Deimination of Myelin Basic Protein. 2. Effect of Methylation of MBP on Its Deimination by Peptidylarginine Deiminase†

Laura B. Pritzker,‡ Shashikant Joshi,‡ George Harauz,§ and Mario A. Moscarello*,‡

Department of Structural Biology & Biochemistry, The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8, and Department of Molecular Biology and Genetics, University of Guelph, Guelph, Ontario, Canada N1G 2W1

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ABSTRACT: Deimination of myelin basic protein (MBP) has been implicated in the chemical pathogenesis of multiple sclerosis (MS). Degradation of bovine MBP by cathepsin D, a myelin-associated protease, was increased when 6 arginyl residues were deiminated and became very rapid when all 18 arginyl residues were deiminated. Since MBP contains a number of modifications, including methylation, phosphorylation, etc., we studied the effect of methylation, an irreversible modification, to determine how this modification affected deimination. Methylation of Arg 106 in bovine MBP (Arg 107 in human), a naturally occurring modification of MBP, has been shown to affect the deimination of arginyl residues in the present study. Since fractionation of MBP into unmethylated, monomethylated, and dimethylated species cannot be done readily on a preparative scale, mass spectrometry with the Q-TOF instrument resolved these species readily since each differed from the other by 14 atomic mass units (amu). Examination of five different hMBP samples, two from normal brain and three from MS brain, revealed that increased deimination of arginyl residues correlated with a decreased methylation of Arg 107 (human sequence). To study this process in vitro, bovine MBP (bMBP) was used. Component 1 (C-1) is the most cationic of the MBP "charge isomers" and the most unmodified, in which all arginyl residues are intact. It was deiminated to various extents with purified bovine brain peptidylarginine deiminase, generating a number of species containing 0−13.7 mol of citrulline/mol of bMBP. Mass spectrometry of each of these species permitted us to determine the influence of methylation of Arg 106 (bovine sequence) on deimination by this enzyme. We found that bMBP with unmethylated arginine was deiminated at a rate of 0.081 mol of citrulline/min, with monomethylarginine, 0.068 mol of citrulline/min, and with dimethylarginine, 0.036 mol of citrulline/min. We suggest that the methylated arginyl residue becomes sequestered in the hydrophobic β-sheet structure and disrupts the three-dimensional structure of the protein so that other arginyl residues are less accessible to peptidylarginine deiminase.

Protein methylation was first identified as a posttranslational modification in 1959 with the discovery of methyllysine in bacterial flagellar protein (*1*) where it has been shown to be involved in chemotaxis involving the transfer of a methyl group to the γ-carboxyl group of glutamic acid residues in an esterification reaction. These methyl groups may be removed by a methylesterase, and therefore the extent of methylation may be varied in response to attractants and repellants (*2*). Methylation of eukaryotic proteins occurs not only on carboxyl groups of glutamic acid residues but also on side chain nitrogens of lysine, arginine, or histidine and on the N-terminal α-amino group. A large number of methylated proteins have been identified, including ankyrin (*3*), G proteins such as Ras (*4*), and RNA binding proteins such as human hetergeneous RNP protein A1 (*5*), and enzymes including phosphoprotein phosphatase 2A (*6*).

Myelin basic protein (MBP)[1] was first found to have a methylated arginine at position 107 when the primary sequence of the human protein was established (*7*); in the bovine protein methylation was reported on the corresponding arginine 106 (*8*). This methylated arginine was later found to be a mixture of monomethylated and symmetrically dimethylated arginine (*9*). Methylation of MBP is carried out by a specific methyltransferase (*10*). The activity of this methyltransferase increases during active myelination (*11*), suggesting that MBP methylation is a process associated with myelination.

Methylation of MBP is also thought to be involved in the maintenance of myelin integrity. In cultured cerebral cells from embryonic mice, inhibition of the MBP (arginine) methyltransferase prevented compact myelin formation (*12*). MBP containing methylated arginine has been shown to increase the association of lipid vesicles into dimers, suggesting that methylated arginine may increase the interaction

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* Corresponding author. Telephone: (416) 813-5920. Fax: (416) 813-5022. E-mail: mam@sickkids.on.ca.

‡ The Hospital for Sick Children.

§ University of Guelph.

[1] Abbreviations: bMBP, bovine myelin basic protein; component 1 (C-1), MBP−Cit_0 in which all arginyl residues are intact; MBP−Cit_{1-18}, MBP with 1−18 residues of citrulline formed by deimination of arginyl residues; MS, multiple sclerosis.

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Table 1: Mass Spectrometric Assignments of Methylated Arginine Human MBP Charge Isomers[a]

charge isomer	citrulline residues/mol	modification	calculated mass	observed mass	relative abundance	methylated/ unmethylated
normal C-1	0	none	18 459.8			
		acetyl	18 501.8	18 501.4	0.58	
		acetyl + methyl	18 515.8	18 515.4	1.0	3.4
		acetyl + 2 methyl	18 529.8	18 531.2	0.98	
MS C-1	0	none	18 459.8			
		acetyl	18 501.8	18 502.7	0.5	
		acetyl + methyl	18 515.8	18 517.1	0.94	3.9
		acetyl + 2 methyl	18 529.8	18 530.3	1.0	
normal C-8	6	none	18 465.8			
		acetyl	18 507.8	18 507.13	0.3	3.5
		acetyl + methyl	18 521.8	18 520.2	0.4	
		acetyl + 2 methyl	18 535.8	18 535.7	0.64	
MS C-8	6	none	18 465.8			
		acetyl	18 507.8	18 507.8	0.66	
		acetyl + methyl	18 521.8	18 523.0	0.96	3.0
		acetyl + 2 methyl	18 535.8	18 535.6	1.0	
Marburg C-8	18	none	18 477.8			
		acetyl	18 519.8	18 519.1	0.3	
		acetyl + methyl	18 533.8	18 531.5	1.0	6.6[b]
		acetyl + 2 methyl	18 547.8	18 544.9	0.98	

[a] Assignment of posttranslational modifications for MBP isolated from human autopsy material. Relative abundance was determined from centroid spectra and the ratio of methylated to unmethylated calculated from those values. Error in mass = 0.3 amu. [b] The high ratio of methylated/unmethylated in Marburg's sample is due to the rapid hydrolysis of the nonmethylated species by cathepsin D (*34*).

between MBP and the myelin lipids (*13*). In subacute combined degeneration of the spinal cord in humans (SCD), demyelination results from a deficiency in vitamin B_{12}, which decreases the amount of the major methyl donor *S*-adenosylmethionine (*14*) with a subsequent decrease in methylation of MBP. Therefore, methylation of MBP at a single arginine is important in the formation and maintenance of compact myelin.

A second posttranslational modification, the deimination of arginyl residues, has been shown to modify the structure and function of numerous proteins; e.g., deimination of the N-terminus of vimentin resulted in a loss of filament-forming ability and induced filament dissasembly (*15*). The deimination of filaggrin resulted in a loss of organized structure as demonstrated by circular dichroism and fluorescence spectroscopy (*16*). Deimination has also been shown to influence subsequent posttranslational modifications; e.g., domain 8 of human trichohyalin (THH-8) was cross-linked more efficiently by transglutaminase 3 after THH-8 had been deiminated (*17*).

As mentioned above, the lack of methylation of a single arginyl residue of MBP has been implicated in myelin instability in subacute combined degeneration of the spinal cord. The conversion of arginine to citrulline by the enzyme peptidylarginine deiminase has been implicated in demyelination in multiple sclerosis (MS) (*18*). In addition, the severity of MS has been correlated with the number of citrullinyl residues in MBP (*19*). In the present study, we demonstrate that human MBP isolated from normal and MS white matter contained greater amounts of citrulline in MBP in which the proportion of unmethylated Arg 107 was increased. In an in vitro analysis, this conclusion was substantiated by demonstrating that the rate at which bovine MBP was deiminated by purified peptidylarginine deiminase (PAD) was substantially less for the dimethylated bMBP than for the mono- or unmethylated bMBP. This study was possible because of the availability of the Q-TOF mass spectrometer which permitted us to resolve unmethylated, monomethylated, and dimethylated bMBP molecules.

EXPERIMENTAL PROCEDURES

All experimental procedures were performed as described in the accompanying paper (*38*).

RESULTS

Myelin basic protein (MBP) has been shown to consist of several components ("charge isomers"), some of which can be resolved by chromatography on carboxymethylcellulose columns at pH 10.6 (*22*, *23*). To study the citrulline content in the unmethylated, monomethyated, and dimethylated species of the human protein, several of the purified components, hMBP−Cit_0 and MBP−Cit_6 from normal brain and MBP−Cit_0, MBP−Cit_6, and MBP−Cit_{18} from MS brain, were subjected to mass spectrometry. These spectra (Figure 1) were highly complex. In each spectrum, three envelopes could be seen differing from each other by 14 amu, i.e., unmethylated, monomethylated, and dimethylated species. The posttranslational modifications assigned to normal C-1 from human (MBP−Cit_0) are shown in Table 1. From the amino acid sequence the mass of completely unmodified human MBP was calculated to be 18 459.8, a mass not found in any of the samples, from which we concluded that unmodified MBP was not found in nature. This charge isomer[2] was found to be acetylated at the N-terminus, giving a mass of 18 501.4. The mono- and dimethylated species were found at 18 515.4 and 18 531.2, respectively.

The spectrum of component 1 (C-1) from an MS patient is shown in Figure 1B. Three peaks were clearly identified in this spectrum also. The methylated species are shown in Table 1. The acetylated, nonmethylated isomer was found

[2] Charge isomer is used in the biological sense to describe a posttranslationally modified MBP. It is not used in the strict mass spectrometric sense.

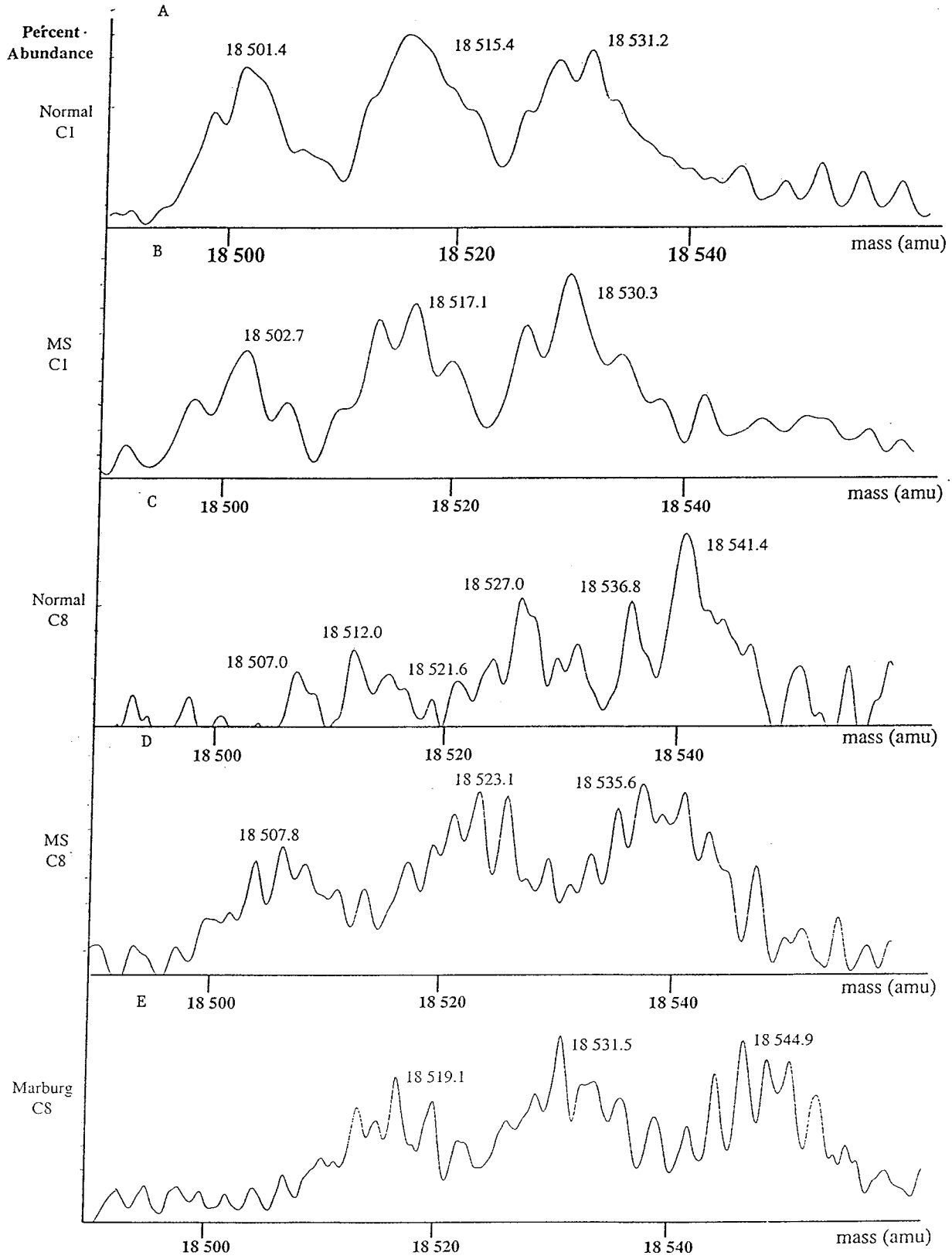

FIGURE 1: 1. Mass spectra of human MBP from normal (N) and multiple sclerosis (MS) white matter: (A) component 1 (N C-1) from normal human white matter; (B) component 1 (MS C-1) from multiple sclerosis white matter; (C) component 8 (N C-8) from normal human white matter; (D) component 8 (MS C-8) from chronic multiple sclerosis white matter; (E) component 8 (MSM C-8) from Marburg's variant of fulminating multiple sclerosis.

at 18 502.7, the acetylated, monomethylated isomer at 18 517.1, and the acetylated, dimethylated isomer at 18 530.3.

Since component 8 (C-8) (hMBP−Cit_6) from normal human brain contains 6 citrullinyl residues, the mass of the acetylated, unmethylated species was at 18 507.0, and the monomethylated C-8 was found at 18 521.6 (Figure 1C) rather than 18 515.4 (Figure 1A). The acetylated, dimethylated species was also shifted by 6 amu and was found at

Table 2: Assignment of Posttranslational Modifications on Bovine C-1

species	calculated mass	measured mass	posttranslational modification
	18 323.5		none
A	18 365.5	18 365.5	MBP + acetyl
B	18 379.5	18 378.6	MBP + acetyl + methyl
C	18 393.5	18 394.2	MBP + acetyl + 2 methyl

18 536.8 (Figure 1C) rather than 18 531.2 (Figure 1A). In C-8 from MS white matter (Figure 1D) the peak representing the acetylated, nonmethylated species (18 507.8) was present, but the envelopes around the acetylated, monomethylated species (18 523.1) and the acetylated, dimethylated species (18 535.6) were more complex, possibly due to deamidations. In the case of the C-8 from Marburg's variant of MS, a protein containing 18 citrullinyl residues, the acetylated, nonmethylated species found at 15 801.4 in C-1 (Figure 1A) was centered at 18 519.1, the acetylated, monomethylated species was centered at 18 531.5, and the acetylated, dimethylated species was centered at 18 544.9.

Deimination of Bovine C-1. To determine the effect of methylation at the single arginyl residue in MBP on deimination, bovine C-1 was purified since it could be obtained in abundant supply. Bovine C-1 has 169 amino acids and a methylated arginine at 106, compared to 170 residues and methylation at 107 for the human protein. Peptidylarginine deiminase (PAD) was purified from bovine brain by the method of Lamensa and Moscarello (*21*).

To determine the effect of monomethylation or dimethylation of arginine 106 on deimination of bMBP by PAD, bovine C-1 was incubated with purified bovine PAD for various lengths of time (0, 0.25, 0.5, 1, 3, 24 h), and the amount of citrulline at each time was determined by amino acid analyses. Citrulline content increased to 2.8 mol/mol of protein in 15 min, and by 3 h it had reached a maximum of 13.7 mol of citrulline/mol of protein. Further incubation up to 24 h did not result in any further increase in citrulline content nor did the addition of fresh enzyme (data not shown). Since the results of amino acid analyses are an average of the citrulline content of all isomers present, i.e., nonmethylated, monomethylated, and dimethylated, the extent of deimination for each of these species could only be resolved by mass spectrometry. Typical spectra of bMBP$-Cit_0$, MBP$-Cit_{2.8}$, and MBP$-Cit_{10.1}$ are collected in Figure 2. Since the conversion of arginine to citrulline represents the only additional modification apart from acetylation and methylation, the spectra were less complex. The time course of deimination is shown in Figure 3.

Assignment of a number of peaks between 18 360 and 18 410 was readily done (Table 2). From the amino acid sequence the mass of completely unmodified bovine MBP C-1 is 18 323.5. As with the human protein this mass was not found in any of the spectra. The addition of an acetyl group at the N-terminus adds 42 amu, resulting in a mass of 18 365.5. Addition of a single methyl group adds 14 amu, giving a mass of 18 379.5. A second methyl group results in a mass of 18 393.5.

Each arginyl residue deiminated increased the mass of the protein by 1 amu. Therefore, the number of citrullines in each of the species A, B, and C at various times of deimination was determined by the increase in mass of each species (Figure 2). For example, the acetylated, nonmethylated MBP had a mass of 18 365 at 0 min, but after 15 min the mass had increased to 18 369, a change of 4 amu, indicating that 4 arginyl residues had been deiminated. The mass of the acetylated, monomethylated MBP increased from 18 380 to 18 383, and the mass of the acetylated, dimethylated MBP increased from 18 394 to 18 398. The relative abundance of each MBP species was determined from the centroid data (Figure 2, upper panels) from which the contribution of each to the total citrulline content was calculated (Table 3). This number was arrived at by summing the abundance of all species and expressing each value as a ratio of this total. The change in mass was then multiplied by this ratio to determine the citrulline contribution of each methylated species. Adding the citrulline contribution of each methylated species gives the total citrulline content in the sample at a given time. This procedure was used for each time of deimination (Table 3). After 15 min, the total citrulline content by this method was 3.5 compared with 2.8 by amino acid analysis. These values are in good agreement since it is known that losses due to acid hydrolysis during preparation of the sample for amino acid analysis occur so that the total citrulline by amino acid analysis was less than that computed from the mass spectra.

Table 3: Citrulline Contribution of Each Methylated Bovine MBP Isomer to the Total Citrulline Content Compared to the Total Citrulline Content by Amino Acid Analysis

		citrulline contribution (from % abundance)		total citrulline	
time (min) of incubation with PAD	arginine	mono-methyl-arginine	dimethyl-arginine	mass spectrometry	amino acid analysis
0	0	0	0	0	0.1
15	1.53	1.34	0.63	3.50	2.8
30	3.19	2.61	0.99	6.79	5.3
60	4.89	4.12	2.23	11.24	10.1
180	6.12	5.30	3.37	14.79	13.5
o/n[a]	4.18	6.95	5.06	16.19	13.7

[a] Overnight.

Table 4: Rate of Deimination of Methylated Arginine 106 in Bovine MBP C-1

bMBP isomer	rate (citrulline/min)
arginine	0.081
monomethyarginine	0.068
dimethylarginine	0.036

The effect of methylation on the time course of deimination in each isomer is shown in Figure 4. The nonmethylated isomer was deiminated to the largest extent, followed by the monomethylated and then the dimethylated isomers. From these curves, the rate at which each of the isomers was deiminated was calculated by linear regression analysis (Table 4). The rate of deimination for the dimethylated isomer (0.036 citrulline/min) was about half that of the nonmethylated isomer (0.081 citrulline/min), and the monomethylated isomer was between the two at 0.068 citrulline/min, demonstrating that methylation at a single arginyl residue in bovine MBP (Arg 106) affected the rate of deimination by PAD.

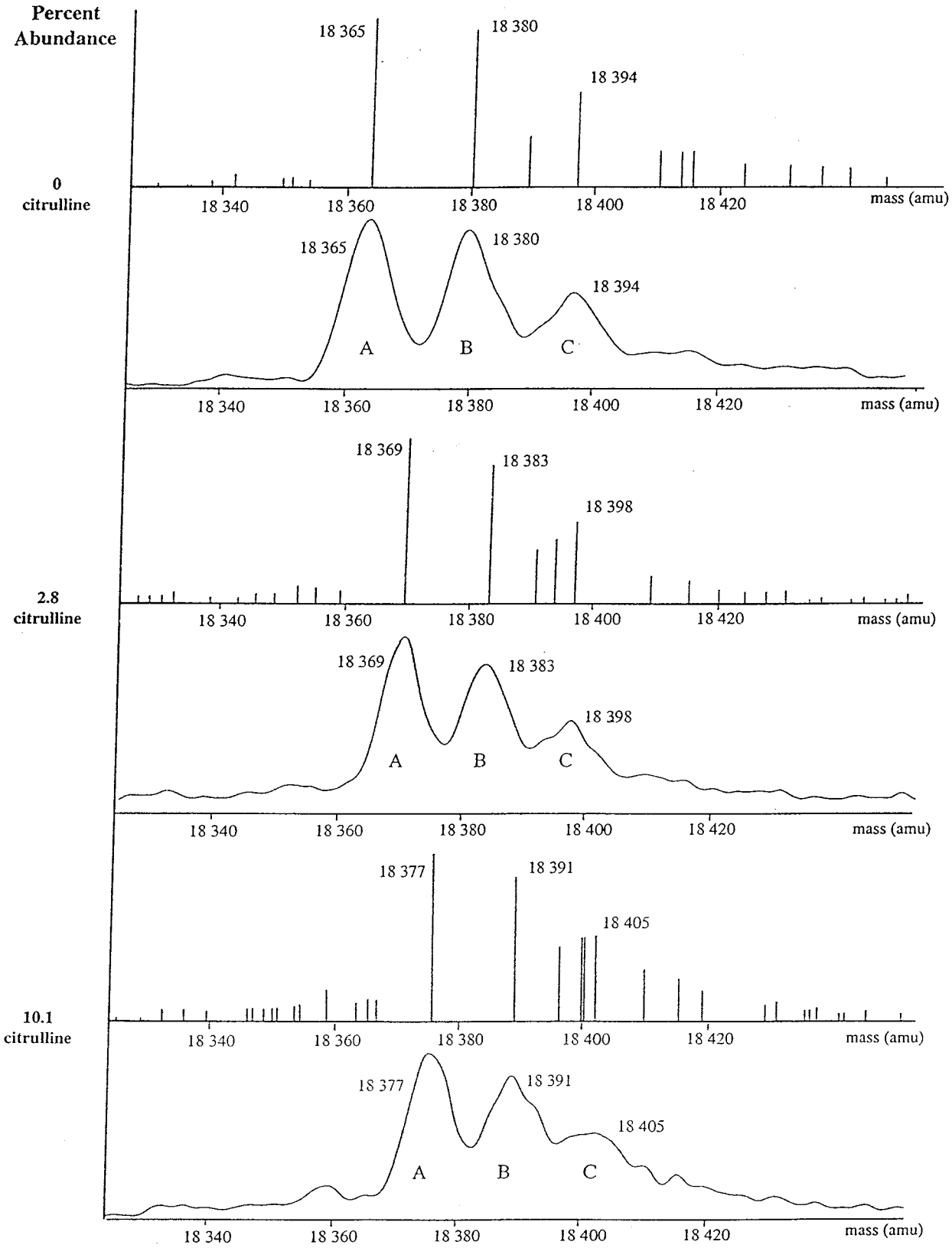

FIGURE 2: 2. Mass spectra of bovine MBP (C-1) deiminated by PAD for various times from 0 to 1 h.

DISCUSSION

In this study, we have demonstrated that the relative amounts of nonmethylated, monomethylated, and dimethylated arginines varied between different charge isomers of human and bovine MBP. In bovine C-1 we found a ratio of 1/0.9/0.6 for nonmethylated, monomethylated, and dimethylated bMBP isomers; however, in human C-1 this ratio was 1.0/2/2, suggesting that the human hMBP was more heavily methylated. Previous studies (*8*) using unfractionated bMBP isolated from bovine spinal cord showed that there was 2−4 times more monomethylarginine than dimethylarginine. A second study using MBP from human brain found that the

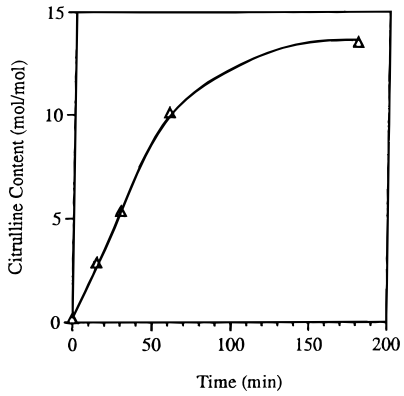

FIGURE 3: 3. Citrulline content of bovine MBP (C-1) deiminated by PAD for various times. At each time, an aliquot of the reaction mixture was removed for amino acid analysis.

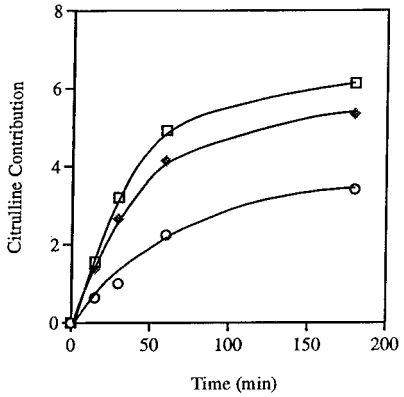

FIGURE 4: 4. Citrulline content for bovine MBP (C-1) deiminated by PAD for various times. The citrulline content in nonmethylated (□), monomethylated (◆), and dimethylated (○) species was obtained from mass spectrometry.

ratio of nonmethylated to monomethylated to dimethylated was 1/6/10. Since the earlier studies were done with unfractionated MBP (comprising all charge isomers), the ratios of methylated species in each of the components (charge isomers) could not be deduced. The present study represents the first time that the relative amounts of unmethylated, monomethylated, and dimethylated species were assessed in specific charge isomers and compared with corresponding charge isomers isolated from human MS material.

The functional significance of methylation varies widely due to the diversity of proteins which have been found to be methylated. Specific methylesterases have been identified for substrates such as protein phosphatase 2A (*24*), but N-methylation is generally considered to be an irreversible reaction as no arginine methylesterases have been identified (*25*). Methylation of proteins has been implicated in cellular stress responses (*26*), in the aging and repair of proteins (*27*), in signal transduction (*28*), and in the modulation of nucleic acid binding in heterogeneous ribonucleoproteins (*29*). Methylation of Arg was found to occur in a Gly-Arg-Gly sequence, which is also found in MBP. We have now shown that methylation affected the rate of deimination of arginyl residues of MBP by peptidylarginine deiminase. Since the presence of increased amounts of citrullinated MBP has been documented in MS, our data suggest that decreased methylation may be a prerequisite for increased rate of deimination.

Methylation has also been hypothesized to affect other posttranslational modifications such as serine phosphorylation in yeast binding RNA protein Np13p (*30*). Arginine methylation may prevent the protein−protein interactions facilitated by serine phosphorylation or may inhibit phosphorylation itself as in phosphorylase. Deimination has also been shown to affect a second posttranslational modification; in a decapeptide corresponding to residues 9−18 of glycogen phosphorylase the presence of citrulline at positions 10 and 16 decreased the activity of protein phosphatase 1 on the peptide (*31*). Therefore, deimination of glycogen phosphorylase decreases its ability to be activated by phosphorylation. In this study the methylation of MBP was also shown to affect a second posttranslational modification, the deimination of arginyl residues.

The methylation of MBP has been hypothesized to be important in the formation of compact myelin. In one study using bovine MBP which is methylated at arginine 106, and carp MBP which is nonmethylated, as well as other cationic proteins such as lysozyme and polyhistidine, it was demonstrated that methylated bMBP promoted the dimerization of myelin vesicles (*13*). In a second study using cultures of cerebral cells from embryonic mice, the methylation of mMBP was inhibited by sinefungin, a methyltransferase inhibitor, and the formation of multilamellar myelin was also inhibited (*12*). The methylation of arginine 106 (bovine sequence) has been suggested to stabilize the "hairpin" formation of bMBP by interacting with lipids, thereby enabling the insertion of bMBP into the myelin sheath (*29*).

Methylation of arginyl residues will increase their hydrophobicity and cause them to sequester themselves away from solvent molecules, thereby disrupting the three-dimensional structure of the protein. This mechanism was demonstrated to be plausible by molecular dynamics simulations (results not shown). Conformational changes in proteins have been demonstrated in other systems. For example, methylation of a single lysine at position 72 in cytochrome *c* decreases the Stokes radius of the molecule, suggesting that the methylated species is more compact than the unmethylated species (*32*). Methylated apocytochrome *c* is more susceptible to cleavage by a yeast cytosolic fraction than unmethylated apocytochrome *c*, suggesting that methylation alters the conformation of the protein (*33*). Since the relative proportions of methylated arginyl residues are decreased in hMBP from MS tissue, less compaction would be induced by this mechanism, thus rendering the structure more accessible to deimination by PAD and subsequent digestion by cathepsin D (*34*).

Abnormal methylation of proteins may produce increased or decreased methylation depending on the activity of methyltransferases. Increased methyltransferase activity has been demonstrated in a number of disease states including hereditary spherocytosis (*3*), chronic renal failure (*35*), and male infertility (*36*). On the other hand, methyltransferase inhibition has been linked to demyelination in human immunodeficiency virus (*37*) and subacute combined degeneration of the spinal cord (*14*). This demyelination is thought to result from the hypomethylation of hMBP, which results in a less compact myelin structure. From the present studies we conclude that decreased methylation of MBP possibly by a shortage of *S*-adenosylmethionine (methyl donor) or downregulation of the methyltransferase gene expression allows the peptidylarginine deiminase greater access to the arginyl groups of MBP.

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