

## Myelin Basic Protein Interaction with Zinc and Phosphate: Fluorescence Studies on the Water-Soluble Form of the Protein

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**ABSTRACT** The interaction of myelin basic protein (MBP) with zinc and phosphate ions has been studied by using the emission properties of the single tryptophan residue of the protein (Trp-115). The studies have been carried out by means of both static and time-resolved fluorescence techniques. The addition of either zinc to MBP in the presence of phosphate or phosphate to MBP in the presence of zinc resulted in an increase of fluorescence intensity and a blue shift of the emission maximum wavelength. Furthermore, a concomitant increase in the scattering was also detected. Anisotropy decay experiments demonstrated that these effects are due to the formation of MBP molecules into large aggregates. A possible physiological role for such interaction is discussed.

### INTRODUCTION

The myelin of the central nervous system contains an encephalitogenic protein, myelin basic protein (MBP), which is believed to play an important role in the formation and maintenance of the multilamellar structure of this membrane (Boggs and Moscarello, 1982; Riccio et al., 1986). This role seems to be strengthened in the presence of selected divalent cations and, in particular, in the presence of zinc. Indeed, zinc ions seem to inhibit the release of MBP from the myelin sheath (Berlet et al., 1987; Earl et al., 1988) and to contribute to the integrity and the compactness of myelin (Inouye and Kirschner, 1984). This role is particularly important considering that MBP dissociation from the myelin sheath is an important aspect of demyelinating processes. In the course of demyelination, MBP is released from the myelin membrane and can sustain an autoimmune response (Warren and Catz, 1987).

It is worthwhile to investigate whether the zinc effect on the myelin structure could be ascribed to direct interaction with the MBP molecule or to stabilization of putative MBP complexes with other myelin components. It has been recently suggested, for example, that zinc ions bridge MBP and proteolipid, an intrinsic protein of the myelin membrane (Earl et al., 1988).

We report here our study on the interaction of zinc ions with purified lipid-free MBP in the presence of phosphate buffer. Experiments were carried out by using the steady-state and time-resolved fluorescence of the single tryptophan residue of the protein.

### MATERIALS AND METHODS

MBP was isolated and purified from the bovine brain and spinal cord according to the procedure of Deibler et al. (1972, 1984). Purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (Liuzzi et al., 1992). Protein concentration was determined spectrophotometrically using a value of  $E_{1\text{cm}}^{1\%} = 5.64$  at 276.4 nm (Liebes et al., 1975). Zinc chloride (purity 98%) was purchased from Fluka Chemie (Buchs, CH). All other chemicals used were analytical grade.

Absorption measurements were recorded on a Jasco PTL-396S spectrophotometer. Steady state fluorescence experiments were carried out on a Perkin Elmer LS50 spectrophotofluorimeter. The cell holder temperature was stabilized at  $20.0 \pm 0.1^\circ\text{C}$  with a circulating bath. Fluorescence titrations were carried out by adding small aliquots (10–20  $\mu\text{l}$ ) of a concentrated stock solution of  $\text{ZnCl}_2$  in water to the protein sample in phosphate buffer.

In some experiments, zinc and/or phosphate were added to a protein solution in HEPES buffer.

Time-resolved fluorescence measurements were performed using the technique of time-correlated single-photon counting with a laser/microchannel plate-based instrumentation, as previously described (Willis et al., 1990b). The data were typically collected at 10 ps/channel in 2048 channels. The excitation wavelength was 295 nm. The decay of the fluorescence intensity  $I(t)$  was analyzed in terms of a sum of exponentials according to the relation:

$$I(t) = \sum \alpha_i e^{-t/\tau_i}$$

where the pre-exponential term  $\alpha$  and the decay constant  $\tau$  are the parameters of the decay function. The experimental curves were analyzed with a non-linear reconvolution procedure implemented to analyze simultaneously multiple decay curves (Willis et al., 1990a). This method was also used to resolve the decay-associated spectra (DAS).

The time-resolved fluorescence anisotropy was measured by monitoring separately the decay of the parallel ( $I_{\text{vv}}$ ) and perpendicular ( $I_{\text{vh}}$ ) components of the emitted light, using a T-format detection system. The excitation wavelength was 300 nm.

The fluorescence anisotropy decay was analyzed by a global analysis of  $I_{\text{vv}}(t)$  and  $I_{\text{vh}}(t)$  curves using the relationships:

$$I_{\text{vv}}(t) = \frac{1}{3} \sum \alpha_i e^{-t/\tau_i} [1 + 2r(t)]$$

$$I_{\text{vh}}(t) = \frac{1}{3} \sum \alpha_i e^{-t/\tau_i} [1 - r(t)]$$

where  $r(t) = \sum \beta_i e^{-t/\theta_i}$

$\beta_i$  = initial anisotropy of the  $i$ -th component

$\theta_i$  = rotational correlation time of the  $i$ -th component

Received for publication 10 September 1993 and in final form 27 January 1994.

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0006-3495/94/04/1174/06 \$2.00

## RESULTS

### Steady-State Fluorescence

As previously reported, the corrected emission spectrum of MBP at neutral pH showed a maximum at 347 nm and a half bandwidth of 60 nm (Cavatorta et al., 1988). These values did not vary with buffer concentrations up to 10 mM regardless of the buffer used (phosphate, cacodylate, Tris, MOPS, and HEPES). Furthermore, the spectral maximum position and the fluorescence quantum efficiency remained approximately constant at a protein concentration between  $10^{-6}$  and  $10^{-5}$ M, where MBP has been reported to be in the monomeric form (Smith, 1985).

The addition of increasing amounts of  $\text{ZnCl}_2$  to a  $7.5 \mu\text{M}$  solution of MBP in 1 mM phosphate buffer caused a progressive increase of the protein fluorescence intensity and a concomitant blue shift of the emission maximum (Fig. 1). The fluorescence intensity reached a plateau value at a  $\text{ZnCl}_2$  concentration of  $550 \mu\text{M}$ , with a spectral maximum of 337 nm. The integrated areas of the fluorescence spectra as a function of the metal to protein molar ratio is also shown (Fig. 2). This zinc effect on protein fluorescence was observed in the phosphate buffer and, to a lesser extent, in Tris; no change in fluorescence was observed by adding zinc to the protein in water or in other buffers.

The same increase in fluorescence and the same blue shift also took place when phosphate was added to a solution containing the protein in the presence of zinc. In this case, to maintain a constant pH, we buffered the solution with 10 mM HEPES, pH 7.5. As mentioned before, HEPES has no effect on protein fluorescence in the presence of zinc. Furthermore, when the metal was preincubated with phosphate buffer and then added to a sample containing the protein alone, no change in fluorescence was detected.

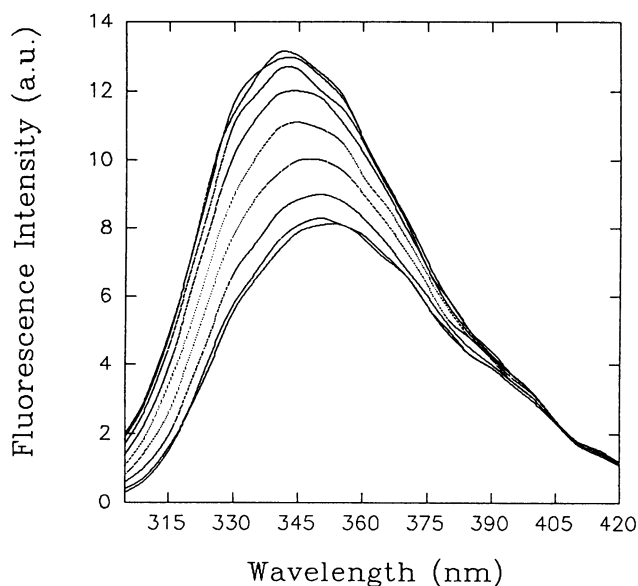


FIGURE 1 Fluorescence emission spectrum of MBP in 1 mM phosphate buffer, pH 7.5 (lowest intensity spectrum), and in the presence of increasing amounts of  $\text{ZnCl}_2$ . The MBP concentration was  $7.5 \times 10^{-6}$ M and the maximum  $\text{ZnCl}_2$  concentration  $5.5 \times 10^{-4}$ M.

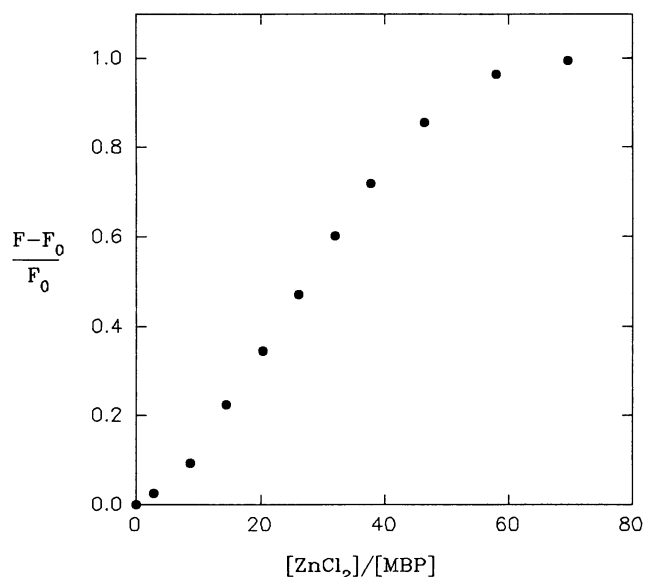


FIGURE 2 Integrated areas ( $F$ ) of the fluorescence spectra reported in Fig. 1, as a function of the metal to protein molar ratio.

In parallel with the changes in fluorescence emission, we also noted a progressive increase in the scattering of the sample as monitored spectrophotometrically at 380 nm (data not shown). This scattering was completely absent in the blank (phosphate plus zinc) and in samples containing the protein and zinc in water or in buffers different from phosphate.

In experiments carried out at higher protein and phosphate concentrations, the addition of zinc resulted in turbidity of the sample. For example, at a protein concentration of  $15 \mu\text{M}$  and 5 mM phosphate, a zinc concentration as low as  $100 \mu\text{M}$  is sufficient to cause opalescence in the cuvette.

To test the importance of zinc ions in the reaction, the effect of EDTA was checked. The addition of 1 mM EDTA to the sample, which is greater than the maximum zinc concentration, completely restored the initial conditions, i.e., the protein fluorescence intensity and the emission maximum wavelength returned to the values of the zinc-free protein and the scattering disappeared. Furthermore, the addition of 1 mM  $\text{Zn}(\text{CH}_3\text{COO})_2$ , instead of  $\text{ZnCl}_2$ , to the sample containing phosphate and protein resulted in a comparable increase in MBP fluorescence, suggesting that the effect is due to the metal cation.

Physiologically occurring metal ions different from zinc did not cause comparable effects on the fluorescence of MBP in the presence of phosphate. In fact,  $\text{Ca}^{2+}$ ,  $\text{Co}^{2+}$ , and  $\text{Mn}^{2+}$  did not affect the emission spectrum of the protein, whereas  $\text{Cu}^{2+}$  strongly quenched the MBP fluorescence intensity without changing the emission maximum wavelength (data not shown). Furthermore, all these metals were unable to induce scattering in the sample.

The zinc effect on the fluorescence showed a marked dependence on pH. In the same conditions as Fig. 1, but at pH 6.8, the fluorescence saturated at  $200 \mu\text{M}$  zinc concentration, but the fluorescence intensity increase was about half of that

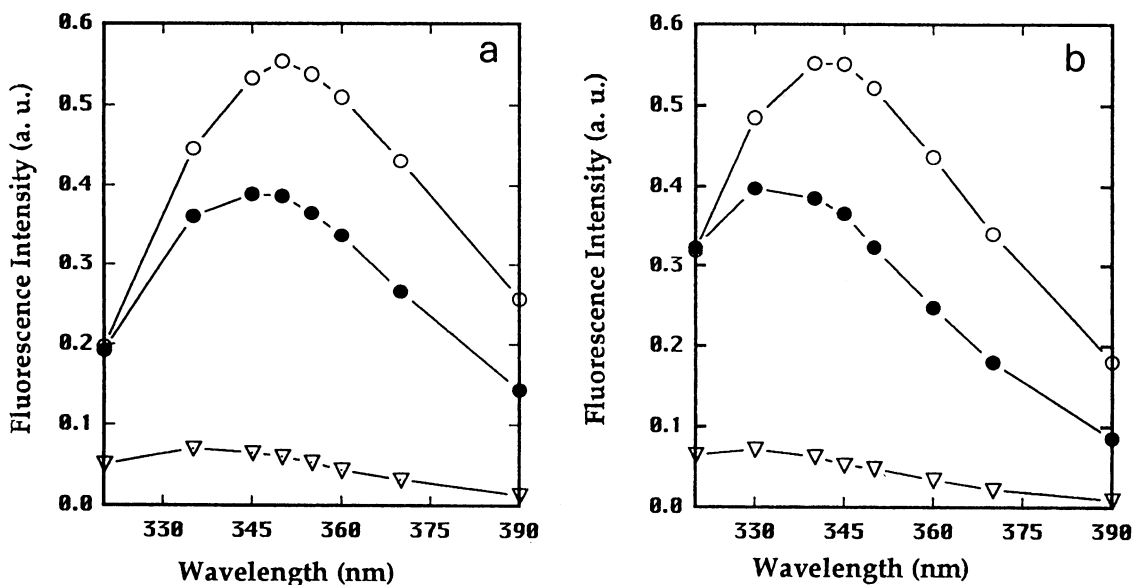


FIGURE 3 DAS for  $7.5 \times 10^{-6}$  M MBP in 1 mM phosphate buffer, pH 7.5 (a), and in the presence of  $5.5 \times 10^{-4}$  M  $\text{ZnCl}_2$  (b). Excitation wavelength is 295 nm. The symbols refer respectively to the long lifetime (○), medium lifetime (●), and short lifetime (▽). The lifetime values are reported in Table 1.

observed at pH 7.5. At pH 5.9, no increase in fluorescence or in scattering was detected at high zinc concentrations. Values of pH higher than 7.5 were not investigated, due to the low solubility of zinc salt.

### Time-resolved fluorescence

The fluorescence decay of  $7.5 \mu\text{M}$  MBP in 1 mM phosphate buffer at pH 7.5 was best fitted by a triple exponential decay model, with decay times of 4.29, 1.72, and 0.33 ns. These values are in good agreement with those previously reported (Cavatorta et al., 1988).

The fluorescence decay was measured at several wavelengths across the emission spectrum, and the decay times did not vary appreciably with wavelength. Hence, the data were combined in a global analysis to produce the DAS, which have maxima at 350 nm (4.29 ns), 348 nm (1.72 ns) and 340 nm (0.33 ns) (Fig. 3 a).

In the presence of  $550 \mu\text{M}$   $\text{ZnCl}_2$ , corresponding to the saturating point in the steady-state titration, the fluorescence lifetimes of MBP are different from those obtained for zinc-

free protein. Again three exponential components were found, but the decay times were 5.58, 2.14, and 0.42 ns. The DAS, in this case, have maxima at 345 nm (5.58 ns) and 330 nm (2.14 and 0.42 ns) (Fig. 3 b). The relative proportions of each decay component did not change appreciably.

The parameters of the fluorescence decay of these two samples are summarized in Table 1.

### Time-resolved anisotropy decays

The anisotropy decays of  $7.5 \mu\text{M}$  MBP in 1 mM phosphate buffer, pH 7.5, are shown in Fig. 4 in the absence (Fig. 4 a) and in the presence (Fig. 4 b) of  $550 \mu\text{M}$   $\text{ZnCl}_2$ .

In the absence of  $\text{ZnCl}_2$ , the anisotropy decay can be described by double exponential kinetics characterized by correlation times of  $1.9 \pm 0.5$  ns and  $0.17 \pm 0.06$  ns, whereas the limiting anisotropy at time zero was 0.15. The statistical parameter serial variance ratio (SVR = 2 for an ideal fit) was 1.77, indicating a good fit of the experimental data, whereas the limiting anisotropy value found in the deconvolution analysis is lower than that of about 0.2 showed by the plot

TABLE 1 (a) Time-resolved fluorescence parameters of MBP, 1 mM phosphate buffer, pH 7.5, using global analysis

$\lambda(\text{nm})$	$\alpha_1$	$\alpha_2$	$\alpha_3$	$F_1$	$F_2$	$F_3$
320	0.15	0.36	0.49	0.45	0.44	0.11
335	0.20	0.40	0.40	0.51	0.41	0.08
345	0.23	0.41	0.36	0.54	0.39	0.07
350	0.24	0.42	0.34	0.55	0.39	0.06
355	0.25	0.43	0.32	0.56	0.38	0.06
360	0.27	0.44	0.29	0.57	0.38	0.05
370	0.29	0.44	0.27	0.59	0.36	0.05
390	0.33	0.45	0.22	0.62	0.35	0.03

The fluorescence decay times, constant over the total range of the wavelengths, are:  $\tau_1 = 4.29 \pm 0.01$  ns,  $\tau_2 = 1.72 \pm 0.01$  ns, and  $\tau_3 = 0.334 \pm 0.003$  ns.  $\alpha_1$ ,  $\alpha_2$ , and  $\alpha_3$  are the normalized preexponential terms.  $F_1$ ,  $F_2$ , and  $F_3$  are the fractional fluorescence of the respective components. SVR = 1.87.

**TABLE 2 (b) Time-resolved fluorescence parameters of MBP, 1 mM phosphate buffer, pH 7.5, in the presence of ZnCl<sub>2</sub>, using global analysis**

$\lambda$ (nm)	$\alpha_1$	$\alpha_2$	$\alpha_3$	F <sub>1</sub>	F <sub>2</sub>	F <sub>3</sub>
320	0.16	0.42	0.42	0.45	0.46	0.09
330	0.20	0.42	0.38	0.51	0.42	0.07
340	0.23	0.42	0.35	0.55	0.38	0.07
345	0.25	0.43	0.32	0.57	0.38	0.05
350	0.26	0.42	0.32	0.58	0.36	0.06
360	0.29	0.42	0.29	0.61	0.34	0.05
370	0.31	0.43	0.26	0.63	0.33	0.04
390	0.34	0.43	0.23	0.66	0.31	0.03

The fluorescence decay times, constant over the total range of the wavelengths, are:  $\tau_1 = 5.58 \pm 0.01$  ns,  $\tau_2 = 2.14 \pm 0.01$  ns, and  $\tau_3 = 0.425 \pm 0.004$  ns.  $\alpha_1$ ,  $\alpha_2$ , and  $\alpha_3$  are the normalized preexponential terms. F<sub>1</sub>, F<sub>2</sub>, and F<sub>3</sub> are the fractoinal fluorescence of the respective components. SVR = 1.77.

(Fig. 4 *a*). This discrepancy reflects the inadequacy of our deconvolution program to analyze a complex decay as that of MBP. We repeated the measurements several times obtaining always the same values. The attempt to fit the anisotropy decay with three exponentials was unsuccessful. Probably the involvement of so many parameters makes the least-square analysis unstable to experimental errors. Nevertheless the values found are in good agreement with those obtained by other investigators (Munro et al., 1979; Lakowicz et al., 1983; Nowak and Berman, 1991).

The anisotropy of MBP in the presence of zinc showed a markedly different behavior. It did not decay to zero but reached a constant value at long times (Fig. 4 *b*). Attempts to fit the decay with an exponential plus a constant value at infinite time failed, because very large errors relative to anisotropy at time zero and to the short correlation time were obtained. We are unable to offer any explanation for these large errors at this time, even if problems connected with the scattering of the sample cannot be excluded. A visual inspection of the decay plot suggests a limiting anisotropy at time zero of about 0.25 and an "infinite time" anisotropy of 0.20.

## DISCUSSION

MBP in all tested buffers and in the absence of Zn<sup>2+</sup> showed a fluorescence spectrum typical of a tryptophan residue in an aqueous environment. In fact, the emission maximum wavelength and the bandwidth were similar to those found for the model system N-acetyl-tryptophanamide under the same conditions (Cavatorta et al., 1988). This result is consistent with the substantially denatured state suggested by several authors for MBP in solution (Nowak and Berman, 1991; Krigbaum and Hsu, 1975; Martenson, 1978).

The triple exponential decay has already been explained (Cavatorta et al., 1988) as indicative of the occurrence of at least three conformers of the protein that are stable on the nanosecond time scale. This behavior could also be assigned to three rotational isomers of tryptophan around the C<sub>a</sub>-C<sub>b</sub> bond (Willis and Szabo, 1992). Because of the lack of NMR results about the rotamers population of the MBP tryptophan residue, an unambiguous assignment of the multiple decay kinetics is not possible at this time. For this reason we only observe that the emission maxima of the DAS are respec-

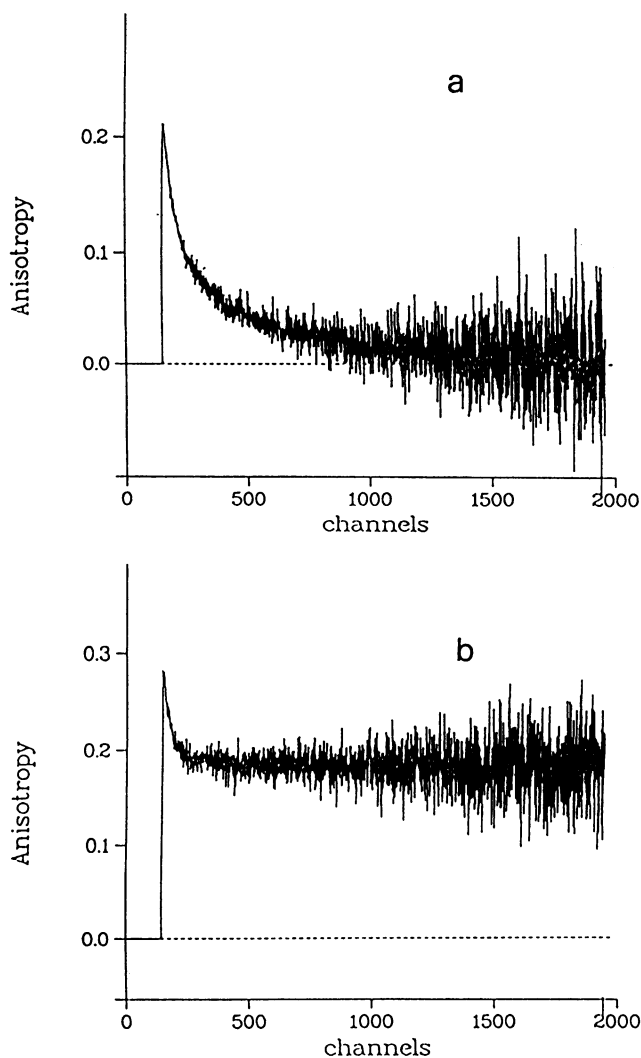


FIGURE 4 Fluorescence anisotropy decay for  $7.5 \times 10^{-6}$ M MBP in 1 mM phosphate buffer pH7.5 (*a*), and in the presence of  $5.5 \times 10^{-4}$ M ZnCl<sub>2</sub> (*b*). Excitation wavelength is 300 nm.

tively 350, 348, and 340 nm, typical of configurations in which the tryptophyl residue is exposed to the solvent.

In general, multiexponential anisotropy decay kinetics are expected for proteins with a single tryptophan residue (Lakowicz, 1983). If the protein is compact, and the tryp-

tophan residue is exposed to the solvent, the longest correlation time would be attributed to the overall rotation of the protein, whereas the shorter ones should correspond to rapid restricted motions of the amino acid on the protein surface. If the protein structure is not compact, segmental motions, which cause ulterior rapid reorientation processes, are also possible. In this case the longest correlation time reflects the motion of the segment containing the fluorophore more than the overall rotation of the protein. The best fit of the MBP fluorescence anisotropy decay provided two correlation times of 1.9 and 0.17 ns. The first value is much shorter than the 7.4 ns predicted for a spherical, rigid protein of 18 kDa molecular mass (Nowak and Berman, 1991), suggesting a high degree of flexibility for the MBP in solution.

The limiting ( $t = 0$ ) anisotropy value extracted from the fit was 0.15, whereas the limiting value reported for a completely immobilized tryptophan residue is 0.33 (Valeur and Weber, 1977). Consequently, the results show that the tryptophan residue undergoes very rapid relaxations, on a time-scale below our instrumental resolution.

Our anisotropy decay data are of the same order of magnitude as those reported by Munro et al. (1979). However, the errors associated with the parameters extracted from our fit were large, and the limiting anisotropy value was considerably lower. This difficulty in adequately fitting the experimental anisotropy decay of MBP probably reflects the very high degree of flexibility of the protein. Such a high flexibility should require a larger number of exponential components to obtain a satisfactory analysis of the data.

The increase in fluorescence intensity and the blue shift observed either upon addition of zinc to MBP in phosphate buffer or upon addition of phosphate to the protein in the presence of zinc clearly indicated an interaction between the protein, the metal, and phosphate ions. The fluorescence increase is saturable under the conditions reported in Fig. 1, suggesting the formation of a well defined compound. These changes in protein fluorescence did not occur when a solution of  $ZnCl_2$  in phosphate was added to the protein alone in water or in HEPES buffer. Such a behavior is probably related to the formation of insoluble compounds like either zinc phosphate or zinc hydroxide, which are unable to react with the protein. The formation of a complex between MBP and zinc ions or MBP and phosphate ions seems to be a prerequisite for the occurrence of the interaction with the third component.

The interaction between MBP, zinc, and phosphate resulted in the formation of MBP molecules into large aggregates. In fact, the increase in scattered light was diagnostic of the formation of large particles. The dimensions of the aggregates increased with protein and phosphate concentration as indicated from the appearance of turbidity. Turbidity introduces artifacts in the correct fluorescence intensity detection, so that a systematic study of the stoichiometry of the reaction is not possible with this technique. Zinc ions were fundamental to the complex formation, because the addition of stoichiometric amounts of EDTA completely restored the initial fluorescence conditions.

The phosphate and zinc effect on protein fluorescence showed a marked dependence on pH, being predominant at physiological pH. This could indicate a specific divalent effect of the salt, because phosphate is a divalent anion only above its second pK value of 7.21 (Weast, 1983). At the same time, the pH behavior can suggest the involvement of histidine residues, inasmuch as histidine is the only amino acid in the protein with a pK close to 7. MBP contains ten histidines and no cysteines. His-X-X-His sequences are involved in binding zinc in so-called zinc-finger proteins (Coleman, 1992). A similar sequence (residues 23–26: His-Ala-Arg-His) is also found in MBP, but the involvement of remaining histidines cannot be excluded.

The triple exponential fluorescence decay shows that MBP conformers or tryptophan rotamers were also maintained when the protein aggregates in the presence of zinc and phosphate ions. The longer lifetimes and the blue shifted emission maxima with respect to the protein in the absence of metal indicate reduced exposure of the tryptophan to the solvent.

Anisotropy decay experiments provide unequivocal proof that zinc promoted the formation of large aggregates. The low limiting anisotropy and the rapid initial decay show that tryptophan undergoes a very fast but not complete reorientation. In fact, the anisotropy did not decay to zero but reached an "infinite time" value of about 0.20. This behavior indicates that the multiprotein complex is too large to experience any appreciable reorientation during the fluorescence lifetime and that the tryptophan residue can rotate only in a restricted environment.

The present data indicate that zinc ions stabilize the "in vitro" self-association of MBP in the presence of phosphate ions. In view of the vulnerability of dissociated MBP to proteolysis, this reaction, if present "in vivo," may play a key role in maintaining the integrity of the protein and, thus, of the myelin sheath. It is noteworthy, in this connection, that zinc in the micromolar range is found in the compacted cytoplasmic space of myelin (Earl et al., 1988), whereas phosphate is present at millimolar concentration in the intracellular space (Guyton, 1981).

At this stage of the experiments, we are unable to define the details of the reaction. The appearance of turbidity at high protein and phosphate concentration seems to indicate that the process is a continuous association with a wide range of aggregate sizes. However, the association could be, "in vivo," a more controlled process, leading to the formation of a stoichiometrically defined complex.

Recently, MBP has been extracted from the myelin membrane with non-ionic detergent in a lipid-bound form (Riccio et al., 1984; Riccio et al., 1990). Such MBP has a more compact structure compared with that dissolved in water. The lipid-bound protein can be incorporated in model membranes like micelles and vesicles and is believed to represent a more realistic picture of the protein in its natural environment.

Studies are in progress in our laboratory on this system to test the suggestion that the endogenous lipids can constitute

an active agent of the association process of MBP in the presence of zinc ions.

The authors wish to acknowledge helpful discussions with Prof. R. T. Sorbi. This research was supported by grants from CNR and MURST, 60% and 40% respectively, Rome, Italy.

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