Phosphorescence Studies of the Interaction of Myelin Basic Protein with Phosphatidylserine Vesicles†

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ABSTRACT: Phosphorescence from the lone tryptophan residue has been studied to monitor the interaction of myelin basic protein with phosphatidylserine vesicles. Spectral shifts in the phosphorescence of the protein in a glycerol–buffer (70:30 w/w) solvent at low temperature are consistent with fluorescence data obtained under ambient conditions, indicating that the tryptophan side chain is exposed to the solvent in the free protein but is buried on interaction with a lipid bilayer. Measurements of the phosphorescence intensity and lifetime as a function of temperature reveal a marked protection of the tryptophan to thermally induced quenching in the presence of phosphatidylserine vesicles. Steady-state anisotropy measurements on the tryptophan phosphorescence were used to follow the slow motions of the protein associated with the synthetic bilayer. The observation that the rotational correlation time for the membrane-associated protein is 4×10^3 times that anticipated for a molecule the size of basic protein reflects its partial intrinsic character in the membrane.

The architecture and dynamics of the myelin sheath, as with other macromolecular assemblies, are determined by the nature and strength of the interactions among the various components that make up the overall structure. Measurements of the molecular dynamics of systems of this type, therefore, not only reveal structural fluctuations that might be of functional significance but also provide insights into the intermolecular interactions and thereby the architecture of the system as well.

Of the components that make up the myelin sheath, basic protein constitutes about one-third of the total protein (Braun & Brostoff, 1977). It has received considerable attention as a result of its ability to elicit the formation of an autoimmune response which produces experimental allergic encephalomyelitis, a pathological state bearing some resemblance to multiple sclerosis (Kies, 1965; Eylar, 1972). The conformation of myelin basic protein (MBP)[1] has been studied in some detail in solution through a variety of physical–chemical techniques. Evidence has been presented favoring either an ordered prolate ellipsoid model for the structure (Eylar & Thompson, 1969; Epand et al., 1974) or a solvated random coil (Chao & Einstein, 1970; Krigbaum & Hsu, 1975). NMR evidence employing natural-abundance ^{13}C has revealed that while parts of the molecular do possess considerable freedom of motion, there does appear to be a more structured core which contains the lone tryptophan in the molecule (Chapman & Moore, 1978).

Less is known about the intereactions of MBP with other components of the myelin sheath. By virtue of its net positive charge under physiological conditions, it interacts with negatively charged phospholipids (Stollery et al., 1980). Additional evidence suggests that it probably penetrates into the bilayer. The protein expands lipid monolayers (Demel et al., 1973; London et al., 1973) and reduces the enthalpy of the lipid phase transition with acidic phospholipids (Papahadjopoulos et al., 1975), and parts of the molecule are selectively protected against trypic digestion in the presence of lipids (London & Vossenberg, 1973). Chemical cross-linking studies have provided evidence that basic protein in the intact myelin structure may exist as a dimer which serves in cross-bridging myelin sheets (Golds & Braun, 1978a,b).

The influence of the interaction between MBP and lipid on the motions of the components has been probed with spin-resonance spectroscopy. Spin-labeled fatty acids were used to show that the mobility of the lipids in synthetic bilayers was primarily retarded in the polar head-group region (Boggs & Moscarello, 1978). Conversely, lipid–protein interactions have been shown to effect the motions of MBP spin-labeled at the two methionine residues, depending on the nature of the lipid (Stollery et al., 1980). However, NMR studies with ^{13}C enrichment of these methionine residues indicates that the side chains are probably exposed to solvent and retain considerable mobility when the protein is associated with lipids (Deber et al., 1978). Regions of the protein more severely restricted in their motions through interaction with the bilayer would be difficult to study due to marked broadening of the NMR lines.

Although MBP possesses a single tryptophan residue which could be expected to serve as a natural probe of the protein motion, as a consequence of lipid–protein and possibly protein–protein interactions, rotational motions would be anticipated to fall outside the nanosecond time range, precluding the use of fluorescence anisotropy measurements. The rotational mobility of membrane proteins has been detected, however, by taking advantage of the long lifetime of the triplet state, employing anistropy measurements on the phosphorescence of the tryptophan residues (Strambini & Galley, 1973, 1976) and of eosin in labeled proteins (Austin et al., 1979) and from the decay in the dichroism of the T–T absorption or absorption of the depleted ground state of eosin-labeled membrane proteins (Cherry & Schneider, 1976).

Phosphorescence anisotropy measurements on the intrinsic chromophores in proteins possess the distinct advantage that concerns over the perturbations introduced with large aromatic probes, particularly with proteins as small as MBP, are avoided. The disadvantage of employing the triplet state of tryptophan as a rotational probe is that the natural lifetime

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[1] Abbreviations used: MBP, myelin basic protein; NMR, nuclear magnetic resonance; PS, phosphatidylserine; T–T, triplet–triplet absorption.

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is very long. However, with the use of viscous solvents at subzero temperatures motions, which in aqueous solution at room temperature would occur in the microsecond time range, can be slowed to the point where rotational correlation times fall into the range of the triplet lifetime (Strambini & Galley, 1976).

Steady-state phosphorescence measurements on the lone tryptophan in myelin basic protein were performed under these conditions in the present study. Phosphorescence spectral and intensity changes provide evidence for burial of the tryptophan side chain on interaction of the protein with phosphatidylserine vesicles, while the anisotropy data indicate a marked restriction in the rotational mobility of the vesicle-associated protein. This suggests an intrinsic role for MBP in the myelin sheath.

Materials and Methods

Preparation of MBP. Myelin was prepared from freshly excised rat brain by the method of Norton & Poduslo (1973). The myelin was partially delipidated with acetone and the basic proteins were acid extracted with 0.1 M KCl according to Lim et al. (1974). The acid-extracted proteins were separated on a preparative gel (Barbarese et al., 1977). Of the four myelin basic proteins, the 14 000-dalton protein was used in the fluorescence and phosphorescence experiments, and the 18 500-dalton protein was employed in some of the phosphorescence anisotropy studies. The proteins were tested for purity on polyacryamide gels and were found to migrate as single components both on $NaDodSO_4$-containing gels (King & Laemmli, 1971) and on an acidic gel (Panyim et al., 1971). The proteins were stored as lyophilized powders at −20 °C and were used within a few days of preparation.

Preparation of Lipid Vesicles. Phosphatidylserine (PS) was obtained from Applied Science Laboratories, Inc., and Serdary Research Laboratories (London, Ontario) as a chloroform solution and kept under nitrogen in a sealed ampule at −20 °C. The samples were tested for purity chromatographically. The vesicles were prepared by evaporating the chloroform under a gentle stream of nitrogen at room temperature and then adding glycerol–phosphate buffer (pH 6.8, 0.15 M KCl) solution which was 70% glycerol (Matheson Coleman and Bell, chromatoquality) by weight. The vesicles formed spontaneously as observed by the immediate appearance of a slight turbidity in the solution. The dispersion was sonicated intermittently for 3 min in an ultrasonic cleaning bath. The vesicles were examined by transmission electron microscopy using negative staining techniques and were found to be polydisperse, the particle diameters being between 300 and 1500 Å with the majority of the vesicles having diameters of ~500–700 Å.

Preparation of Lipid Vesicle–Protein Complexes. Solutions of the purified myelin basic protein were made in the 70:30 w/w glycerol–phosphate buffer solution at 1–2 mg/mL and added to 5–10 mg of dry lipid. Again, vesicle formation was spontaneous and the resulting dispersions were sonicated intermittently for a total of 3 min at 30-s intervals. Emission experiments were carried out on the samples the same day.

Emission Experiments. Fluorescence and phosphorescence data were obtained with an apparatus described previously (Purkey & Galley, 1970). The excitation wavelength from a 110-W high-pressure Hg arc (Illumination Industries, Nutley, NJ) was selected with a monochromator (Bausch & Lomb, 250 mm) with a 10-nm band-pass, or at temperatures (>−60 °C) at which the phosphorescence was weak, the exciting light was simply filtered with 5 cm of 0.01 M benzoic acid solution. This maximized the tryptophan phosphorescence intensity but simultaneously eliminated excitation of tyrosine residues in the protein when required.

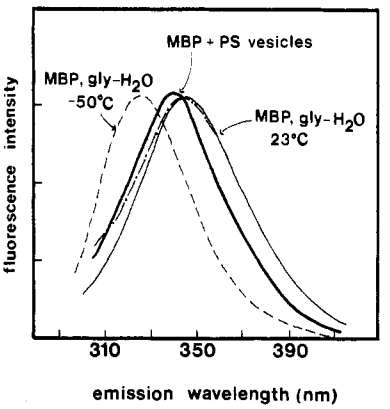

FIGURE 1: Fluorescence spectra of MBP. (—) 5×10^{-5} M MBP in 70:30 w/w glycerol–phosphate buffer (pH 6.8), λ_{ex} = 300 nm. (·--) Same as above with λ_{ex} = 280 nm. (---) Same as above with λ_{ex} = 300 nm; T = −50 °C. (—) MBP in the presence of the PS vesicles in 70:30 glycerol–buffer at 23 °C. The relative intensities of the various spectra cannot be compared.

For emission anisotropy experiments the excitation and emission beams were polarized with UV-transmitting linear polarizing film (Polaroid HNB'P). The phosphorescence anisotropy A was calculated from $A = (I_{\parallel} - G \cdot I_{\perp})/(I_{\parallel} + 2G \cdot I_{\perp})$ in which $I_{\parallel}$ and $I_{\perp}$ are the phosphorescence intensities polarized parallel and perpendicular to the vertically polarized exciting light, respectively. The correction factor G was obtained from the ratio of vertically to horizontally polarized emission excited with horizontally polarized light (Azumi & McGlynn, 1962).

Emission was observed with the samples contained in 2 mm i.d. Spectrosil tubes which were centered in a quartz Dewar equipped with Spectrosil windows. The temperature of the sample was controlled by the circulation of precooled N_2 through a side arm in the Dewar. The temperature of the sample was monitored with a copper–constantan thermocouple taped against the sample tube.

Results

Tryptophan Emission of Myelin Basic Protein in Solution. The fluorescence spectrum of the small rat myelin basic protein in 70:30 (w/w) glycerol–phosphate buffer (pH 6.8) at room temperature appears in Figure 1. The wavelength maximum of the emission in the presence of glycerol is at 342 nm, blue shifted by 6 nm in comparison with the fluorescence observed for the protein in purely aqueous solution (Burnett & Eylar, 1971; Jones & Rumsby, 1975). These room-temperature wavelength maxima for the protein in either solvent do not appear as far to the red as the corresponding spectra for free tryptophan. The data are consistent with the exposure of the lone tryptophan residue in the protein to the polar solvent with the wavelength maximum occurring to the blue in the presence of glycerol due to the somewhat low solvent polarity. However, the lack of a correspondence between these emission maxima and those observed for free tryptophan indicates that the exposure of the tryptophan residue in the protein is not complete or that there are additional interactions of the aromatic side chain with the protein itself.

With the protein in the glycerol–buffer solvent, the solvent-exposed nature of the tryptophan residue can be readily confirmed from the temperature dependence of the fluorescence spectrum in that the solution does not freeze at 0 °C. The spectra in Figure 1 and the data in Figure 2 reveal that the spectrum retreates to the blue as the viscosity of the solvent is increased with decreasing temperature. This retreat occurs

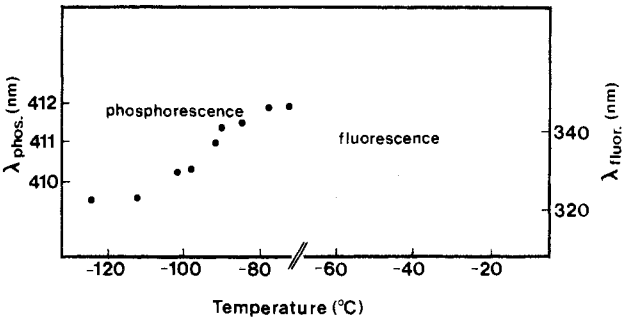

FIGURE 2: Emission wavelength maxima for MBP in 70:30 glycerol–buffer as a function of temperature. The red shift observed with increasing temperature is a consequence of relaxation of the polar solvent.

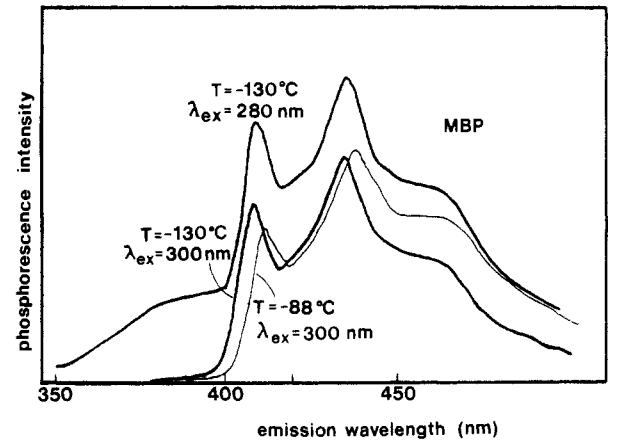

FIGURE 3: Phosphorescence spectra of 10^{-4} M MBP in 70:30 glycerol–buffer.

until a temperature is attained at which solvent reorganization about the chromophore can no longer occur during the nanosecond excited-state lifetime (Lippert, 1957; Bakhshiev & Piterskaya, 1966; Eisinger & Navon, 1969). On the assumption that the spectrum attained at low temperature is reasonably representative of the spectrum that would be observed at room temperature immediately following excitation, then subsequent relaxation of the mobile solvent must result in a 22-nm red shift to produce the 342-nm room-temperature fluorescence of MBP in the glycerol–water solvent.

These solvent-induced effects observed in the fluorescence of the protein in the glycerol–buffer are also apparent from phosphorescence measurements but at lower temperatures. Excitation at 300 nm results selectively in the appearance of the tryptophan component of the phosphorescence of MBP. At the temperature (−130 °C) for which the spectrum appears in Figure 3, the particular solvent employed is sufficiently rigid that solvent-relaxation effects do not manifest themselves in the spectra (Milton et al., 1978) so that comparisons can be made with tryptophan in other static environments. The 409- and 436-nm wavelength maxima observed for MBP in Figure 3 are at longer wavelengths than the 407- and 434-nm maxima observed for free tryptophan (Konev, 1967; Longworth, 1971), again suggesting additional interactions of the indole side chain with the protein itself. These wavelength maxima, however, are within the range of values found for solvent-exposed tryptophans in peptides and proteins (Ross et al., 1980; Hershberger et al., 1980).

At higher temperatures a red shift occurs in the tryptophan phosphorescence spectrum of MBP over a rather narrow temperature range. The phosphorescence shift appearing in Figure 2 is considerably smaller and occurs at a lower temperature than that observed in the fluorescence spectrum. The smaller red shift arises as a consequence of a smaller change in the electronic structure of tryptophan when excitation is to the excited triplet as compared to the excited singlet state. The lower temperature and therefore higher solvent viscosity at which the red shift occurs in the phosphorescence, on the other hand, derives from the duration (seconds) of the triplet-state lifetime which allows slow relaxation effects to appear (Galley & Purkey, 1970).

The sensitivity of the tryptophan phosphorescence of myelin basic protein to thermally induced quenching provides an alternate measure of the ability of the solvent to interact with the chromophore, bearing in mind that quenching by solvent-mediated processes is a function not only of the location of the residue with respect to the solvent but also of the protein flexibility as well (Saviotti & Galley, 1974; Weber & Lakowicz, 1973; Eftink & Ghiron, 1975). It is evident from Figure 5 and earlier work (Domanus et al., 1980) that the phosphorescence is readily quenched with increasing temperature over the temperature range at which the solvent-relaxation red shift is observed. A comparison of the temperature dependence of the phosphorescence lifetime of the lone tryptophan in MBP with that of other systems reveals that, as anticipated from the spectral data, the susceptibility of the residue to quenching is much more akin to that observed for free tryptophan than for buried residues in globular proteins.

Tyrosine Emission of Myelin Basic Protein in Solution. The room-temperature fluorescence spectrum of the small basic protein depicted in Figure 1 displays only a 2-nm blue shift with very little change in shape with excitation at 280 nm. These minor perturbations are observed with the protein dissolved in either the purely aqueous or the glycerol–buffer solvent and reveal that tyrosine dose not contribute greatly to the fluorescence. The spectrum for the small basic protein (14 000 daltons) obtained with excitation at 280 nm does not exhibit the very prominent tyrosine shoulder reported by Jones and Rumsby (1975) for the large protein (18 500 daltons) which contains four tyrosine residues rather than the two in the small protein (Dunkley & Carnegie, 1974).

The evidence for a tyrosine contribution for the small basic protein is, however, definitive in the phosphorescence. The low-temperature phosphorescence spectrum displays a distinct tyrosine component with excitation at 280 nm. This appears in Figure 3 as a structureless emission between 350 and 450 nm (Longworth, 1961) which is absent from the spectrum obtained with 300-nm excitation. The contribution of this tyrosine component is significantly larger relative to the tryptophan in the large rat basic protein (unpublished observations).

Tryptophan Emission of Basic Protein in the Presence of PS Vesicles. The interaction of myelin basic protein in the glycerol–buffer solvent with PS vesicles results in the spectral shifts which mimic those observed on the addition of NaDodSO$_4$ to an aqueous solution of MBP at room temperature (Jones & Rumsby, 1975). The room temperature flurescence spectrum (Figure 1) is shifted to the blue in the presence of PS vesicles and becomes independent of temperature. The absence of the solvent-relaxation red shift indicates that the lone tryptophan in MBP becomes shielded from the polar solvent when the protein interacts with the lipid bilayer. It should be noted that while the 335-nm temperature-independent emission maximum observed in the presence of PS vesicles is to the blue of the spectrum of the free protein at room temperature, it is 15 nm to the red of the spectrum (320 nm) observed for the free protein at low temperatures where the solvent is polar and rigid. This type of comparison of the

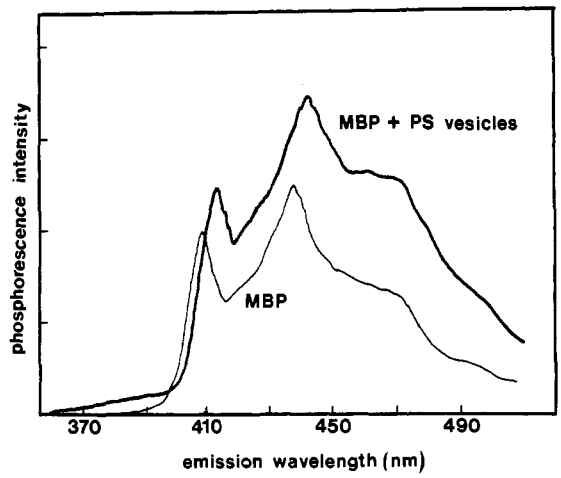

FIGURE 4: Phosphorescence spectra of MBP of the lone tryptophan in MBP in the presence of phosphatidylserine vesicles.

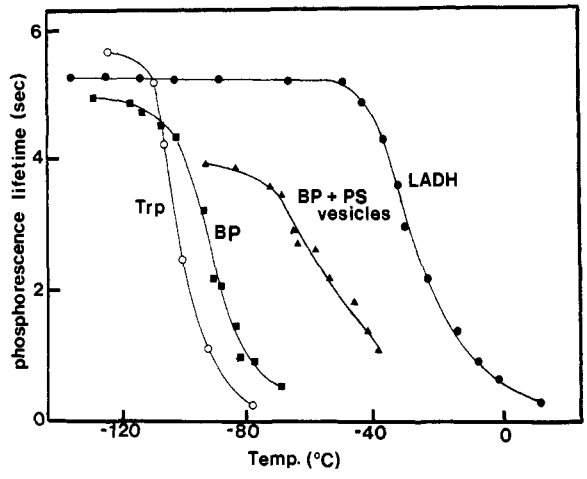

FIGURE 5: Temperature dependence of the phosphorescence lifetime of tryptophan free in solution and in a number of protein environments. The data for liver alcohol dehydrogenase (LADH) and free tryptophan are from earlier work (Domanus et al., 1980). The solvent was 70:30 (w/w) glycerol–buffer in all cases.

spectra for the free and bound protein at low temperature indicates that the tryptophan in the vesicle-associated MBP is influenced by a local environment with a higher polarizability and/or lower polarity than the polar solvent (Purkey & Galley, 1970).

Corresponding spectral changes in the tryptophan phosphorescence wavelength maxima, albeit smaller in magnitude than for fluorescence, reflect the transfer of the tryptophan side chain from a more polar to a less polar and/or more polarizable environment on interaction of MBP with PS vesicles. The wavelength maxima of the tryptophan phosphorescence of MBP in the presence of PS vesicles (Figure 4) occur at 411 and 439 nm or 2–3 nm to the red of the spectrum for the free protein in rigid medium, i.e., in the absence of solvent relaxation below −120 °C. Furthermore, as with the fluorescence, the spectrum in the presence of liposomes is independent of temperature throughout the region in which a solvent-relaxation red shift is observed for the free protein (Figure 2).

A comparison in Figure 5 of the tryptophan phosphorescence lifetime for MBP in the presence of vesicles with that observed for the protein in glycerol–buffer alone reveals that the tryptophan is afforded considerable protection against thermally induced quenching. At −70 °C the phosphorescence lifetime of the solvent-exposed tryptophan in the free protein can be seen to have fallen by a factor of 10 while that of the vesicle-bound protein remains essentially unaltered at this temperature from its low-temperature 3.8-s limiting value. The transition in the tryptophan phosphorescence lifetime that does occur for the liposome-associated protein above −70 °C can be also compared in Figure 5 with the corresponding data for liver alcohol dehydrogenase (Domanus et al., 1980). It is evident that the protection afforded the tryptophan in liposome-bound MBP is considerably less than that experienced by buried tryptophans in globular proteins which possess sufficient local rigidity to display long-lived phosphorescence at ambient temperatures (Saviotti & Galley, 1974).

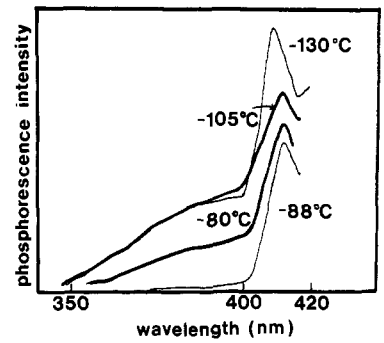

FIGURE 6: Tyrosine phosphorescence of MBP in the absence and presence of PS vesicles as a function of temperature; λ_{ex} = 280 nm. The results indicate protection of the tyrosine phosphorescence in small MBP to quenching in the presence of PS vesicles.

Tyrosine Emission of Vesicle-Associated MBP. One or both of the tyrosine residues in the small rat MBP are similarly influenced by a change in local environment when the protein is associated with PS vesicles. Phosphorescence spectra of free and liposome-bound MBP, excited at a wavelength which is absorbed by tyrosine, are displayed in Figure 6. It is apparent from the spectra for the free protein at −130 and −88 °C that the tyrosine phosphorescence, as was observed with other proteins (McCarville & McGlynn, 1969; Saviotti & Galley, 1974), is more readily quenched than tryptophan. The corresponding spectra for the protein associated with PS vesicles, on the other hand, reveal that despite the decreased quenching of tryptophan, the tyrosine to tryptophan ratio remains higher than that for the free protein, indicating an even greater relative protection of one or both tyrosine residues in small MBP. The spectral and phosphorescence quenching data provide evidence which suggests that myelin BP interacts with PS vesicles in such a way that both the lone tryptophan and at least one of the two tyrosines interact with the bilayer. This, however, does not reveal the extent to which the protein penetrates into the bilayer or the degree to which it is immobilized by it. In order to bring evidence to bear on this latter point, we performed phosphorescence measurements which are sensitive to motions on a slow time scale and which can be carried out on the native protein emission (Strambini & Galley, 1973, 1976, 1980).

Steady-State Tryptophan Phosphorescence Anisotropy Measurements of MBP in the Presence and Absence of PS Vesicles. The tryptophan residues in the protein were selectively excited (λ_{ex} >295 nm), and the steady-state phosphorescence anisotropy was monitored at the 410-nm maximum in the phosphorescence. A plot of anisotropy A as a function of temperature for both the free MBP and vesicle-bound MBP in glycerol–buffer appears in Figure 7. The limiting anisotropy observed for the free protein at low temperatures (A_0 = −0.14) is characteristic of tryptophan both in rigid medium (Konev, 1967) and in a number of proteins under these conditions (Strambini & Galley, 1976). It indicates the absence of detectable rotational motions in the

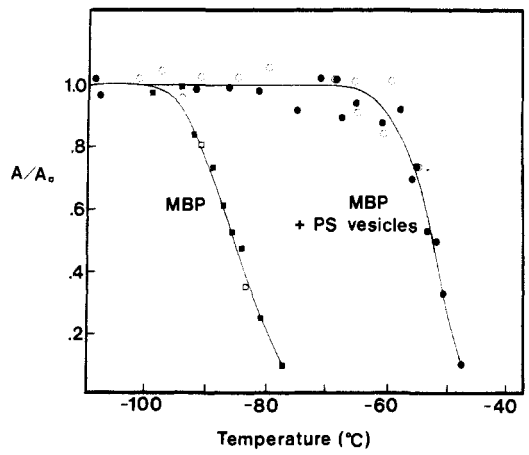

FIGURE 7: Normalized phosphorescence anisotropy A/A_0 as a function of temperature for free and bound MBP. The transition for the vesicle-associated protein at a higher temperature results from a marked reduction in rotational mobility.

sample below −87 °C. The value of A_0 for MBP associated with PS vesicles ($A_0 = -0.085$) was consistently lower than that observed for the free protein as a consequence of depolarization due to scattering in the somewhat turbid vesicle suspension.

A decline in the steady-state anisotropy is observed at a considerably higher temperature for the vesicle-associated protein than for the lipid-free protein in the glycerol–buffer solvent. Reference to the Perrin equation, $A/A_0 = 1/[1 + \tau(T)/\phi(T)]$, reveals that the temperature profiles in Figure 7 are a reflection of the temperature dependence of both the excited-state lifetime, τ, and the rotational correlation time, ϕ. As indicated previously (Figure 4), differences in the quenching susceptibility of the tryptophan residue in the free and bound protein do result in distinctly different values of τ above −90 °C. However, with increasing temperature the more rapidly declining lifetime observed for the free protein would ten in fact to minimize any real difference in the rotational behavior of MBP in the free and bound forms. The quite distinct temperature transitions for the free and bound protein, therefore, represent a very large difference in rotational mobility of the protein in these two states.

From values of A/A_0 and the phosphorescence lifetime τ, ϕ was calculated as a function of temperature for free MBP in the glycerol–buffer solvent and appears in Figure 8 along with the corresponding values obtained with *Staphylococcus* nuclease and human serum albumin, both of which also possess single tryptophan residues. These rotational correlation times are governed by the hydrodynamic volumes v for the proteins involved and the viscosity η of the medium in which they move [$\phi = v\eta(T)/kT$)]. Although the steepness of the transitions in Figure 7 is dominated by the temperature dependence of the solvent viscosity, variations in ϕ at a given viscosity or temperature are a reflection of differences in hydrodynamic volumes between the proteins.

The rotational correlation times obtained from the fluorescence anisotropy data for the lone tryptophan in *Staphylococcus* nuclease at room temperature in aqueous solution (Brochon et al., 1974; Munro et al., 1979) indicate that overall motion of the globular protein is responsible for the depolarization. The 4-fold difference between the values of ϕ for MBP with those for bovine serum albumin (Figure 8) at a given temperature is consistent with their difference in molecular weight and therefore with hydrodynamic volumes which are determined by overall globular structures. This trend is consistent with recent phosphorescence anisotropy data with

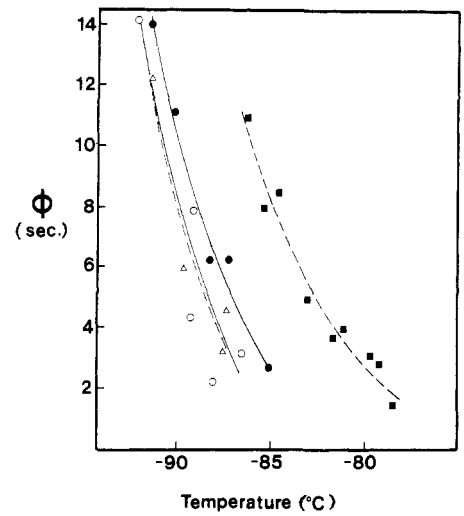

FIGURE 8: Protein rotational correlation times from the protein tryptophan phosphorescence anisotropy. (● and ○) MBP; (△) *Staphylococcus* nuclease; (■) BSA. Solvent: 70:30 (w/w) glycerol–phosphate buffer (pH 6.8).

other proteins (Strambini & Galley, 1976). A comparison between the values of ϕ for MBP and those obtained for *Staphylococcus* nuclease at a given temperature reveals that the hydrodynamic volumes for these two proteins are very similar. In view of the closeness in molecular weight of these two proteins, this suggests that overall motion of MBP or of a sizable part of its structure is at the origin of the phosphorescence depolarization for this protein as well.

The rotational correlation time for MBP when associated with PS vesicles remains infinitely long throughout the temperature region over which the free protein undergoes depolarization. In some experiments an initial decrease in A/A_0 at about −77 °C, which then returned to its maximum value at higher temperatures, was observed. This decrease and recovery of A/A_0 prior to the principal depolarization at higher temperatures (Figure 7) appeared to be associated with the presence of protein which was not bound to the PS vesicles. The motion of this protein initially contributes to a decline in A/A_0 but is selectively quenched with increasing temperature, leaving only the longer lived tryptophan phosphorescence from the liposome-associated MBP to contribute to the anisotropy. The principal depolarization associated with the liposome-bound protein is only observed at temperatures >−60 °C. Rotational correlation times for the bound protein were calculated from the relative change in anisotropy and the phosphorescence lifetimes in the transition region. Given the availability of viscosity data for the 70:30 w/w glycerol/buffer solvent in this temperature range (Kim, 1977), an effective hydrodynamic volume corresponding to the observed values of ϕ was calculated. The effective hydrodynamic volume derived from the motion observed for the liposome-bound MBP in this way is $(2.3 \pm 5) \times 10^{-17}$ cm^3. This would correspond to a spherical particle with a diameter of ~350 Å.

Discussion

The observation in the present study that myelin basic protein is severely restricted in its rotational motions when associated with phosphatidylserine vesicles points to the utility of measurements of the native phosphorescence of protein systems of this type. By virtue of the short (nanosecond) lifetime of the excited singlet state, particularly those of the native chromophores in proteins, the rotational motions associated with macromolecular assemblies, such as that seen in the present study, occur too slowly to be effectively probed

with fluorescence anisotropy experiments. The long lifetime of the triplet state obviates this problem (Strambini & Galley, 1973, 1976; Razi Naqvi et al., 1973; Cherry et al., 1976). On the other hand, the triplet state of tryptophan in proteins is often so long that the motions need to be slowed even further with viscous solvents to bring them into a time range wherein they can be monitored with steady-state phosphorescence anisotropy measurements (Strambini & Galley, 1976).

The large difference in rotational mobility between free MBP and vesicle-bound MBP made it impossible to extract rotational correlation times from the steady-state anisotropy data under identical conditions and directly compare them. Depolarization of the tryptophan phosphorescence of the free protein, for which the motions are much more rapid, was detected at a lower temperature and therfore much higher solvent viscosity than that for the bound protein. The correlation times for free MBP were simply compared with those of *Staphylococcus* nuclease to demonstrate that the values obtained were reasonable for a protein of this size. Hydrodynamic volumes for these two proteins were not computed from the rotational correlation times extracted from the data in that the viscosity of the solvent used has not been measured at these low temperatures. Munro et al. (1979) have carried out fluorescence anisotropy measurements on the tryptophans in these proteins at room temperature. Their observation of a more rapid decay in the anisotropy for MBP than for *Staphylococcus* nuclease indicates the presence of internal motions of the tryptophan side chain in the myelin protein. The absence of a significant difference between the behavior of *Staphylococcus* nuclease and that of MBP in our experiments implies that (a) tryptophan internal motions become less significant in comparison with overall rotations in the glycerol–buffer solvent at low temperatures and (b) the tryptophan internal motions are anisotropic, and because of the difference in singlet–singlet and triplet–singlet transition moment directions in the indole chromophore (Konev, 1967), fluorescence and phosphorescence anisotropy measurements preferentially monitor in-plane and out-of-plane motions, respectively. Time-dependent phosphorescence anisotropy measurements as a function of temperature and with a number of solvents are currently under way to decide between these alternatives.

The apparent hydrodynamic volume for the vesicle-associated MBP was calculated from experimental values of ϕ by using viscosity data for the solvent (Kim, 1977) which exists for the temperature range over which depolarization was observed in this case. However, the calculation from the relation $v = \phi kT/\eta$ of an hydrodynamic volume v with a diameter of 350 Å for the membrane-associated protein must be considered with caution. It has not been established from the present preliminary data whether it is indeed solvent which is restricting the motion of a tightly associated lipid–protein complex or assembly or if it is restrictions imposed by the bilayer on the protein itself which are involved. The calculated v provides a measure of the size of an assembly that *would be required* to account for the observed motions. The fact that the hydrodynamic volume calculated in this way is 4×10^3 times that anticipated for a globular protein of molecular weight 18 000 emphasizes the extent of the immobilization resulting from the interaction of MBP with PS vesicles. It is evident that myelin basic protein cannot be regarded as an extrinsic membrane protein which binds at the surface of the bilayer through a flexible linkage. It must be at least partially embedded in the bilayer and interacting strongly with it through lipid–protein interactions. While side-chain motion of parts of the membrane-associated protein such as those of the two methionine residues in the molecule appears to persist (Deber et al., 1978), that part of the protein containing the tryptophan residue must be strongly membrane associated. The finding that the tryptophan-containing portion of MBP is resistant to tryptic digestion when reconstituted with myelin-extracted lipids demonstrated this association (London & Vossenberg, 1973), although little protection was found with pure phosphatidylserine in the liquid-crystalline state.

Despite the interactions of the myelin basic protein with the bilayer, the motions that are observed do not result from the overall tumbling of the vesicles in solution since aggregation of vesicles in the presence of MBP (Smith, 1977) has been observed to occur. The aggregated vesicles are clearly larger than the 350-Å "particle" extracted from the anisotropy data. There must be some motions of the protein, or of the tryptophan side chain in the protein, with respect to the lipid vesicles. It is likely that it is the local membrane viscosity that dictates the time scale of the motions that are observed. Further studies will be required in which the membrane viscosity is measured under conditions of the presenct experiments in order to more precisely define whether the data reflect the motion of monomer MBP associated with liposomes in the gel state or if the restricted motion results in part from the dimerization of MBP which has been proposed as an integral part of the myelin structure (Golds & Braun, 1978a,b).

The addition of phosphorescence spectral data to that of fluorescence has aided in establishing that the changes in tryptophan environment accompanying the association of MBP with lipid occur in a similar manner under the conditions of the anisotropy measurements as they do at room temperature. The solvent-relaxation red shift for the free protein mimics that seen in the fluorescence at a much higher temperature, confirming the solvent-exposed nature of the tryptophan. In addition, the low-temperature tryptophan phosphorescence red shift observed on association of MBP with PS vesicles is consistent with the transfer of the side chain to a solvent-free environment. It is evident from this type of comparison that the nature of association of protein and membrane is retained in the glycerol–buffer solvent throughout the temperature region in which rotational behavior was monitored.

The sensitivity of the phosphorescence quenching of the tryptophan in MBP to the immediate environment of the protein provides an illustration of another useful aspect of triplet-state spectroscopy in probing membrane structure. Association of the protein with PS vesicles clearly results in considerable protection of the chromophore to thermally induced quenching. The experiments were carried out in air-saturated solutions so that oxygen quenching undoubtedly is a significant factor. It has been demonstrated that aromatic chromophores are less susceptible to quenching when incorporated into micelles (Kalyanasundaram et al., 1977; Turro et al., 1978). However, in the absence of more detailed studies of this type with synthetic bilayer systems containing known oxygen concentrations, it remains unclear whether the degree of protection experienced with vesicle-associated MBP is a consequence of the tryptophan side chain becoming embedded in lipid in the gel state or if the chromophore is buried in a protein environment as a result of a lipid-induced conformational change (Keniry & Smith, 1979) or through protein–protein association.

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