Nowak, T., Haywood, P. L., & Barondes, S. H. (1976) *Biochem. Biophys. Res. Commun. 68*, 650-657.
Ochs, D. (1983) *Anal. Biochem. 135*, 470-474.
O'Farrell, P. H. (1974) *J. Biol. Chem. 250*, 4007-4021.
O'Farrell, P. Z., Goodman, H. M., & O'Farrell, P. H. (1977) *Cell 12*, 1133-1142.
Rademacher, T. W. Parekh, R. B., & Dwek, R. A. (1988) *Annu. Rev. Biochem. 57*, 785-838.
Redinbaugh, M. G., & Campbell, W. H. (1985) *Anal. Biochem. 147*, 144-147.
Roberson, M. M., Ceri, H., Shadle, P. J., & Barondes, S. H. (1981) *J. Supramol. Struct. Cell. Biochem. 15*, 395-402.
Roberts, D. D., & Ginsburg, V. (1988) *Arch. Biochem. Biophys. 267*, 405-415.
Schachter, H. (1984) *Clin. Biochem. 17*, 3-14.
Sen-Majumdar, A., Murthy, U., & Das, M. (1986) *Biochemistry 25*, 627-634.
Sharon, N. (1984) *Trends Biochem. Sci. 9*, 198-202.
Smith, J. W., & Knauer, D. J. (1987) *Anal. Biochem. 160*, 105-114.
Tasheva, B., & Dessev, G. (1983) *Anal. Biochem. 129*, 98-102.
Thomas, K. A., & Gimenez-Gallego, G. (1986) *Trends Biochem. Sci. 11*, 81-84.
Thomson, A. W., & Fowler, E. F. (1981) *Agents Actions 11*, 265-273.
Tschopp, J., Masson, D., Schäfer, S., Peitsch, M., & Preissner, K. T. (1988) *Biochemistry 27*, 4103-4109.
Vehmeyer, K., Brandt, W., Nagel, G. A., & Gabius, H.-J. (1988) *Life Sci. 43*, 1591-1596.
Warburg, O., & Christian, W. (1942) *Biochem. Z. 310*, 384-421.
Wolpe, S. D., Davatelis, G., Sherry, B., Beutler, B., Hesse, D. G., Nguyen, H. T., Moldawer, L. L., Nathan, C. F., Lowry, S. F., & Cerami, A. (1988) *J. Exp. Med. 167*, 570-581.

Secondary Structure of Charge Isomers of Myelin Basic Protein before and after Phosphorylation†

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ABSTRACT: Human myelin basic protein (MBP) was fractionated into several of its charge isomers (components). Of these, the secondary structures of four isomers before and after phosphorylation have been studied by circular dichroism (CD). None of the four showed any α-helical structure. All of the components showed varying amounts of β-structure, random structure, and turns. Component 1 (C-1), the most cationic of the components, showed 13%; component 2 (C-2) had 19%; C-3, 17%; and C-4, 24% of β-structure. Each of the four components was phosphorylated with protein kinase C, from human brain. The extent of phosphorylation varied considerably from 2.8 ± 0.6 mol of PO_4/mol of protein in C-1 to 5.2 ± 0.8 mol of PO_4/mol of protein in C-4. The effect of phosphorylation on the secondary structure was to induce β-structure in all the components. The largest change in β-structure was in C-1 and the least in C-4. The surprising result is that although the components were phosphorylated to different extents, the amount of β-structure in all four components increased to a final proportion of 35-40%. Treatment of phosphorylated C-1 with acid phosphatase removed 50% of the total radioactivity. Although the remainder represented approximately 1 mol of PO_4/mol of protein, the proportion of β-structure was unaltered. We concluded that a single phosphorylation site identified as residues 5-13 represented a critical size for stabilization of β-structure of MBP in solution and that phosphorylation at the other sites had little influence on secondary structure.

Myelin basic protein (MBP) represents the major extrinsic protein of the myelin membrane, accounting for about 35% of the total protein responsible for the organization of the unique multilayered structure of myelin (Brady et al., 1981a; Epand, 1988). In model systems, the addition of MBP to vesicles composed of phosphatidylglycerol resulted in the formation of "crystalline" multilayers (Brady et al., 1981a). When compared to MBP isolated from myelin of victims of multiple sclerosis (Brady et al., 1981b), the latter was shown to be much less effective in organizing the formation of multilayers, implying that the MBP isolated from the MS material was less cationic than that isolated from normal brain. Since these observations could be explained by an increase in the relative proportions of the less cationic components, a detailed study of the charge microheterogeneity of MBP seemed appropriate.

Although purified MBP migrated as a single band on SDS-PAGE, on alkaline gels it could be resolved into 6-10 bands (components) on the basis of charge (Chou et al., 1976). These components were resolved on CM-52 columns at pH 10.6 as described originally by Chou et al. (1976). Since each component differed from the other by a single charge, we referred to them as charge isomers (Cheifetz & Moscarello, 1985). Thus, component 1, which was eluted last off the

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CM-52 column, was the most cationic (most highly positively charged); component 2 differed from component 1 by the loss of one net positive charge and therefore eluted from the column just prior to component 1. Component 3 differed from component 1 by the loss of two net positive charges, and so on for the other components.

The source of this charge microheterogeneity is generally considered to arise from the loss of a C-terminal Arg, deamidation of glutamine or asparagine, and phosphorylation. However, the charge microheterogeneity is not well-defined so that any combination of the above-mentioned factors as well as others, e.g., substitution of Arg by citrulline (Wood & Moscarello, 1989), can result in formation of the large number of charge isomers observed on alkaline gels.

Phosphorylation represents a well-defined source of charge microheterogeneity. Thus, phosphorylation of component 1 with 1 mol of phosphate/mol of MBP will convert component 1 into component 3, the result of the addition of two negative charges at alkaline pH. Phosphorylation of component 1 in vitro was shown to markedly decrease its ability to aggregate lipid vesicles (Cheifetz & Moscarello, 1985), and the formation of multilayers, as measured by X-ray diffraction, was reduced by a factor of 2 (Brady et al., 1985). It was clear that a small change in the net charge of the protein as generated by phosphorylation had large effects on the ability of the protein to interact with and organize lipids into multilayers. Phosphorylation of MBP has been observed in vivo (Chou et al., 1978) and in the isolated myelin membrane in vitro (Schulz et al., 1988). However, neither deamidation nor the loss of the C-terminal arginine has been reported to occur in vivo. The role of charge microheterogeneity has not been elucidated although natural and model membrane studies suggest that localized changes in charge microheterogeneity affect both compaction of myelin and movement of ions (Moscarello et al., 1985; Cheifetz et al., 1985).

In the present paper, we report our studies on the secondary structures of some of the charge isomers of MBP and the effect that phosphorylation has on these structures. Previously, it appeared that the different charged isomers had altered interactions with lipid primarily as a result of their differing charge (Brady et al., 1985). However, the present study implicates a role for charges in determining protein conformation, in particular β-structure. These altered conformational properties, particularly those arising as a result of phosphorylation, may determine the mechanism by which MBP interacts with a lipid bilayer.

In the model presented by Stoner (1984), phosphorylation of specific residues was of considerable importance in the stabilization of the β-structure. For example, hydrogen bonding between a specific residue such as Arg-107 or Lys-105 with phosphothreonine-99, or Arg-31 with phosphoserine-166, and other similar interactions were important in stabilizing the "hydrophobic box" structure predicted by this analysis (Stoner, 1984). In the studies reported here, we demonstrate not only that β-structure is induced and maintained by phosphorylation but also that a single critical phosphate is instrumental in maintaining the β-structure. Interestingly, this critical phosphorylation site, which was located in the N-terminal peptide consisting of residues 5–13, was not considered in the model proposed (Stoner, 1984).

Materials and Methods

Materials

Bovine brain L-α-phosphatidylserine was purchased from Avanti Polar Lipids. ATP, protease inhibitor, phenylmethanesulfonyl fluoride (PMSF), and L-α-dioleoylphosphatidylglycerol (diolein) were from Sigma. [γ-^{32}P]ATP (8–10 Ci mmol^{-1}) was obtained from Dupont–New England Nuclear. All reagents for acrylamide gel electrophoresis were obtained from Bio-Rad (Richmond, CA). Postmortem normal human brains were obtained from the Canadian Brain Tissue Bank, Toronto.

All other chemicals used were of analytical or HPLC grade.

Methods

Isolation of Components (Charged Isomers) from Human Myelin Basic Protein. The components of MBP were isolated as described previously (Chou et al., 1976; Cheifetz & Moscarello, 1985). Approximately 100 mg of MBP dissolved in 6 mL of 0.08 M sodium glycinate buffer, pH 9.6, containing 6 M urea (ultrapure Schwarz/Mann) was loaded onto a preequilibrated 0.9 × 30 cm column of Whatman CM-52 (carboxymethyl)cellulose cation exchanger at a flow rate of 12 mL/h. The column was washed with the same buffer to remove unadsorbed protein. A linear sodium chloride gradient (0–0.2 M) in a 0.08 M sodium glycinate/2 M urea buffer, pH 10.6, was used to elute the bound protein selectively from the column on the basis of net charge. The isomers were eluted in order of increasing net positive charge and were numbered according to the convention established by Deibler and Martenson (1973). The purified components were desalted by dialyzing overnight with four changes of water and were lyophilized and stored at −20 °C until further use.

Alkaline Urea Gel. Disc gels were used to check the purity of the components and also to assess the relative amounts of the phosphorylated derivatives (Cheifetz & Moscarello, 1985). Briefly, the gels were prepared as follows: 5% acrylamide/bis(acrylamide) (30:1 w/w) in 0.01 M glycinate/8 M urea buffer, pH 10.6, was polymerized in (1.0 cm × 0.6 cm) gel tubes with TEMED/ammonium persulfate. The gels were overlayed with water and left overnight at room temperature to set. Prior to loading of the proteins, the gels were electrophoresed for 1 h at 3.75 mA/gel in the 0.05 M sodium glycinate, pH 10.6, running buffer. This was found to enhance the resolution of the protein on the gel. Approximately 50 μg of protein dissolved in 4.0 M urea was loaded on the gels and then electrophoresed for 3–5 h at 3.75 mA/gel. The gels were then removed from the gel tubes and stained for 10 min in 0.5% amido black/7% acetic acid followed by destaining with several changes of 7% acetic acid.

Preparation of Soluble Human Brain White Matter Protein Kinase C. Protein kinase C from white matter of normal human brain was prepared according to the procedure of Wolf et al. (1984) with some modifications. Briefly, 5 g of white matter stored at −70 °C was homogenized in 8 volumes of 20 mM Tris buffer, pH 7.4, and 10 mM DTT containing 1 mM PMSF and 1 mM $CaCl_2$. The homogenate was centrifuged at 25000g for 20 min, and the supernatant was discarded. The pellet was rehomogenized in 5 volumes of same buffer except 0.1 mM $CaCl_2$ replaced 1.0 mM $CaCl_2$. The homogenate was centrifuged for 20 min at 25000g, and the supernatant was discarded. The pellet was resuspended in 5 volumes of homogenizing buffer containing 5 mM EGTA and 2 mM EDTA, and the suspension was allowed to stand for 1 h on ice and then centrifuged at 100000g for 1 h. The supernatant was collected and treated as a soluble protein kinase C preparation. This preparation was stable over a week when stored at 4 °C.

Human brain white matter soluble protein kinase C prepared by this procedure yielded an enzyme with a specific activity of 5–10 nmol of phosphate min^{-1} (mg of protein)$^{-1}$ when human myelin basic protein was used as a substrate. An increase in activity of 3–4-fold was observed in the presence

of Ca^{2+} and phosphatidylserine. The optimum phosphorylation of basic protein was obtained in the presence of 10 mM $MgCl_2$, 20 μg/mL phosphatidylserine, 2 μg/mL diolein, and 500 μM $CaCl_2$ at pH 7.5.

Phosphorylation of Myelin Basic Protein Components. The final reaction volume of 250 μL contained 500 μg of one of the MBP components plus 20 μg of soluble protein kinase C, 100 μM ATP (2×10^6 cpm [γ-^{32}P]ATP), 20 mM Tris buffer, pH 7.5, 500 μM $CaCl_2$, 20 μg of phosphatidylserine, and 2 μg of diolein. The reaction mixture was incubated at 30 °C for 1 h, and the reaction was stopped by rapid cooling to 0 °C, followed by addition of an equal volume of 0.1% trifluoroacetic acid (TFA). The phosphorylated protein was separated from the reaction mixture for each component by reversed-phase HPLC on a C:18 μBondapak reversed-phase column (Waters). The column was equilibrated with 0.05% TFA, and the protein was eluted by using a linear gradient from 0 to 60% aqueous acetonitrile. The radioactive phosphorylated protein peak was counted, lyophilized, and resuspended in water.

Acid Phosphatase Treatment of Phosphorylated C-1. C-1 containing 6×10^5 cpm of ^{32}P was dissolved in 0.1 M sodium acetate buffer, pH 4.8, at a concentration of 150 μg/mL and was treated with 2 units/mL (7 μg of protein) potato acid phosphatase (Sigma, specific activity 270 units/mg of protein). The reaction was carried out for 1 h or overnight at 37 °C. The incubation mixture was applied to a Dowex-50 × 8 column in the H^+ form. The radioactive phosphate was recovered in the void volume of the column.

Peptide Maps. A proteinase Lys C digest was prepared from phosphorylated C-1 previously treated with acid phosphatase. In this experiment, the phosphorylated C-1 was treated with acid phosphatase overnight under the conditions described above, and the protein was separated from the sodium acetate and radioactive phosphate by reversed-phase HPLC. Approximately 400 μg of protein was digested with Lys C (enzyme:protein ratio = 1:50) in ammonium bicarbonate buffer at pH 8.0. The digest was lyophilized and applied to a reversed-phase C:18 μBondapak column in 0.05% TFA and eluted with a linear gradient of 0–60% acetonitrile, at a flow rate of 0.5 mL/min. The radioactive peptide was collected and subjected to amino acid analysis in a Waters Pico Tag amino acid analyzer after hydrolysis in 5.7 N HCl in the gas phase for 24 h. The peptide was identified from the amino acid composition.

Circular Dichroism (CD). Circular dichroism spectra of phosphorylated and nonphosphorylated components of basic protein were recorded in the wavelength range of 250–190 nm on an Aviv Model 6IDS solid-state CD instrument (Aviv Associates, Lakewood, NJ). The instrument was interfaced with an AT&T computer which was used for all mathematical calculations. The spectra were measured by using a 1-mm sample cell that was maintained at 25 °C with a thermostated cell holder. The CD data were expressed as the mean residue ellipticity, [θ] (degrees centimeter squared per decimole), using

$$[\theta]_\lambda = \theta_{exp} MRW / 10dC$$

107.5 as the mean residue weight of the protein (MRW), where $[\theta]_\lambda$ is the mean residue ellipticity, λ is the wavelength of irradiation, θ_{exp} is the observed ellipticity in degrees, d is the path length in centimeters, and C is the concentration of protein in grams per cubic centimeter.

Secondary Structure Estimation. Data points at 1-nm intervals between 190 and 240 nm were analyzed by the least-squares curve-fitting program to obtain estimates of the amount of each type of secondary structure in the different components. The sum of the fractions of different structures,

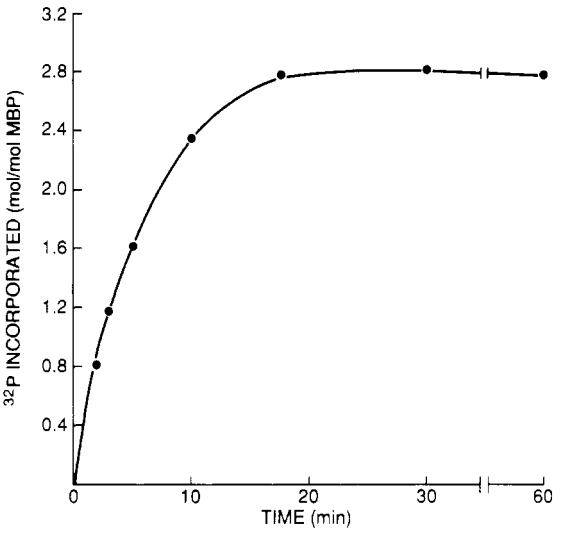

FIGURE 1: Phosphorylation of MBP by human brain protein kinase C. Fifty micrograms of MBP was incubated at 30 °C in a final volume of 0.2 mL of 20 mM Tris buffer at pH 7.5 containing 10 mM $MgCl_2$, 500 μM $CaCl_2$, 100 μM ATP (1×10^6 cpm of [γ-^{32}P]ATP), 20 μg of phosphatidylserine, and 2 μg of diolein. The reaction was started by the addition of 25 μL (15 μg of protein) of soluble protein kinase C. At the end of the each incubation time period, the reaction was stopped by addition of 3.0 mL of 10% trichloroacetic acid. Phosphorylated MBP was precipitated with TCA and filtered through the Amicon filtration unit using GF/C filters. The filters were washed 3 times with 10% TCA, and the radioactivity of the filter was quantitated by scintillation spectroscopy.

α-helix, β-structure, random structure, and turns, was constrained to add up to 1.0. The reference data were from 15 water-soluble proteins provided by Professor J. T. Yang (Chang et al., 1978). The least-squares curve-fitting program was adapted to the AVIV instrument by James Laurino.

Protein concentrations were determined by a micro-Lowry procedure as modified by Peterson et al. (1977). For calculation of the mean residue ellipticity, the protein concentration was measured by amino acid analysis in the Waters Pico Tag system after hydrolysis in 5.7 N HCl in the gas phase.

RESULTS

Phosphorylation of Myelin Basic Protein (MBP). In order to conserve components, the time course of phosphorylation was followed with unfractionated MBP, i.e., MBP which had not been resolved into its various charge isomers. When unfractionated MBP was phosphorylated as described under Methods, the phosphorylation was rapid for the first 10 min and attained maximal values by 20 min and then remained constant for 1 h. The maximum incorporation was 2.8 mol of phosphate/mol of MBP (Figure 1). For subsequent phosphorylations, a time of 60 min of incubation was adopted.

When phosphorylation of the isolated components was carried out by the same procedure, the amount of phosphate incorporated for components 1–4, respectively, was 2.8 ± 0.6, 3.7 ± 0.5, 3.2 ± 0.5, and 5.2 ± 0.8 (means and standard deviations from three experiments) mol of phosphate/mol of MBP. Interestingly, phosphorylation of component 1 (C-1) was the lowest at 2.8 mol of phosphate/mol of MBP, even though C-1 is considered to be the least posttranslationally modified component. Component 2 and component 3 (which is partially phosphorylated in vivo) incorporated 3.7 and 3.2 mol of phosphate/mol of MBP, respectively. Surprisingly, the greatest phosphorylation was observed with C-4 which incorporated 5.2 ± 0.8 mol of phosphate/mol of MBP.

The alkaline urea gels of the components are shown in Figure 2. Component 1, the most cationic, showed the fastest

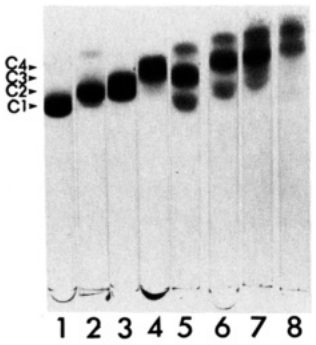

FIGURE 2: Alkaline polyacrylamide gel electrophoresis of phosphorylated and unphosphorylated components of MBP. Purified components were phosphorylated as described under Materials and Methods. Twenty-five micrograms of each component was loaded on disk gels. Lanes 1–4 represent unphosphorylated components C-1–C-4, and lane 5–8 represent phosphorylated components C-1–C-4.

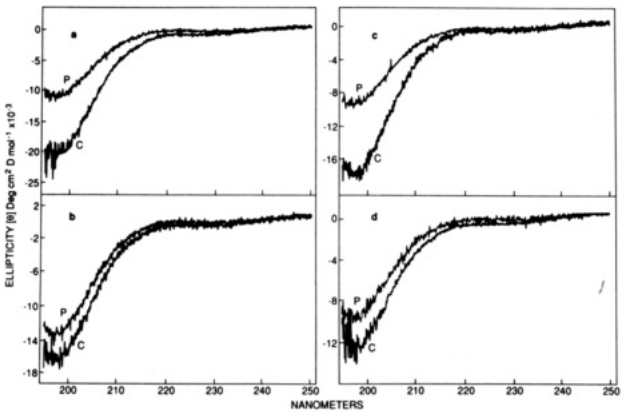

FIGURE 3: Circular dichroism (CD) spectroscopy of unphosphorylated and phosphorylated components of MBP. Figure 3a compares the CD spectra of phosphorylated (P) and unphosphorylated (C) component C-1. Similarly, Figure 3b–d compares the spectra of phosphorylated and unphosphorylated components C-2, C-3, and C-4. The proteins were dissolved in distilled water (150 μg/mL), and the CD spectra were taken as described under Materials and Methods.

mobility (gel 1). When phosphorylated (gel 5), the mobility changed, demonstrating modification of the component. A small amount of the C-1 was not phosphorylated, but most migrated with the mobility of C-3 with a smaller amount of a more highly phosphorylated species, probably C-5. Phosphorylation of component 2 (gel 2) generated several less cationic species (gel 6) as did the phosphorylation of components 3 and 4 (gels 7 and 8, respectively). Therefore, the phosphorylation of the individual components of MBP by protein kinase C produced several phosphorylated species, although in each case one major phosphorylated species was obtained (less evident for C-4).

Secondary Structure of Charge Isomers 1–4 before and after Phosphorylation. The secondary structures of charge isomers 1–4 isolated from normal human basic protein are shown in Figure 3a–d. In Figure 3a, the spectrum from 250 to 190 nm of component 1 (the most cationic charge isomer) is compared to the CD spectrum of this component, phosphorylated with protein kinase C as described under Methods. The spectra are generally similar in shape. Below 200 nm, the ellipticity of the phosphorylated material increased from a value of -20×10^3 deg cm^2 dmol^{-1} for the unphosphorylated C-1 to -11×10^3 deg cm^2 dmol^{-1} for phosphorylated C-1, representing an increase in secondary structure.

Similar spectra were recorded for the other charge isomers (Figure 3b–d). In Figure 3c, the spectra for phosphorylated and nonphosphorylated component 3 are shown. These are qualitatively similar to the spectra for component 1 (Figure 3a). The ellipticity of the phosphorylated component increased by 50% over that of the nonphosphorylated component.

Table I: Relative Proportions of α-Helix, β-Structure, Random Structure, and Turns in Charge Isomers of Human Myelin Basic Protein[a]

charge isomer	f_α	f_β	f_r	f_t
		Prior to Phosphorylation		
C-1	0	0.13 ± 0.01	0.64 ± 0.02	0.23 ± 0.02
C-2	0	0.19	0.57	0.24
C-3	0	0.17	0.59	0.24
C-4	0	0.24 ± 0.05	0.57 ± 0.06	0.19 ± 0.02
		After Phosphorylation		
C-1	0	0.37 ± 0.05	0.43 ± 0.08	0.20 ± 0.03
C-2	0	0.40	0.40	0.20
C-3	0	0.39	0.41	0.20
C-4	0	0.41 ± 0.04	0.40 ± 0.002	0.19 ± 0.01

[a] The relative proportions of secondary structure were obtained from an analysis of data points at 1-nm intervals between 190 and 240 nm by the least-squares method. The sum of the fractions of each structure was constrained to add up to 1.0. The reference data were obtained from 15 water-soluble proteins provided by Yang (Chang et al., 1978). The values for C-1 and C-4 are the means and standard errors of the mean from six independent preparations of the components. The values for C-2 and C-3 are single determinations from a single set of spectra. However, they are in good agreement with two additional sets of spectra. f_α = proportion of α-helix, f_β = proportion of β-structure, f_r = proportion of random structure, and f_t = proportion of turns.

The spectra for components 2 and 4 are shown in Figure 3b,d. The ellipticities of the nonphosphorylated components 2 and 4 were -16×10^3 and -12×10^3 deg cm^2/dmol, respectively, suggesting that component 4 had a greater degree of secondary structure than the other components. In both cases, phosphorylation resulted in a smaller change of ellipticity than components, C-1 and C-3. In the case of C-2, the proportion of β-structure increased from 0.19 (nonphosphorylated) to 0.40 (phosphorylated), while in the case of C-4 it increased from 0.24 (nonphosphorylated) to 0.41 (phosphorylated).

The relative proportions of α-helix, β-structure, random structure, and turns are shown in Table I. None of the components showed any α-helical conformation in solution. However, all showed significant amounts of β-structure, random structure, and turns. Components 1, 2, and 3 showed low proportions of β-structure, 0.13–0.19, while component 4 was considerably higher at 0.24. The calculations in Table I for C-1 and C-4 are the means and standard errors from six independent preparations of components. Although those shown for C-2 and C-3 are from a single set of spectra, the results are in parallel with those of C-1 and C-4 spectra.

Phosphorylation of the components increased the proportion of β-structure in all components (Table I). The proportion of β-structure in C-1 increased from 0.13 ± 0.01 to 0.37 ± 0.05, about 3-fold. Similar increases were observed for C-2 and C-3 while C-4 increased from 0.24 ± 0.05 to 0.41 ± 0.04, representing the smallest increase. The increase in β-structure did not correlate with the extent of phosphorylation, since C-1 incorporated 2.8 mol of PO_4/mol of MBP while C-4 incorporated 5.2 mol of PO_4/mol of MBP.

Component 1 showed a large change in β-structure on phosphorylation from 0.13 ± 0.01 before phosphorylation to 0.37 ± 0.05 after phosphorylation. Since this change suggested an increase in secondary structure as a result of phosphorylation, phosphorylated C-1 dissolved in 0.1 M sodium acetate buffer, pH 4.8, was treated with acid phosphatase for 60 min as described under Methods, and the CD spectra of protein solutions at pH 4.8 were taken again to study the reversibility

Table II: Relative Proportions of α-Helix, β-Structure, Random Structure, and Turns of Component 1 from Human MBP

component 1	rel proportions of secondary structures[b]			
	f_α	f_β	f_r	f_t
phosphorylated[a]	0	0.45	0.38	0.17
acid phosphatase (1 h)	0	0.38	0.43	0.20

[a] Both phosphorylated and nonphosphorylated C-1 were dissolved in 0.1 M sodium acetate buffer, pH 4.8, for these experiments. [b] See legend to Table I.

of the change. The results are shown in Table II. Digestion with acid phosphatase had little effect on the relative proportions of the various structures, decreasing the proportion of β-structure from 0.45 to 0.38. After the CD spectra had been run, the phosphorylated C-1 sample, treated with acid phosphatase, was applied to a Dowex-50 column (H^+ form) in a Pasteur pipet as described under Methods. Any inorganic phosphate released from the phosphorylated MBP should appear in the void volume of the column. Approximately 50% of the radioactivity originally bound to C-1 was recovered in the void volume, demonstrating that half of the phosphate was enzyme-labile while the remainder was not. No further release of phosphate was obtained even when phosphorylated C-1 was incubated with acid phosphatase overnight. We concluded that the phosphate which was not available to the enzyme occupied a protected site, which was important in the stabilization of the β-structure in the protein. Although other sites could be phosphorylated, these were not involved in the formation and/or maintenance of β-structure.

Identification of Sites of Phosphorylation in C-1. The identification of a critical phosphorylation site protected from acid phosphatase treatment was determined by isolation of peptides from the proteinase Lys-C digest resolved by HPLC as described under Methods. The peptide map is shown in Figure 4. The ^{32}P-labeled radioactive peptide after acid phosphatase treatment was eluted at 15 min (marked with an asterisk in Figure 4). This site was identified as residues 5–13 by amino acid analysis. It contains two potential phosphorylation sites at seryl residues 7 and 12. As this site was not available to acid phosphatase, it may be presumed to be a protected site involved in the maintenance of the secondary structure. Details of the distribution of phosphorylated sites in C-1 prior to acid phosphatase treatment will be described in a second paper (Ramwani and Moscarello, unpublished results).

Discussion

The solution conformation of MBP has been investigated extensively (Eylar & Thompson, 1969; Chao & Einstein, 1970; Palmer & Dawson, 1969). These studies were in agreement that little or no α-helical structure was present in the molecule on the basis of optical rotary dispersion studies. Circular dichroism studies (Smith, 1977) showed there was little change in secondary/tertiary structure over the pH range of 5.6–9.2. However, X-ray scattering studies (Epand et al., 1974) demonstrated that the molecule contained considerable secondary structure, although no specific α-helical or β-structure was identified. Immunochemical studies (Whitaker et al., 1977) showed that antibody to peptides 43–88 reacted well with the peptide but showed little or no reaction with intact basic protein. They concluded that this region of the molecule possessed a specific secondary structure. In a recent study of the conformation of MBP in solution and in lipid vesicles by Fourier-transform infrared spectroscopy, a considerable amount of β-structure was found in MBP bound to lipid

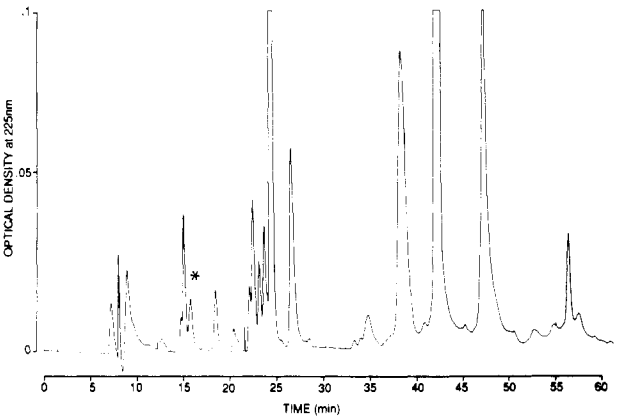

FIGURE 4: Peptide map of proteinase Lys-C digest of phosphorylated component C-1. Four hundred micrograms of phosphorylated component C-1 was treated overnight with acid phosphatase and then digested with proteinase Lys-C. The peptides were separated by reversed-phase HPLC as described under Methods. Among all the peptides eluted, only the peptide eluting at 15 min (shown by the star) was radioactive. By amino acid analysis, it was identified to be the peptide consisting of residues 5–13 of the human sequence.

vesicles (Surewicz et al., 1987). This observation provides the first experimental evidence for predicted models of MBP in which considerable β-structure was proposed (Stoner, 1984).

In all of the above-mentioned studies, the MBP used was unfractionated; i.e., although it could be shown to be pure by several methods such as a single band on SDS–PAGE and a single boundary in the analytical ultracentrifuge, in none of the above-mentioned studies was the MBP resolved into its various components (charge isomers). An earlier study by Jones and Epand (1980) did not detect any conformational differences among the components of MBP. In the present study using human brain MBP components and improved instrumentation, we have demonstrated that the solution conformation is different for some of the components.

An analysis of the relative proportions of α-helix, β-structure, random structure, and turns revealed that none of components 1–4 (Table I) had any α-helical structure. However, all components showed varying amounts of all of the other structures. In order of increasing amounts of β-structue C-1 < C-2 = C-3 < C-4, while the order for random structure was C-1 > C-2 = C-3 > C-4. Although the accuracy of β-structure estimates by CD is not very high (Chang et al., 1978), our results definitely indicate conformational differences among the components of MBP, and the estimate of the relative amounts of β-structure among the basic protein components is more reliable.

When the components were subjected to phosphorylation by the soluble form of protein kinase C, C-4 showed the lowest increase in the proportion of β-structure, demonstrating that it was largely in this conformation as isolated. The amount of β-structure in all other components changed considerably. Thus, the amount of β-structure in C-1 increased from 0.13 $\pm$ 0.01 to 0.37 $\pm$ 0.05 after phosphorylation, and in all components, the final proportion of β-structure varied within a narrow range of 0.37–0.41. Clearly, the different amounts of phosphate incorporated do not correlate with the amount of β-structure in each component. In his predicted model, Stoner (1984) has implicated salt bridges between phosphorylated amino acids and positively charged Arg and Lys residues as a mechanism by which the β-structure can be stabilized. The sites proposed for stabilization of the β-structure by phosphorylation were in the tri-proline region or near the C-terminus of the molecule. Neither Chan et al. (1987) nor the present studies were able to demonstrate significant

phosphorylation in vitro in the tri-proline region of the molecule. This tri-proline region must be highly structured since a synthetic peptide, Arg-Thr-Pro-Pro-Pro-Ser-Gly, which corresponds to residues 97–103 could be phosphorylated in vitro (Chan et al., 1986). The sites phosphorylated by a purified calcium-activated, phospholipid-dependent, and cAMP-dependent protein kinase were located in the N- and C-terminal regions of the molecule, i.e., in domain A, residues 1–83, and domain C, residues 120–170 (Chan et al., 1987). The inability of acid phosphatase to reverse the changes in the CD spectrum and the inability to remove more than 50% of the total $^{32}PO_4$ of phosphorylated C-1 support the view that phosphorylation at a critical site is responsible for the increase in β-structure observed. This site consists of residues 5–13, which contains Ser at positions 7 and 12 of the sequence. Although both of these sites were phosphorylated, 75% of the radioactivity was found on Ser-7 (Ramwani and Moscarello, unpublished experiments). Once formed, the phosphate stabilizing the β-structure is not accessible to acid phosphatase.

In summary, several interesting properties of MBP were revealed by the present study: (i) the charge isomers of MBP have different amounts of secondary structures, primarily β-structure in solution; (ii) phosphorylation of C-1–4 in vitro with soluble protein kinase C induces 35–40% β-structure in all charge isomers, regardless of the previously existing extent of phosphorylation; (iii) the largest increase in β-structure was observed with C-1 which incorporated the least amount of phosphate (2.8 ± 0.6 mol of P/mol of MBP); (iv) acid phosphatase was able to remove 50% of the phosphate from C-1, but only a small effect on β-structure content was observed; (v) the site inaccessible to acid phosphatase was in the peptide consisting of residues 5–13.

References

Brady, G. W., Murthy, N. S., Fein, D. B., Wood, D. D., & Moscarello, M. A. (1981a) *Biophys. J. 34*, 345–350.

Brady, G. W., Fein, D. B., Wood, D. D., & Moscarello, M. A. (1981b) *FEBS Lett. 125*, 159–160.

Brady, G. W., Fein, D. B., Wood, D. D., & Moscarello, M. A. (1985) *Biochem. Biophys. Res. Commun. 126*, 1161–1165.

Chan, J. K. F., Stoner, G. L., Hashim, G. A., & Huang, K. P. (1986) *Biochem. Biophys. Res. Commun. 134*, 1358–1364.

Chan, J. K. F., Moscarello, M. A., Stoner, G. L., & Webster, H. de F. (1987) *Biochem. Biophys. Res. Commun. 144*, 1285–1287.

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

Chao, I. P., & Einstein, E. R. (1970) *J. Neurochem. 17*, 1121–1132.

Cheifetz, S., & Moscarello, M. A. (1985) *Biochemistry 24*, 1909–1914.

Cheifetz, S., Boggs, J. M., & Moscarello, M. A. (1985) *Biochemistry 24*, 5170–5175.

Chou, F. C-H., Chou, C.-H. J., Shapira, R., & Kibler, R. F. (1976) *J. Biol. Chem. 251*, 2671–2676.

Chou, C.-H. J., Chou, C.-H. F., Kowalski, T. J., Shapira, R., & Kiebler, R. F. (1978) *J. Neurochem. 30*, 745–750.

Deibler, G. F., & Martenson, R. E. (1973) *J. Biol. Chem. 248*, 2392–2396.

Epand, R. M. (1988) in *Neurobiological Research* (Marangos, P. J., Campbell, I., & Cohen, R. M., Eds.) Vol. 2, pp 231–265, Academic Press, Orlando.

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

Eylar, E. H., & Thompson, M. (1969) *Arch. Biochem. Biophys. 129*, 469–479.

Jones, A. J., & Epand, R. M. (1980) *Biochim. Biophys. Acta 625*, 165–178.

Moscarello, M. A., Brady, G. W., Fein, D. B., Wood, D. D., & Cruz, T. F. (1985) *J. Neurosci. Res. 15*, 87–100.

Palmer, F. B., & Dawson, R. M. C. (1969) *Biochem. J. 111*, 629–636.

Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.

Schulz, P., Cruz, T. F., & Moscarello, M. A. (1988) *Biochemistry 27*, 7793–7799.

Smith, R. (1977) *Biochim. Biophys. Acta 491*, 581–590.

Stoner, G. L. (1984) *J. Neurochem. 43*, 433–447.

Surewicz, W., Moscarello, M. A., & Mantsch, H. (1987) *Biochemistry 26*, 3881–3886.

Whitaker, J. N., Chou, C.-H., Chou, F. C.-H., & Kibler, R. F. (1977) *J. Exp. Med. 146*, 317–331.

Wolf, M., Sahyon, N., Levine, H., III, & Cuatrecasas, P. (1984) *Biochem. Biophys. Res. Commun. 122*, 1268–1275.

Wood, D. D., & Moscarello, M. A. (1989) *J. Biol. Chem. 264*, 5121–5127.