Wolf (1982) requires much higher w_0 values. This holds for basic proteins such as lysozyme (Grandi et al., 1981) and for a basic peptide [ACTH-(1–24)] as well (unpublished results). The net charge of the peptide chain therefore is not mainly responsible for the observed phenomena. It may reflect, instead, a specific interaction of the protein with the water entrapped in the micelle, the unusual physical properties of which, especially at low w_0 values, are attributed to the interaction with the ionic head groups of the surfactant [for a review, see Fendler (1982)]. When MBP is solubilized in Nikkol, a nonionic surfactant,

much lower solubility is obtained even at a higher concentration of the surfactant. Furthermore, optimal solubilization is shifted toward a higher w_0 value of 10.0 (Figure 2B). In AOT reverse micelles, the presence of bound water (six or seven water molecules per AOT molecule), as opposed to free water, has been reported by various spectroscopic techniques (Wong et al., 1976; Thompson & Gierasch, 1984; Maitra, 1984). Therefore, it may be safely assumed that at optimal solubilization of MBP in AOT (w_0 = 5.6) the amount of free water present is negligible. The solubility curve indicates then a preferential solvation of MBP by bound water. Also, a change of conformation of the 170 amino acid peptide chain, due to specific interactions with this type of solvent in close vicinity to the surfactant polar head groups, may lead to a more energetically favorable conformation driven by entropic forces and conferring on MBP the maximal solubility described herein.

In this connection, it is worthwhile to note the dielectric studies of Gent et al. (1970), who reported the presence of highly organized water of "ice-like" character in myelin, comparable to biological interfacial water (Kuntz & Kauzmann, 1974). Finally, it is interesting that the other major protein of myelin, the Folch-Pi proteolipid, which is insoluble in aqueous solvents, also displays optimal solubilization in reverse micelles at the low value of w_0 = 5.6 (Delahodde et al., 1984). There may thus be a similarity in the mechanism of interaction of both proteins with the interface provided by this system, probably bearing a resemblance to the native myelin environment.

Structural Changes of MBP. There is an obvious change of MBP structure, upon incorporation in reverse micelles, from a flexible, extended chain to a more ordered α-helical structure as indicated by circular dichroism spectra (Figure 4). Such an increase in structural order was predicted from sequence analysis by Martenson (1981) and experimentally observed in the presence of lipids by Keniry & Smith (1981) and in dodecylphosphocholine micelles by Mendz et al. (1984). The data presented in this work are in excellent agreement with the above-mentioned results.

Two additional points deserve comment. Increasing detectably the amount of free water in the system above w_0 = 7.0 does not modify the folding of MBP. Also, the conformation of MBP is not measurably affected by changes in AOT concentration, ruling out any direct interaction between the surfactant monomers and MBP. In contrast, changing the surfactant from anionic (AOT) to nonionic (Nikkol) results in a decrease of the percent of induced α-helix by about 50%. The preferential binding of interfacial water by MBP can explain both results in part. In such a case, additional free water would remain at distance in a larger water core, leaving the folded protein structure unperturbed. Furthermore, as the Nikkol uncharged oxyethylene groups are unable to bind water at the interface with the same strength as the charged AOT molecules, a less ordered structure is induced. Another possibility, which does not preclude the first one, is a tight contact of the charged MBP side chains with the AOT polar head groups inducing the observed α-helicity. These contacts would then be weakened in the case of Nikkol as a surfactant, with a less folded protein as a consequence.

Reactivity of MBP in Reverse Micelles. An important question in the understanding of MBP–lipid interaction is the localization of peptide residues or segments coming in contact with the polar head groups. In this respect, it is important to note that the most recent reports (Harris & Findlay, 1983; Frazer & Deber, 1984; Weise, 1985; Mendz et al., 1984) argue against lipid bilayer penetration by extensive parts of the polypeptide chain. Nevertheless, strong head-group interaction is not precluded (Sedzik et al., 1984).

Our results with MBP encapsulated into reverse micelles show that segments of the polypeptide chain are in contact with water. For example, lysine ϵ-amino groups were found accessible to the water-soluble fluorescent reagent *o*-phthaldehyde, and the binding of hemin by micellar solutions of the protein could be performed, as described by Vacher et al. (1984), indicating that the adjacent residues to the Phe–Phe 44–45 bond were also accessible to aqueous solvents (unpublished results).

As expected, the sole tryptophan residue of MBP (Trp-117) was found to be completely accessible to oxidation by *N*-bromosuccinimide in aqueous solutions. However, upon insertion of MBP into reverse micelles, the indole ring became inaccessible to the reagent, the high consumption of reagents probably indicating a nonspecific side reaction with tyrosines (Spande & Witkop, 1967). In contrast, the model tryptophan compound (NATA), tested in identical micellar solutions, was fully reactive with *N*-bromosuccinimide, ruling out any nonspecific interaction of Trp with AOT.

The difference in accessibility of the indole ring to water in NATA and MBP, both solubilized in micellar solutions, is further evidenced by comparison of fluorescence emission spectra, upon excitation at 295 nm, as a function of w_0. The observed blue shift of Trp-117 in AOT reverse micelles as compared to aqueous solutions and its invariance to increasing amounts of water indicate its shielding from the aqueous solvent. This behavior contrasts with the continuous variation of the wavelength of maximum fluorescence emission for NATA as a function of w_0, indicating in the latter case a progressively higher polar environment of the chromophore and/or changes in the physical properties of entrapped water, such as dielectric constant and viscosity. It has to be underlined that, even at the highest water to surfactant molar ratio measured (w_0 = 22.4), the maximum wavelength of fluorescence emission is still to the blue of a fully exposed indole derivative. The blue-shifted emission maximum observed in reverse micelles compares well with previous measurements by Vadas et al. (1981), using MBP in 70% aqueous glycerol at 20 °C (λ_{max} 342 nm) and in phosphatidylserine vesicles (λ_{max} 335 nm). Since the viscosity of water measured in reverse micelles, at low w_0 values, corresponds to that of a 78% v/v glycerol aqueous solution (Tsuji et al., 1983), we may conclude that in micellar solutions Trp-117 is inaccessible as a result of the conformational change described above. This is supported also by the fluorescence emission maximum of MBP in Nikkol micelles at w_0 = 5.6 (λ_{max} = 341 nm). Thus, there is a correlation between the absence of charged head groups, an increased accessibility of Trp-117 to solvent, and a loss of α helix of MBP in Nikkol. Finally, any interaction of the indole ring with the apolar solvent would result in a shift to the much shorter wavelength of about 325 nm (Lakowicz, 1983) and can be ruled out.

Dynamics of Trp-117 in Reverse Micelles. In reverse micelles, the tryptophan environment within the protein becomes more apolar or more constrained in the nanosecond time scale, considering the blue shift of the emission maximum. Steady-state and time-resolved fluorescence anisotropy measurements demonstrate that these changes are accompanied by severe restrictions of the tryptophan rotational motion upon incorporation of MBP in reverse micelles. This motion, in micelles, is clearly hindered, indicating that the fluorophore rotates within a restricted volume inside the protein matrix

(Lakowicz et al., 1983; Ichiye & Karplus, 1983) in striking contrast to its motion in water (Munro et al., 1979). The term $A_1 \exp(-t/\phi_1)$ of the anisotropy decay in eq 2 represents the rotation within the restricted volume. The second term A_2 is the coefficient of an exponential with an infinite correlation time. It arises from a slow rotation in common with the protein in the micelle. This slow motion originates from the rotation of the micelle as a whole and not from an independent rotation of the protein, owing to the high viscosity of entrapped water (Tsuji et al., 1983). Actually, the calculated effective hydrodynamic radius of AOT micelles occupied by MBP was found to have values ranging from 42 to 57 Å for w_0 of 5.6 and 22.4, respectively (Chatenay et al., 1985). Corresponding correlation time values, calculated from the Stokes–Einstein relation, at 20 °C ($\eta_{isooctane}$ = 0.54 cP), would range from 42 to $\simeq$100 ns, which, on the time scale of the fluorophore emission, are indeed infinite. It can be remarked that the amplitude of the infinite component remains constant, indicating that the average distribution angle of Trp-117 does not change with increased amounts of water in the micelle. In contrast, the rotational motion of the fluorophore within the restricted volume becomes faster with increased w_0. Therefore, increasing the water content of micelles does not measurably modify the protein conformation at least in the vicinity of Trp-117 but only the internal protein dynamics.

The fluorescence decay behavior of MBP deserves some comments. Total fluorescence intensity decays of Trp-117, either in water or in reverse micelles, are quite complex but can be adequately fitted by biexponential functions. In water, the existence of a flexible, extended, polypeptide chain would provide, as previously reported by Ross et al. (1981) for ACTH-(1–24), a distribution of slightly different tryptophan environments and therefore lead to a distribution of fluorescence lifetimes. In reverse micelles, in contrast, a more ordered structure of MBP, generating rotational barriers to the Trp-117 motion in the nanosecond time scale, is observed. As pointed out by Wahl (1975), the existence of such barriers would lead to complex decay kinetics. It would provide at the same time an additional dynamic quenching.

In this work, we have shown that the rotation barriers for Trp-117 remain unaffected by increased amounts of water whereas its rotational motion within the restricted volume becomes faster. This, in turn, enhances the collision probability of the fluorophore with the rotation barriers and decreases the mean excited-state lifetime to values even lower than that observed in pure water.

In summary, the results presented here demonstrate that MBP, inserted in reverse micelles, displays a set of distinctive characteristics that may mirror some of its properties in the aqueous myelin spaces. (i) It is a water-soluble protein, but unlike other water-soluble proteins, it exhibits an atypically high affinity for interfacial water. (ii) Although segments of the protein react with water-soluble reagents at any w_0 values studied, other segments are shielded from the solvent (namely, in the vicinity of Trp-117) and seem to be immobilized close to surfactant polar heads. (iii) The ordered conformation of encapsulated MBP is unaffected by the water content of the system, indicating severe restrictions in the dynamics of the polypeptide chain, although a more relaxed environment of the fluorophore is observed at higher water content.

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Registry No. AOT, 10041-19-7; isooctane, 540-84-1.

REFERENCES

Benson, J. R., & Hare, P. E. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 619–622.

Berlman, I. B. (1971) in *Handbook of Fluorescence Spectra of Aromatic Molecules*, p 220, Academic Press, New York.

Birch, D. J. S., & Imhof, R. E. (1981) *Rev. Sci. Instrum. 52*, 1206–1212.

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

Braun, P. (1984) in *Myelin* (Morell, P., Ed.) pp 97–113, Plenum, New York.

Chang, C. T., Wu, C. S. C., & Yang, J. T. (1978) *Anal. Biochem. 91*, 13–31.

Chatenay, D., Urbach, W., Cazabat, A. M., Vacher, M., & Waks, M. (1985) *Biophys. J.* (in press).

Chen, Y. H., Yang, J. T., & Chan, K. H. (1974) *Biochemistry 13*, 3350–3359.

Chen, L. A., Dale, R. E., Ross, S., & Brand, L. (1977) *J. Biol. Chem. 252*, 2163–2169.

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

Delahodde, A., Vacher, M., Nicot, C., & Waks, M. (1984) *FEBS Lett. 172*, 343–347.

Eylar, E. H. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1425–1431.

Fendler, J. H. (1982) *Membrane Mimetic Chemistry*, pp 55–71, Wiley, New York.

Frazer, P. E., & Deber, C. M. (1984) *J. Biol. Chem. 259*, 8689–8692.

Gent, W. L. G., Grant, E. M., & Tucker, S. W. (1970) *Biopolymers 9*, 124–126.

Golds, E. E., & Braun, P. E. (1976) *J. Biol. Chem. 251*, 4729–4735.

Grandi, C., Smith, R. E., & Luisi, P. L. (1981) *J. Biol. Chem. 256*, 837–843.

Harris, R., & Findlay, J. B. C. (1983) *Biochim. Biophys. Acta 732*, 75–82.

Ichiye, T., & Karplus, M. (1983) *Biochemistry 22*, 2884–2890.

Imre, V. E., & Luisi, P. L. (1982) *Biochem. Biophys. Res. Commun. 107*, 538–545.

Keniry, M., & Smith, R. (1981) *Biochim. Biophys. Acta 668*, 107–118.

Kirschner, D. A., Ganser, A. L., & Caspar, D. L. D. (1984) in *Myelin* (Morell, P., Ed.) pp 51–91, Plenum, New York.

Kuntz, I. D., & Kauzmann, W. (1974) *Adv. Protein Chem. 28*, 239–345.

Lakowicz, J. R. (1983) in *Principles of Fluorescence Spectroscopy*, p 346, Plenum, New York.

Lakowicz, J. R., Maliwal, B. P., Cherek, M., & Balter, A. (1983) *Biochemistry 22*, 1741–1752.

Laursen, R. A., Samiullah, M., & Lees, M. B. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2912–2916.

Lees, M. B., Chao, B. H., Lin, L. F. H., Samiullah, M., & Laursen, R. A. (1983) *Arch. Biochem. Biophys. 226*, 643–656.

Lipari, G., & Szabo, A. (1980) *Biophys. J. 30*, 486–506.

Luisi, P. L., & Wolf, R. (1982) in *Solution Behavior of Surfactants* (Mittal, P., & Fendler, J. M., Eds.) pp 887–905, Plenum, New York.

Luisi, P. L., Henninger, F., Joppich, M., Dossena, A., & Casnati, G. (1977) *Biochem. Biophys. Res. Commun. 74*, 1384–1389.
Luisi, P. L., Meier, P., Imre, V. E., & Pande, A. (1984) in *Reverse Micelles* (Luisi, P. L., & Straub, B. E., Eds.) Plenum, New York.
Maitra, A. (1984) *J. Phys. Chem. 88*, 5122–5125.
Martenson, R. E. (1978) *J. Biol. Chem. 253*, 8887–8893.
Martenson, R. E. (1981) *J. Neurochem. 36*, 1543–1560.
Mendz, G. L., Moore, W. J., Brown, L. R., & Martenson, R. E. (1984) *Biochemistry 23*, 6041–6046.
Munro, I., Pecht, I., & Stryer, L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 56–60.
Ong, R. L., & Yu, R. K. (1984) *J. Neurosci. Res. 12*, 377–393.
Ross, J. B. A., Rousslang, K. W., & Brand, L. (1981) *Biochemistry 20*, 4361–4369.
Sedzik, J., Blaurock, A. E., & Hoechli, M. (1984) *J. Mol. Biol. 174*, 385–409.
Spande, T. F., & Witkop, B. (1967) *Methods. Enzymol. 11*, 498–506.
Spencer, R. D., & Weber, G. (1970) *J. Chem. Phys. 52*, 1654–1663.
Stoffel, W., Hillen, H., Schröder, W., & Deutzmann, R. (1983) *Hoppe-Seyler's Physiol. Chem. 365*, 1455–1466.
Stoffel, W., Hillen, H., & Giersiefen, H. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5012–5016.
Thompson, F., & Gierasch, L. M. (1984) *J. Am. Chem. Soc. 106*, 3648–3652.
Tsuji, K., Sunamoto, J., & Fendler, J. H. (1983) *Bull. Chem. Soc. Jpn. 56*, 2889–2893.
Vacher, M., Nicot, C., Pflumm, M., Luchins, J., Beychok, S., & Waks, M. (1984) *Arch. Biochem. Biophys. 231*, 86–94.
Vadas, E. B., Melancon, P., Braun, P. E., & Galley, W. C. (1981) *Biochemistry 20*, 3110–3116.
Valeur, B., & Weber, G. (1977) *Photochem. Photobiol. 25*, 441–444.
Vincent, M., & Gallay, J. (1984) *Biochemistry 23*, 6514–6522.
Vincent, M., de Foresta, B., Gallay, J., & Alfsen, A. (1982) *Biochemistry 21*, 708–716.
Wahl, P. H. (1975) *Chem. Phys. 7*, 220–228.
Wahl, P. H., Auchet, J. C., & Donzel, B. (1974) *Rev. Sci. Instrum. 45*, 28–32.
Weise, M. J. (1985) *J. Neurochem. 44*, 163–170.
Wong, M., Thomas, J. K., & Grätzel, M. (1976) *J. Am. Chem. Soc. 98*, 2391–2397.