Determination of the Sites of Posttranslational Modifications in the Charge Isomers of Bovine Myelin Basic Protein by Capillary Electrophoresis-Mass Spectroscopy†

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Received September 22, 1997; Revised Manuscript Received December 4, 1997

ABSTRACT: The posttranslational modifications in each of the 18.5 kDa bovine myelin basic protein charge isomers C-1 to C-6 have been determined by the use of capillary electrophoresis-mass spectroscopy. The pattern of modifications is viewed as being unique to each charge isomer and is thought to reflect a specific placement and function for each isomer in the myelin membrane. Several of the sites of posttranslational phosphorylation were found to differ from a number of the reported sites that were phosphorylated in vitro by various kinases. These differences suggest that an extremely cautious approach be taken in identifying in vivo posttranslationally modified amino acid residues from residues that have been modified in vitro by various kinases. We have identified the following posttranslationally phosphorylated and deamidated, modified sites in the bovine MBP components C1−C6. C1 has no modification; C2 represents a deamidation of Gln 146; in C3, Thr 97 and Ser 164 are phosphorylated; in C4, Ser 54, Thr 97, and Ser 160 are phosphorylated; in C5 Ser 7, Ser 54, Thr 97, and Ser 164 are phosphorylated; and in C6, Ser 7, Ser 54, Thr 97, Ser 160, and Ser 164 are phosphorylated.

The role of posttranslational modification such as acylation, phosphorylation, methylation, glycosylation, deamidation, and attachment of fatty acids has been shown to play a significant role in directing the function of various proteins. In CNS myelin basic protein, these modifications include (1) the phosphorylation of specific serine and threonine residues, (2) the acylation of the N-terminal amino acid, (3) the methylation of a specific arginine residue, (4) the deamidation of a glutamine residue, and (5) the conversion of arginine residues to citrulline in MBP components C6 and C8. All of these modifications contribute to the introduction of charge microheterogeneity into the protein. The identification of the spectrum of individual in vivo sites modified in each of the charge isomers has not been reported. We now identify many of these sites, in bovine MBP, by a technique that combines capillary electrophoresis and mass spectroscopy.

Bovine CNS myelin basic protein (MBP)[1] was initially purified and sequenced by Eylar et al. (*1*, *2*, *3*). Shortly thereafter, the amino acid sequences of other common animal species were reported by Kies, Deibler, and Martenson (*4*). The human sequence was reported by Carnegie (*5*). Initial recognition that MBP, as isolated, is microheterogeneous was recognized to be the consequence of posttranslational modifications from the attachment of phosphate groups, loss of terminal arginine residues (*6*), and other modifications including the methylation of arginine residue 106, which yield the monomethyl and dimethyl derivatives (*7*), the deamidation of a glutamine residue (*8*, *11*), the conversion of arginine residues in components C6 and C8 to citrulline by the enzyme peptidyl arginine deiminase (*9*), and the loss of one or two terminal arginine residues (*6*, *11*). Using CMC cellulose chromatography columns, the separation of these charge isomers on a preparative scale was reported by Martenson et al. and Chou et al. (*10*, *11*) and were designated as components C1−C8 in order of their elution from the column. The identification of the in vivo posttranslational modification sites on unfractionated and components C1−C3 MBP was achieved using rabbit MBP peptide maps and amino acid analysis of the peptides. Using this approach, the following modified sites in rabbit MBP, were identified: Ser 7, Ser 56, Thr 96, Ser 113, and Ser 163 (*12*). Chou et al. (*11*) identified Thr 97 and Ser 164 as being phosphorylated in bovine MBP. These findings differ somewhat from phosphorylation sites reported, based on in vitro studies using various protein kinases on bovine MBP (*13*).

Previous studies concerned with the sites of phosphorylation of CNS myelin basic protein were done on unfractionated MBP, with the exception of those reports from Martenson et al. (*12*), Chou et al. (*11*), Agrawal et al. (*13*), and Moscarello et al. (*9*, *14*). The in vitro studies were done on unfractionated MBP. Therefore, the location of phosphate moieties on the six charge isomers of bovine MBP, and other species-derived MBP, have not been assessed previously on the individual, chromatographically separated charge isomers. The previous in vitro and in vivo studies were time

† We gratefully acknowledge support of this work by the National Institutes of Health under Grant R01 GM49500 and the National Science Foundation under Grant BIR-9513878.

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[1] Abbreviations: MBP, myelin basic protein; CMC, carboxy methyl cellulose; MAPK, mitogen activated protein kinase; MAP, mitogen activated kinase; TPCK, L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone; CE, capillary electrophoresis; MS, mass spectroscopy; TOF, time-of-flight; TIE, total ion electropherogram.

S0006-2960(97)02347-7 CCC: $15.00
Published on Web 02/04/1998

consuming and were limited in scope. A review on the phosphorylation of myelin proteins by Ulmer appeared in 1988 (*15*).

The posttranslational modification of proteins has acquired increasing importance as their role in signal transduction and influence on protein conformation has been discerned. Myelin basic protein has been shown to be an effective substrate for mitogen-activated protein kinase (MAPK) (*16*). The MAP kinase cascade is conserved in all eukaryotes, and thus, the ability of MBP to function as a substrate may be indicative of its role in a signal transduction pathway. Other suggested roles for the phosphorylation of MBP include sorting newly synthesized MBPs at their site of synthesis and routing them to the site of insertion into the membrane (*17*), and/or involvement in the compaction and maintenance of the membrane (*18*, *19*).

To extend our understanding of the role played by posttranslational modification in MBP, we assessed these modifications in the bovine MBP charge isomer components. In this manuscript, we present a direct approach for the identification of the amino acid residues modified by in vivo posttranslational modifications in six of the MBP charge isomers. This technique can be used to determine the sites of posttranslational modification in most moderate sized proteins.

The use of mass spectrometry to identify some of the amino acid residues of bovine MBP that are phosphorylated in vitro by MAP kinase was reported by Erickson et al. (*16*). Gibson et al. (*20*) subjected the human MBP peptide fragment 45−89 to mass spectral analysis and were unable to confirm the phosphorylation of Ser 56.

EXPERIMENTAL SECTION

Materials. Ammonium bicarbonate, ammonium acetate, and formic acid were obtained from Sigma Chemical Co. (St. Louis, MO). TPCK-treated trypsin was obtained from Promega (Madison, WI). Praestol was generously donated by Stockhausen GMBH (Krefeld, Germany). All chemicals were used without further purification. Water was filtered and deionized by a Milli-Q purification system (Millipore Corp., Bedford, MA). Fused silica capillaries were obtained from Polymicro Technologies (Phoenix AZ).

Sample Preparation. Bovine myelin basic protein was isolated and purified according to the procedure of Deibler et al. (*22*). Charge isomer components were separated on a CMC column according to the procedure of Chou et al. and Martenson (*10*, *11*).The fractions containing components C1−C8 were separated and their purity assessed by polyacrylamide gel electrophoresis at basic pH, Figures 1 and 2. The purified fractions were exhaustively dialyzed against distilled water and then lyophilized.

For each of the fractions, an amount equal to 20 μg of MBP was digested for 24 h at 37 °C, with trypsin using a protein:enzyme ratio of 50:1 in an ammonium bicarbonate buffer, pH 8.2. The digests were then taken to dryness under a vacuum and reconstituted in the capillary electrophoresis buffer to give a solution with a charge isomer concentration ca. 1×10^{-5} M.

The trypsin digest of each charge isomer was separated using an in-house constructed capillary electrophoresis apparatus consisting of a ±30 kV high voltage power supply

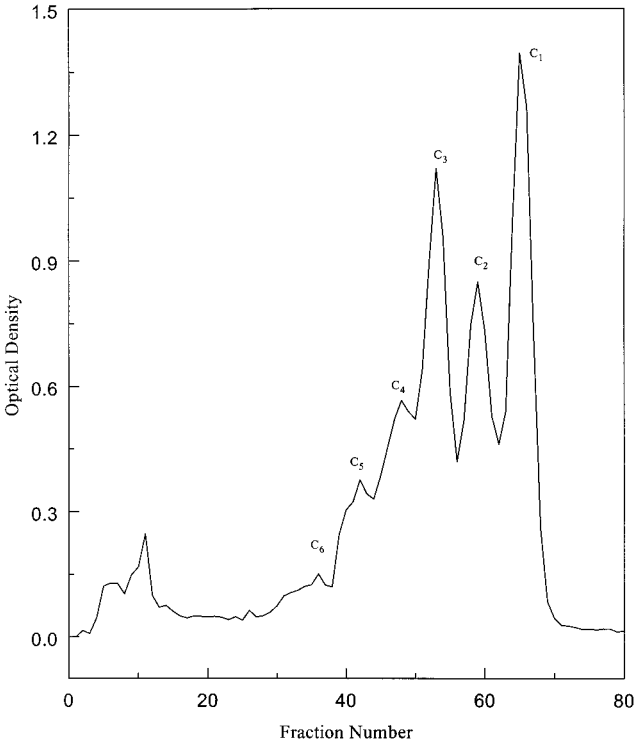


FIGURE 1: Separation of bovine MBP charge isomers on a CMC column at pH 10.5. Elution was with a linear 0 to 0.2 M NaCl gradient.

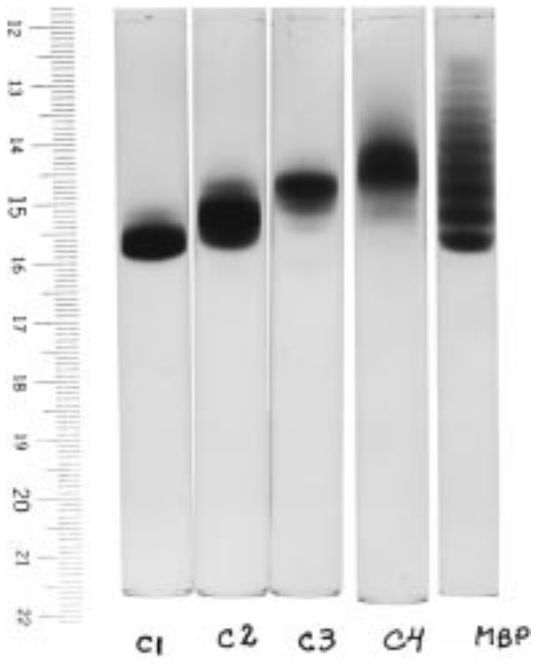


FIGURE 2: Basic polyacrylamide gel electrophoresis, pH 10.5, of some of the CMC column separated bovine MBP charge isomer components stained with 0.5% amido black. Procedure according to Deibler et al. (*21*).

(model CZE 1000R, Spellman High Voltage Electronics Corp., Plainview, NY), a variable wavelength UV detector (model SC100, Thermo Separation Products, Fremont, CA), and a positively coated capillary column. The entire separation normally required approximately 15 min and was monitored at a wavelength of 200 nm. The data were collected by a computer (model P5-66, Gateway 2000 computer, Gateway Inc., Sioux City, SD). The samples were

Table 1: Experimental and Theoretical Data for the Tryptic Peptides of Bovine Myelin Basic Protein

sequence		theoretical mass	experimental mass	modified peptide mass value
T1, 1−4	AAQK	416.5	not detected	
T2, 5−5	R	174.2	173.9	
T2+3, 5−9	RPSQR	642.7	643.0	723.1
T4, 10−11	SK	233.3	233.6	
T5, 12−23	YLASASTMDHAR	1322.5	1322.2	
T6, 24−29	HGFLPR	725.8	726.4	
T7, 30−31	HR	311.3	311.4	312.5
T8, 32−41	DTGILDSLGR	1046.1	1046.1	
T9, 42−47	FFGSDR	727.8	727.3	
T10, 48−51	GAPK	371.4	371.8	
T11, 52−52	R	174.2	173.9	
T12, 52−56	RGSGK	503.6	504.1	
T13, 57−63	DGHHAAR	762.8	763.2	
T14, 64−73	TTHYGSLPQK	1131.3	1131.0	
T15+16, 74−90	AQGHRPQDENPVVHFFK	2006.2	2006.0	
T17, 91−96	NIVTPR	698.8	698.6	699.5
T18, 97−104	TPPPSQGK	810.9	811.7	891.2
T19, 105−106	GR	231.3	231.3	
T20, 107−112	GLSLSR	631.7	632.5	
T21+22, 112−129	FSWGAEGQKPGFGYGGR	1801.0	1801.2	
T23, 130−134	ASDYK	582.6	582.5	
T24, 135−138	SAHK	441.5	442.7	
T25, 139−141	GLK	316.4	317.0	
T26, 142−151	GHDAQGTLSK	1013.1	1013.4	996.8
T27, 152−154	IFK	406.5	406.6	
T28, 155−158	LGGR	401.5	402.3	
T29, 159−161	DSR	376.4	376.3	456.0
T30+31, 162−169	SGSPMARR	861.0	861.6	940.2

electrokinetically injected into the capillary by applying several thousand volts for 10 s to the solution in the sample vial that also contained the capillary end and the electrode. A UV detection window was made by burning off 0.5 cm of the polyimide coating ca. 6 cm distance from the anodic end.

Preparation of Capillary Tubes. The adsorption of the positively charged peptides onto the inner capillary wall was reduced by covalently attaching positively charged small molecules to the wall of a noncovalently bound polymer. We have previously reported that such coatings successfully minimized the attachment of peptides or proteins to the capillary wall (*23*, *24*). The coating material used in the present study was a solution of the commercial polymer Praestol (*25*, *26*). The capillary tubes, 40 μm i.d., 105 μm o.d., and 60 cm length, were sequentially treated with 1 N NaOH for 4 h, deionized water for 10 min, 5% aqueous Praestol solution for 4 h, deionized water for 10 min, and finally with capillary electrophoresis buffer for 30 min. The buffer consisted of 100 mM formic acid and 5 mM ammonium acetate at pH 3.0. The column was then conditioned for 30 min. The applied electric field was reversed relative to the bare fused silica capillary because the positive layer generated an electroosmotic flow toward the anodic end. The applied electric field was ca. −300 V/cm.

On-Line CE/MS System. An ion trap storage reflectron time-of-flight mass spectrometer (IT/re/TOF MS) described in previous work was used as the detector for the CE separated peptides (*23*). The apparatus consists of a quadrupole ion trap storage device (model 1251, R.M. Jordan Co., Grass Valley, CA) and a reflectron TOF mass analyzer (model D1450). Typically, externally produced ESI ions were trapped and stored for a period of 0.5 s by an applied RF field on the ring electrode. A helium buffer gas was used to enhance ion trapping, before the ions were ejected into the TOF spectrometer. Detection was achieved with a 40 mm triple MCP detector (Model C-2501, R.M. Jordan Co.). The pressure inside the TOF tube was 4×10^{-7} Torr, while the approximate pressure in the trap was 10^{-3} Torr when the He buffer gas was added to the trap. One advantage of this device is that the RF field can be tuned up for a specific mass range of ions in order to enhance their detection efficiency; e.g., in the MBP tryptic digest, where there are many low mass peptides, the RF voltage was lowered to 1000 V for the on-line CE/MS experiment.

The mass data were collected and processed using a 250 MHz transient recorder (model 9846, Precision Instruments Inc., Knoxville, TN) connected to a Gateway personal computer model P5-66. The data acquisition system could acquire a single mass spectrum up to 25 Hz, using in-house developed custom software. A time-of-flight range of 0−150 μs was generally used as the mass acquisition period. This corresponds to an approximate m/z range of 0−1500 Da.

The anodic end of the CE capillary was used as a microelectrospray source to render the capillary end conductive and produce a stable spray plume. To accomplish this, the end of the polyimide coating was removed, etched with a concentrated HF solution to a diameter of ca. 50 μm, and silver coated. The coated tip was inserted into a 2 cm length of stainless steel tubing (125 μm i.d., 250 μm o.d.) connected to the microelectrospray power supply. This provided an electrical contact for both microelectrospray and capillary electrophoresis. The power supply of the capillary electrophoresis was set at ca. −12 kV, while the microspray needle was set at a value of ca. 3.5 kV, leading to a total charge separation of ca. −15 kV. The liquid stream inside the coated capillary, determined by the electroosmotic and

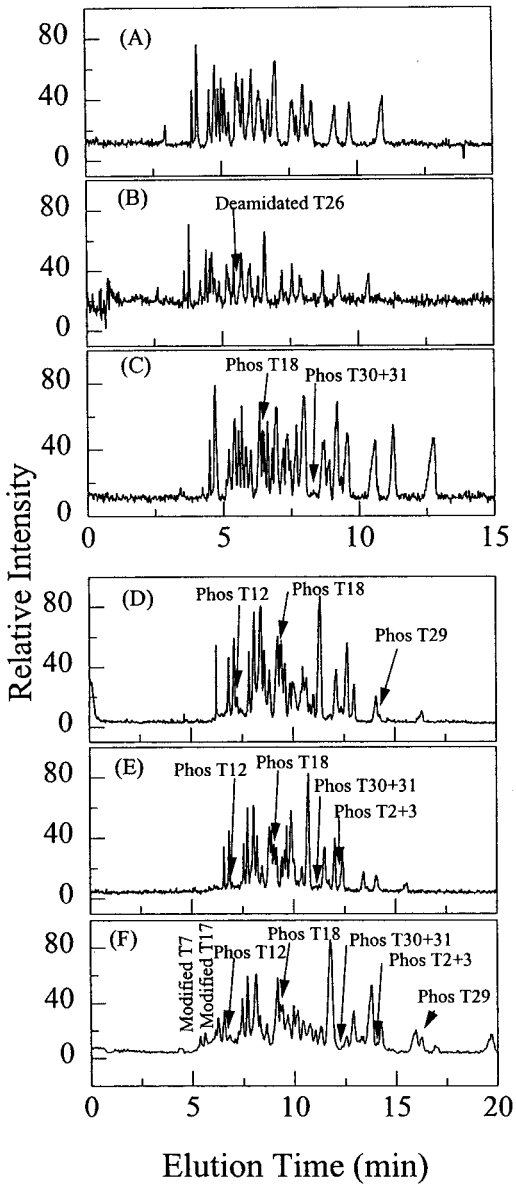


FIGURE 3: Total ion electropherograms (TIEs) of the six charge isomers of bovine MBP: (A) MBP-C1, (B) MBP-C2, MBP-C3, (D) MBP-C4, (E) MBP-C5, (F) MBP-C6 separation conditions: the capillary was Praestol coated, 60 cm long, 40 μm i.d., 105 μm o.d. Buffer: 100 mM formic acid and 5 mM ammonium acetate, pH 3.0, $V_{CE} = -12\,000$ V, $V_{ESI} = 3500$ V, mass acquisition speed, 2 Hz.

electrophoretic flows, was sufficient to form a stable microelectrospray background during this sheathless condition.

RESULTS

Digestion of bovine MBP with trypsin should yield 30 peptide fragments. The total ion electropherogram (TIE) of MBP−C1 mass data for the tryptic peptides formed is presented in Table 1, and the TIE is shown in Figure 3. The theoretical and experimental masses obtained are listed, and based on the known primary structure, it was possible to account for more than 95% of the total protein sequence. The major 30 peptides in the tryptic digest of C1 were also present in C2. There is no mass shift of 79 mass units, that can be discerned and would account for a phosphorylation of a serine or threonine residue. A careful search of the TIEs and 3D diagram revealed a peak in the C2 digest that is absent in the C1 digest. This peak is indicated by an arrow in the C2 TIE. The mass spectrum corresponding to this peptide (T26) and the normal spectrum are given in Figure 4. The mass difference of 8.4 Da in peptide T26 is attributed to the doubly charged ion that gives a 16.8 Da difference for the singly charged molecular ion. This is interpreted as indicating that the C2-T26 fragment has undergone deamidation of the glutamine 146 residue in this peptide. Although the identification of glutamine and asparagine residues by mass spectrometry is not always straightforward, in the present instance, we believe this interpretation to be correct based on the following arguments. First, there was no evidence for deamidation in the T-26 fragments from any of the other charge isomers, and Gln 146 has been reported as being deamidated when the bovine MBP was sequenced by standard methods (*11*). Second, the amino acid sequence in this region that includes Gln 146 conforms to the generalized assignment of sequence composition with a propensity for β-turn formation which would allow the side-chain functional group on appropriate residues to interact. Third, it has been reported by Bischoff et al. (*27*) that the susceptibility for deamidation of Asn was markedly enhanced when it was followed by a Gly residue. By analogy this should also apply to Gln when followed by Gly.

Last, the ability of a glutamine residue to act as an acyl donor with a specific lysine residue in β endorphin by forming an isopeptide bond has been reported in the literature (*28*). Thus, the T26 peptide molecular weight showing a loss of 16 amu can be explained by the argument presented.

The TIE pattern of MBP-C3 is distinct from that of C1. Two additional peaks, attributed to the phosphorylation of peptide T18 and T30+31, are clearly discernible in the mass fragments of C3 but absent in C1. A comparison of the mass spectra of the phosphorylated and non phosphorylated forms indicates a mass shift of 79 Da in peptides T18 and T30+31 as shown in Figure 5. These phosphorylated sites are assigned to Thr 97 and Ser 164.

For peptides T12, T18, and T29 derived from MBP-C4, the mass fragments obtained indicate the presence of phosphate groups (indicated by the arrows). The phosphorylated peptides are assigned to T18, T29, and T12. The phosphorylated fragment T30+31 present in component C3 is absent in component C4.

The MBP-C5 tryptic digest shows the absence of a phosphorylated T29 fragment in the C5-TIE. The T2+3, T18, T30+31, and T12 peptide fragments show that they are phosphorylated. However, the phosphorylated peptide T29 is absent although it is relatively abundant in MBP-C4.

In MBP-C6, five phosphorylated peptides are present. In addition, there is a mass shift of 1 Da in peptides T17 and T7. The mass shift of 1 Da is ascribed to a change in the C-terminal arginine C═N−H to a C═O, which could arise from the conversion of an arginine residue to a citrulline residue. While such a modification has not been reported previously for arginine residues in this segment of MBP, this modification has been well documented in the C-terminal region of MBP-C8 (*42*).

DISCUSSION

The role of posttranslational phosphorylation, glycosylation, acylation, and other modifications in modifying the

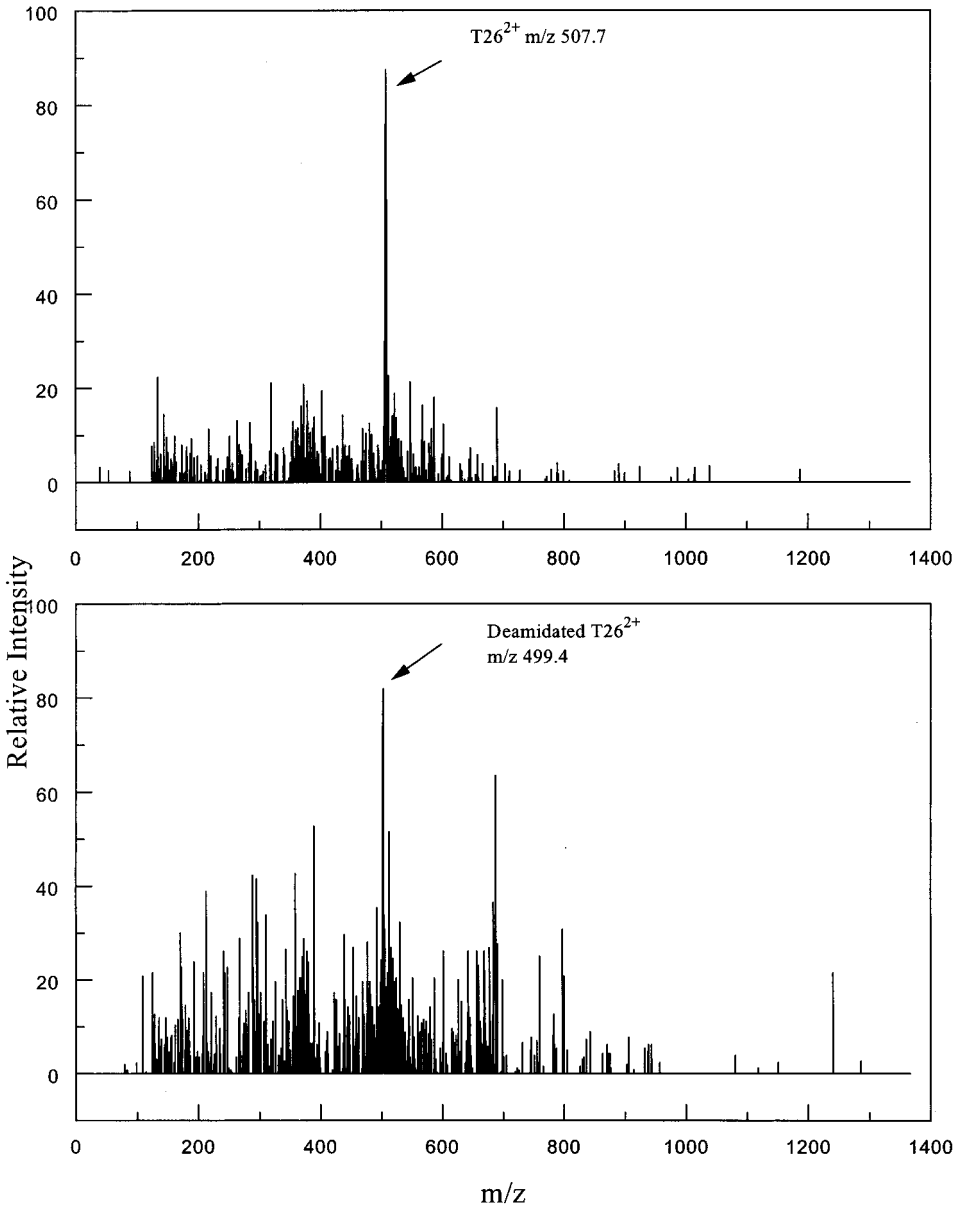


FIGURE 4: Mass Spectra of the T26 fragment ion from MBP-C1 (A) and the corresponding deamidated fragment ion from MBP-C2 (B) obtained in the CE/MS experiment.

structure and biochemical activity in many proteins is well documented, but in many instances is not well understood. In particular, the role of the extensive phosphorylation of MBP in higher animals has not been elucidated. A number of laboratories have attempted to determine the in vivo phosphorylated sites by treating the purified MBP with various kinases. Ulmer has reviewed these studies (*15*). We view the extrapolation of such results to the identification of in vivo phosphorylated sites as being subject to major errors.

The primary structures of the major MBP from more primitive species such as sharks, dogfish, and rays have been reported (*29*−*31*). However, no determination of posttranslational modifications present in these MBPs has been reported. Very little is known about the MBP primary structures, number of charge isomers, and number of gene products from animals that are lower on the evolutionary scale. The majority of MBP sequences that have been reported have been based on mammalian species. Studies from this laboratory indicate that MBP from alligator, red-eared turtle, and dogfish contain about three charge isomer components as opposed to about eight in mammalian species (*32*). To establish a common reference point, that permits the overlap of amino acid sequences common to the primary structure of any MBP, we have designated the human MBP sequence as the primary reference standard for which the homology of all amino acid residues in any MBP species can be referred. This permits a consistent alignment system of all amino acid residues for the comparison of sequence homology and sites of posttranslational modification in any species of MBP, and for this study, it allows for a facile comparison of modified human, bovine, and rabbit MBPs. For each sequence, we maintain its unique numbering designation of each amino acid residue but align structures based on the human sequence. The appropriate residue number in a specific species sequence is indicated as a subscript throughout the aligned sequences of the three MBP proteins. The assessment of MBP in vivo phosphorylation was reported by Martenson et al. (*12*) using unfractionated rabbit brain MBP. In a joint publication, Agrawal and

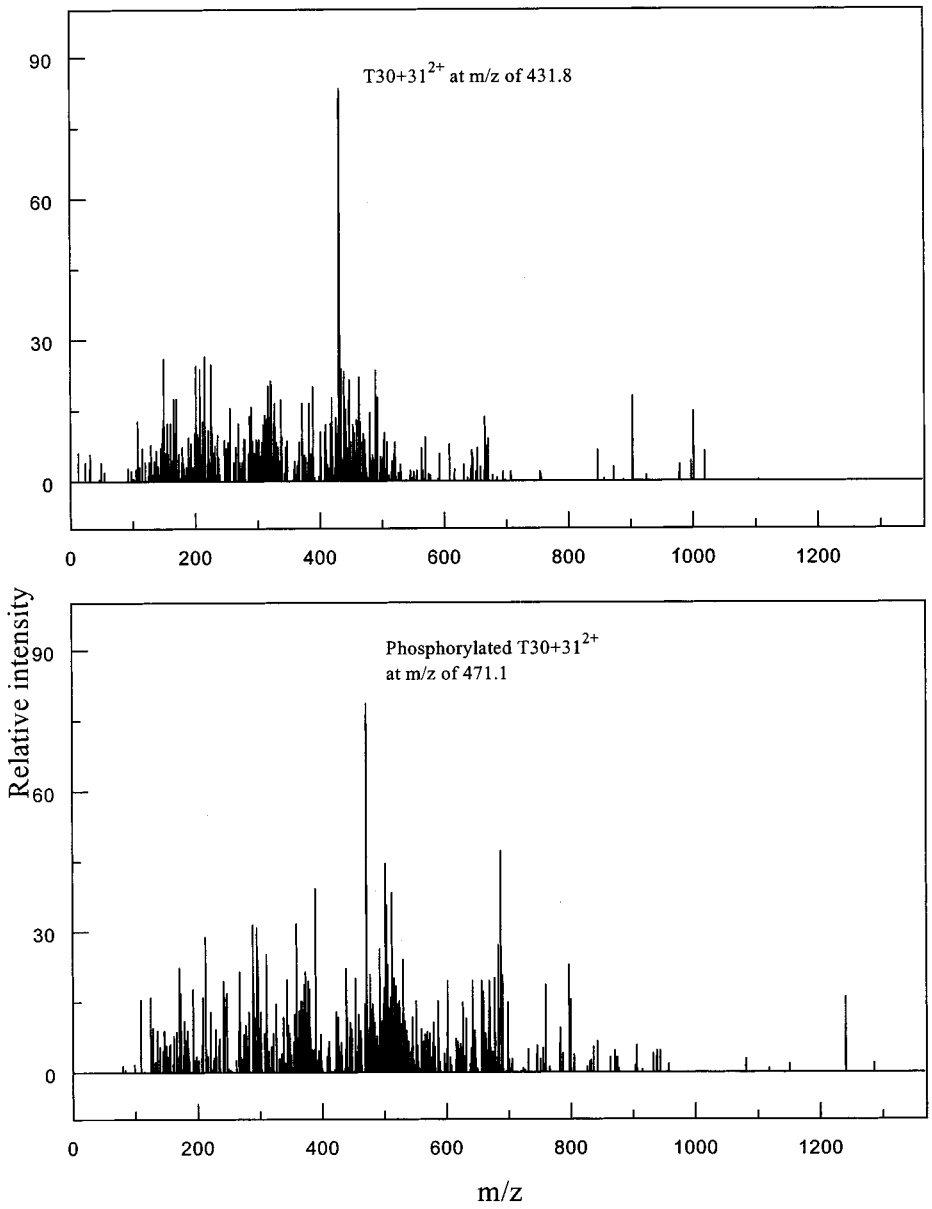


FIGURE 5: Mass spectra of the T30+31 fragment ion from MBP-C1 (A) and the corresponding phosphorylated fragment ion from MBP-C3 (B) obtained in the CE/MS experiment.

Martenson (*13*) studied the incorporation of [^{32}P]orthophosphate into rabbit brain and separated the radioactive MBP formed, using basic gel electrophoresis. In this study, they showed that no radioactivity was present in components 1 and 2. They found that the radioactivity was localized in components 3, 5, and 7. They did not identify the specific sites of phosphorylation. Subsequently, the five sites of phosphorylation in rabbit MBP, namely Ser 7, Ser 56, Thr 96, Ser 113, and Ser 163, were identified but were not correlated with the specific charge isomers that constitute the purified MBP. In a report earlier than the Martenson report on rabbit MBP, Chou et al. (*11*) had identified Thr 97 and Ser 164 as being phosphorylated in bovine MBP. Using our capillary electrophoresis-mass spectroscopy technique we have also assigned the sites of phosphorylation and deamidation to specific charge isomers of bovine MBP. In Table 2, below, we contrast our results with those reported in the Martenson et al. (*12*) paper on rabbit MBP, and those reported in the Chou (*11*) paper on bovine MBP. For comparison, the sites of in vitro phosphorylation of bovine MBP using protein kinase enzymes is also provided. In Figure 6, the primary sequences of the human, bovine, and rabbit MBP sequences are compared and numbered so that the equivalent sites of phosphorylation can be readily identified. In the assessment of modifications of the charge isomers from bovine MBP using the capillary electrophoresis-mass spectroscopy methodology, we find, in agreement with the findings for rabbit MBP, that component C1 is unmodified except for the N-terminal acyl group. Component C2 has been deamidated at glutamine 146 but our data shows no indication of deamidation at glutamine 102 as reported by Chou et al. (*11*). The identification of the sites of phosphorylation of component C3 were found at threonine 97 and serine 164 in agreement with the report by Chou et al. on the unfractionated bovine MBP. There is no indication for the phosphorylation of Thr 33 as found in the in vitro studies by Kishimoto et al. (*33*). Component C4 has residues Ser 54, Thr 97, and Ser 161 phosphorylated. The Ser 159 site was not identified as being phosphorylated in the rabbit MBP. However, Ser 159 was reported to be phosphorylated

Table 2: Sites of Phosphorylated Residues and Deamidation in Myelin Basic Protein

present data	Martenson (*12*) rabbit data	Chou (*11*) bovine	Kishimoto (*32*) protein kinase A and C
Ser 7 (7)[a]	Ser 7 (7)		Ser 7 (7)
Ser 54 (56)	Ser 56 (56)		Ser 10 (12)
Thr 97 (98)	Thr 96 (98)	Thr 98 (98)	Thr 33 (35)
			Ser 45* (F45) (kinase C only)
			Ser54 (56)
			Ser 109 (110)
	Ser 113 (115)		Ser 114 (115)
			Ser 131 (132)
			Ser 150 (151)
Ser 160 (161)			Ser 160 (161)
Ser 164 (165)	Ser 163 (165)	Ser 164 (165)	Ser 164 (165)
deamidation of Gln 146 (147)			

[a] Numbers in parentheses indicate the equivalent sequence site in the human MBP.

in vitro by protein kinase A and C (*33*). Component 5 had Ser 7 phosphorylated along with Ser 54, Ser 164, and Thr 97. Lastly, component 6 had Ser 7, 54, 160, 164, and Thr 97 phosphorylated. Although Thr 97 is found to be phosphorylated in components 3, 4, 5, and 6, phosphorylation of Ser 54 is identified only in components 4, 5, and 6, while Ser 164 is phosphorylated only in components 3, 5, and 6. This is in contrast to the report by Gibson et al. (*20*) who reported that Ser 56 in human MBP was not phosphorylated. Ser 56 in human MBP is equivalent to Ser 54 in bovine MBP. Phosphorylation of Ser 7 was noted only in components 5 and 6. This is rather noteworthy since it may be indicative of some structural change that has occurred in the folding of MBP as a consequence of charge reduction, and this change now exposes Ser 7 so that it becomes a kinase substrate. Since Ser 7 occurs at the very beginning of the N-terminal region where it should be quite exposed, it is somewhat surprising to find that it is only phosphorylated in components 5 and 6. Since the time sequence of phosphorylation at each particular serine or threonine residue is not known, it may be that components C5 and C6 are phosphorylated before components C3 and C4. Clearly, the Thr residue at position 97 is properly positioned to make it a good substrate for MAP kinase and appears to be one of the first sites in MBP to be phosphorylated. A recent report by Yon et al. (*34*) demonstrated the presence of MBP in brain sections stained by immunogold and visualized by electron microscopy using a specific antibody for phosphorylated Thr 97. In contrast, our data differs from the reports of Kishimoto et al. (*33*), Carnegie et al. (*35*, *36*), Shoji et al. (*37*), and Turner et al. (*38*) as shown in Table 3.

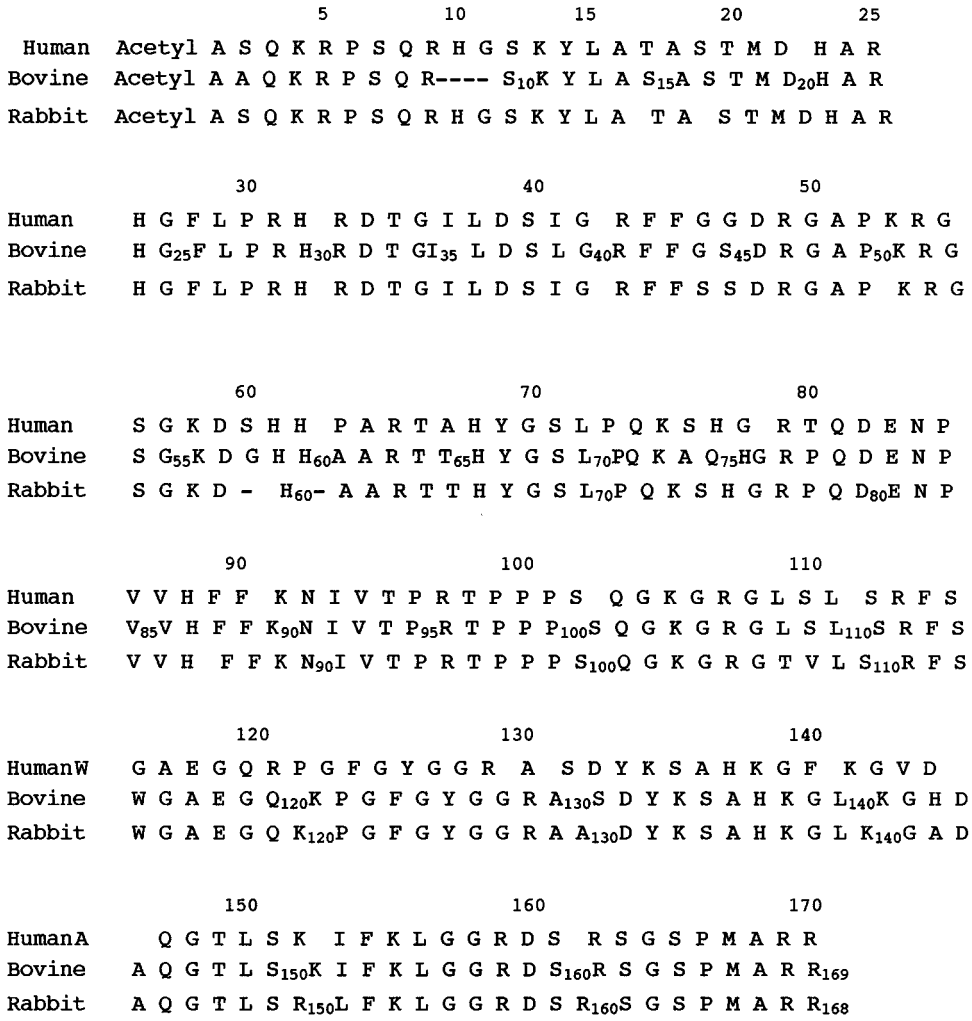
It appears that several phosphorylation sites observed from in vitro kinase phosphorylations are not phosphorylated *in vivo*. No evidence to support the phosphorylation of Ser 111 or Ser 114 could be found in the mass spectral data of MBP charge isomers. This leads to a caution in extrapolating the results of such in vitro experiments to in vivo conclusions. It is of considerable importance to understand why the sequence of charge reduction and phosphorylation, of the charge isomers, progresses in the rather unique manner that it does. Assessment of our data is summarized in Table 3.

Our data support the literature report (*11*) that the charge reduction from isomer C1 to C2 is accomplished by a deamidation. We did not find evidence for its presence in isomers C3−C6. Isomer C3 has a reduction of positive charges achieved by the introduction of phosphate groups at Thr 98 and Ser 165. We were unable to find a reduction of positive charges by one phosphate and a deamidation sequence. Isomer C2 was the only isomer in which it was possible to clearly discern the deamidation of a glutamine residue. Isomer C4 in which three positive charges have been neutralized by the phosphorylation of Ser 56 and Ser 161 along with Thr 98 follows the pattern of not building upon the C3 phosphorylations. In this instance, as in the case of C3, the phosphorylation of Thr 97 is carried over but Ser 164 is not and is replaced by the phosphorylation of Ser 160 plus the additional phosphorylation of Ser 54. Component C5 has a new phosphorylated site at Ser 7, and the phosphorylated Ser 160 is replaced by a phosphorylated Ser 164. The sites at Ser 54 and Thr 97 are also retained. Lastly, component C6 incorporates all of the previously phosphorylated sites but shows no evidence for deamidation of asparagine 82. Our assessment of the sites of phosphorylation, deamidation, and methylation is somewhat limited by the sensitivity of current instrumentation. Nevertheless, there is a clearly unique pattern of charge reduction, and we interpret this uniqueness as indicative of a specific role and placement for each charge isomer in the membrane. This would be in accord with the known presence of MBP in oligodendroglia prior to myelin sheath formation (*39*). The uniqueness placement concept is also supported by the report of McLaurin et al. (*40*) in which component C8 was localized in the intraperiod line of myelin electron micrographs.

The phosphorylation of Thr 97 in the charge isomers C3−C6 and its mode of phosphorylation by MAP kinase provides a strong implication for a possible role of these isomers in some signal transduction process. Deibler et al. (*22*) have reported that treatment of a mixture of components C2 and C3 with thrombin yielded an MBP fragment containing only the phosphorylated Thr 97 residue. In the present study, we find that component C3 contains not only phosphorylated Thr 97, but also phosphorylated Ser 164. It may be that, experimentally, the Deibler et al. methodology was not sensitive enough to detect the phosphorylation of Ser 164 or that our preparation of component C3 was contaminated with some small amount of component C4 and C5. However, if there was such contamination, we should have also detected the presence of Ser 54 and Ser 160 in these mass spectra. These phosphorylated residues do not appear to be present at our present levels of sensitivity.

Schulz et al. (*41*) isolated myelin with varying levels of compaction from human brain. When these myelin preparations were phosphorylated by endogenous myelin kinase, 70% of the radioactive ^{32}P was incorporated into Ser 102. The remaining 30% of the radioactivity was distributed to three other sites. Ser 102 is not one of the in vivo phosphorylation sites.

This study has demonstrated that the combination of capillary electrophoresis linked to mass spectroscopy provides a facile and accurate method for the determination of posttranslational modification of proteins. In this specific instance, it was shown that the charge isomers of bovine MBP are not modified in a sequential manner but that modifications of each charge isomer are specific to that isomer. We view this as providing support for the concept



*Note that alignment follows human sequence but numbering follows the parent sequence.

FIGURE 6: The amino acid sequences of MBP from human, bovine, and rabbit brain with amino acid residues aligned with the human MBP sequence, but numbered according to the primary structure of each species.

Table 3

MBP charge isomer fraction	charge reduction
C1—no modification	0
C2—deamidation of glutamine 147	−1
C3—phosphorylation Thr 97, Ser 164	−2
C4—phosphorylation Ser54, Thr 97, Ser 160	−3
C5—phosphorylation Ser 7, Ser 54, Thr 98, Ser 164	−4
C6—phosphorylation Ser 7, Ser 54, Thr97, Ser 160, Ser 164	−5

that each charge isomer has a specialized function in the assembly of an optimized, biochemically functional myelin membrane, and that the myelin membrane may not be optimized with respect to function in more primitive animal species. Nevertheless, these lower vertebrates do make CNS myelin which is sufficiently functional to permit reasonable compaction and functioning of the membrane. The myelin from these species may be analogous to the immature myelin of higher animals. As maturation proceeds, additional MBPs are synthesized and incorporated to carry out other functions that may not be related to myelin compaction but might contribute to some signal transduction mechanism. Whether lower species, such as dogfish and sharks, contain phosphorylated forms of MBP is still to be determined. Dogfish MBP is reportedly not phosphorylated (*20*). Clearly, the role of phosphorylation and other posttanslational modifications of MBP is an important focus for which much additional information will be needed to understand the biological function of this unique system of protein molecules.

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BI972347T