Multiple Sclerosis

AN IMPORTANT ROLE FOR POST-TRANSLATIONAL MODIFICATIONS OF MYELIN BASIC PROTEIN IN PATHOGENESIS*

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Myelin basic protein (MBP) represents a candidate autoantigen in multiple sclerosis (MS). We isolated MBP from normal and MS human white matter and purified six components (charge isomers) to compare the post-translational modifications on each. The sites and extent of methylation, deimination, and phosphorylation were documented for all tryptic peptides by mass spectrometry. We found that mono and dimethylated arginine 107 was increased in MS samples; deimination of arginine occurred at a number of sites and was elevated in MS; phosphorylation was observed in 10 peptides in normal samples but was greatly reduced or absent in most peptides from MS samples. Data obtained with MBP isolated from fresh brain obtained from a spontaneously demyelinating mouse model supported the view that the changes observed in human brain were probably related to pathogenesis of demyelination, *i.e.* we found decreased phosphorylation and decreased amounts of glycogen synthesis kinase in brain homogenates using specific antibodies. This study represents the first to define post-translational modifications in demyelinating disease and suggest an important role in pathogenesis. ***Molecular & Cellular Proteomics 2:453–462, 2003.***

Multiple sclerosis (MS)[1] is the most common demyelinating disease of the human central nervous system. It is multifactorial, requiring genetic, environmental (possibly viral), and immunological factors (1). Genetic screens of MS populations have failed to uncover a major susceptibility locus (2), suggesting that MS is a polygenic disease with each gene of small effect.

Myelin basic protein (MBP), a major myelin protein that accounts for 35% of the total myelin protein, is a strong candidate autoantigen (3). It is a 170-amino acid protein in the human, containing 19 arginyl and 12 lysyl residues, which accounts for its basic character. It is devoid of cysteinyl residues, with a high proportion of disorder promoting amino acids such as A, R, G, Q, S, P, E, and K. It is a member of an expanding group of proteins that include the amyloid and prion proteins, considered to be intrinsically disordered (4), in which the disordered state is the functional state. Because of this, post-translational modifications determine the nature and extent of secondary structure, permitting the protein to adopt multiple conformations for a variety of binding events (5). Because interactions between the positive arginyl and lysyl residues and the negatively charged phosphate groups of the membrane phospholipids are essential to the structure of compact myelin, changes in positive charge of MBP would decrease the strength of these interactions (6).

MBP is an unusual protein that has never been crystallized due to the myriad of post-translational modifications it possesses. These include phosphorylation, deamidation, deimination, arginine methylation, and N-terminal acylation. These give rise to a family of microheteromers or "charge isomers," several of which can be resolved by chromatography (7). The effects of some of these modifications on protein structure and protein-lipid interactions has been studied in our laboratories. Phosphorylation by protein kinase C *in vitro* resulted in a large increase in β-structure as determined by circular dichroism. Although several sites were phosphorylated by protein kinase C, phosphorylation at serine 7 was particularly important for the stabilization of the β-structure (8). Strong interaction between arginine 5 and 9 with the phosphate on serine 7 was responsible for the stabilization of structure.

Deimination of arginyl residues by the action of peptidylarginine deiminase (EC 7.5-3.15) generates citrulline, an important irreversible modification of MBP. In a series of studies, we demonstrated that 20% of the MBP isolated from normal human adults was citrullinated, compared with 45% in chronic MS and 90% in fulminating MS (9–11). In *in vitro* studies we showed that citrullinated MBP enhanced T cell responsiveness (12), altered conformation (11), and induced vesicle fragmentation (13). Clearly this modification, which has been studied most extensively, has important implications for myelin membrane stability.

The effects of other modifications such as methylation of arginine 107 as the mono-methyl or symmetric dimethyl arginine (14, 15) have not been studied as extensively. Methyla-

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Received, August 21, 2002; and in revised form, June 19, 2003
Published, MCP Papers in Press, June 25, 2003, DOI 10.1074/mcp.M200050-MCP200

[1] The abbreviations used are: MS, multiple sclerosis; MBP, myelin basic protein; GSK, glycogen synthesis kinase; DM20, myelin proteolipid protein isoform; ND4, transgenic mice containing 70 copies of DM20 cDNA; PLP, proteolipid protein.

tion of MBP by vitamin B_{12} administration reversed the myelinolysis of subacute combined degeneration of the spinal cord, suggesting an important role for methylation in myelination (16). A role for arginine methylation in signaling and transcriptional activation has been postulated (17–20). The recent demonstration that the enzyme responsible for methylating arginine, protein methyl transferase-5, acts in concert with Janus kinase (18, 19), which phosphorylates STAT-1 transcription factor in signaling activity of the interferon receptor (18). Because interferon β is used widely in the treatment of MS, elucidation of the role played by methylation may have therapeutic relevance.

Whereas an assessment of the post-translational modifications in the "charge isomers" of MBP from bovine and dogfish have been reported (21, 22), no similar analysis has been carried out for the human MBP "charge isomers." As a first step in defining the roles of these three major modifications, we have isolated several of the MBP charge isomers, protease digested each, resolved the peptides by liquid chromatography, and identified the modification(s) in each by mass spectrometry. We demonstrated that MS MBP was less phosphorylated, more methylated, and more deiminated than MBP from normal tissue. We suggest that these modifications of MBP, which affect charge (phosphorylation, deimination), conformation, and hydrogen bonding (methylation), compromise its ability to form stable myelin multilayers. Because similar changes were found in a spontaneously demyelinating transgenic model, the changes documented are unlikely to represent artifacts of disease or post-mortem autolysis. We suggest that MS may represent a new class of diseases called "post-translational" diseases.

EXPERIMENTAL PROCEDURES

Isolation of MBP and Purification of Components

Sample Collection—All samples were collected from human brain at autopsy. Normal brain was obtained from violent or sudden cardiac death not involving brain injury. The white matter was carefully dissected from the gray matter, frozen in liquid nitrogen, and stored at −70 °C. Only non-involved white matter was used, *i.e.* plaque tissue was carefully avoided. Ages of normal samples varied from 25 to 75 years, and wherever possible normals were age-matched with MS samples. This procedure was routinely done at the Canadian Brain Tissue Bank, from which most of the samples were obtained. A few samples were obtained from the Rocky Mountain Brain Bank in Colorado. Only white matter from autopsies carried out 8–10 h postmortem was used. In addition to age and sex, clinical histories, including drug therapy and pathological reports, were obtained on all patients. Histopathology was done on each sample at the brain bank to confirm the diagnosis. None of the samples had any evidence of additional neurological disease. Histopathology of the normal samples confirmed the complete absence of disease.

Purification of Components—Isolation of MBP was carried out essentially as described for bovine MBP (6). Briefly, the frozen tissue was extracted with chloroform-methanol, followed by acetone, at 4 °C to remove lipid. The solids were immediately extracted with 0.2 N H_2SO_4 overnight at 4 °C. The acid extracts were combined with an equal volume of absolute ethanol and allowed to precipitate at −20 °C overnight. The precipitated material represents the total MBP fraction, which was subsequently dissolved in 0.1 M glycine buffer, pH 9.6, containing 6 M urea and applied to a CM52 column. The C-8 fraction was collected in the flow-through and further purified on high-pressure liquid chromatography (10). The charge isomers C-5 → C-1 were eluted from the column with a sodium chloride gradient 0 → 0.2 M in the glycine-urea buffer, pH 10.6. The least cationic C-5 eluted first, followed by C-5 → C-1 in that order. The most cationic, least modified charge isomer was C-1. Protein concentrations were determined by amino acid analyses after hydrolysis in 5.7 N HCl.

Nomenclature—All "charge isomers" were derived from the 18.5-kDa isoform. Therefore "charge isomer" is used in the biological sense and not the mass spectrometric sense, because they vary in mass depending on the nature of the post-translational modification. "Charge isomers" from normal human brain are abbreviated as C-1N, C-2N, C-3N, etc. The corresponding "charge isomers" from MS tissue are represented as C-1MS, C-2MS, C-3MS, etc.

Western Blots

Western blots were carried out by the method of Towbin *et al.* (23). The antibodies used in this study included a polyclonal anti-MBP IgG, a monoclonal antibody P12 reactive with phosphorylated threonine 98 (24), a monoclonal antibody that recognizes phosphoserine, and a monoclonal that recognizes all subunits (α, β, γ) of glycogen synthesis kinase (GSK).

Transgenic Mice

The transgenic mice (ND4) used in these studies were CD-1 containing 70 copies of the cDNA for DM20, a myelin proteolipid. They are normal at birth, but at 3 months of age they demyelinate spontaneously, which becomes progressively worse from 3 to 10 months, after which the animals are moribund (25).

Capillary Electrophoresis Mass Spectrometry

Each charge isomer (1 mg) was digested with trypsin (Promega, Madison, WI) at a ratio of 1:50 trypsin:protein overnight at 37 °C in 100 mM ammonium bicarbonate buffer, pH 8.0. The digests were vacuum dried and reconstituted in deionized water.

Separation of the tryptic peptides was performed on an in-house constructed polybrene capillary electrophoresis apparatus with a 50-cm long capillary (26). The capillary electrophoresis buffer solution consisted of 100 mM formic acid that was adjusted to pH 2.7 with ammonia. The tryptic peptides were loaded into the capillary using an electrokinetic injection method that applied -2.5 kV for 10 s. A voltage of 12 kV was applied across the capillary to perform the separation. The separated peptides were fed directly into the mass spectrometer as described previously. An ion trap storage/reflectron time-of-flight mass spectrometer was used to detect the separated peptides (27). A three-dimensional contour map, which displays individual mass spectra along with elution profiles, was used to pinpoint and integrate specific peaks (28).

TABLE I
Relative proportions of components isolated by cation exchange chromatography
Relative proportions = (amount of each charge isomer)/column charge. *n*, number of samples in study. Each sample is from a separate brain.

MBP	Charge Isomer $\mu g/OD_{280}$						
	n	C1	C2	C3	C4	C-5	C8
Normal	6	0.22 ± 0.01	0.12 ± 0.01	0.18 ± 0.015	0.11 ± 0.02	0.11 ± 0.017	0.22 ± 0.05
MS	8	0.15 ± 0.035	0.11 ± 0.005	0.15 ± 0.02	0.10 ± 0.02	0.12 ± 0.015	0.35 ± 0.03

Peptide Peak Assignment

Initial assignments of each peptide peak were performed using the FindMod tool (28), which is available at www.expasy.ch/tools/findmod/. The experimental $[M+H]^+$ values were compared with the theoretical average peptide masses with ±150 ppm mass tolerance. To confirm the assignments, tandem mass spectrometry fragmentation of each peak was performed as described in previous work (27). In addition, in those instances in which the post-translational modifications resulted in a decrease or increase of 1 or 2 atomic mass units, the assignment was confirmed by the mobility of the ion undergoing electrophoresis. Details of all the mass spectral methods can be found in the various publications indicated above.

RESULTS

Relative Proportions of Charge Isomers

The proportions of the various charge isomers (Table I) are expressed as the amount of each (μg) divided by the column charge (OD_{280} units). The amounts of C-2, C-3, C-4, and C-5 between normal and MS material were not significantly different. C-1 from MS was decreased compared with normal, whereas C-8 from MS was increased. This was consistent with our previous observations (10).

Methylated Arginine

Only arginine 107 occurs as the unmethylated, monomethylated, and symmetrically dimethylated forms. The relative abundance (intensity of methylated peptide/intensity of nonmethylated peptide) was obtained for each species. The intensities from dimethylated forms were approximately twice those of monomethylated peptides. The number of samples reflects the number of different brains used. Each number in Table II is the mean of all the brains. Some variation was observed from brain to brain and was due to the differences in duration and severity of disease of the MS patients, which varied from 4 years (severe) to 30 years (benign).

The integrated total ion counts of monomethylated, dimethylated, and unmethylated peptides have been calculated to compare the relative intensities of methylated peptides. The integration was performed on the three-dimensional contour map (28). GLSLSR (residues 108–113) was used for the unmodified standard, while GRGLSLSR (residues 106–113) with arginine 107 methylated was used for the methylated standards. Peptides containing methylated arginine are not cleaved by trypsin (14), probably due to steric hindrance by the bulky methyl group.

TABLE II
Mono- and dimethylation of arginine 107 in MBP charge isomers from normal and MS brains (relative intensities)
Relative intensity = (intensity of methylated arginine)/(intensity of nonmethylated arginine). *, methylated arginyl residue.

Component	Monomethylation (GR*G SLSR)	Dimethylation (GR*G SLSR)	Number of Brains
C-1 (N)	1.6	1.8	3
C-1 (MS)	1.1	1.1	2
C-2 (N)	1.31	2.4	3
C-2 (MS)	0.67	0.97	2
C-3 (N)	0.98	1.68	3
C-3 (MS)	0.69	1.28	2
C-4 (N)	1.23	2.44	16
C-4 (MS)	2.47	4.20	7
C-5 (N)	0.85	2.14	8
C-5 (MS)	2.49	4.29	6
C-8 (N)	1.14	1.9	3
C-8 (MS)	1.26	1.94	2

In the case of C-1, C-2, and C-3, the intensities of the mass spectral peaks were low, so these numbers must be interpreted with caution. In C-4 and C-5 of the MS samples, the intensities of the methylated peaks were 1–3 times higher than the corresponding normal charge isomers, and more samples were available, giving considerable strength to these numbers. In both C-4 and C-5 of the MS samples, the ratios (methylated/unmethylated) were 2–3 times greater than the corresponding ratios from normal C-4 and C-5, suggesting that a greater portion of the MBP from MS samples was methylated.

We have recently completed a study of the activity of the enzyme protein methyl transferase 5, which methylates MBP symmetrically. This could only be done in the spontaneously demyelinating transgenic mouse model. In a developmental study from 0.5 to 6 months of age, a 3-fold increase in methylation of MBP was observed before signs of disease, suggesting that this post-translational modification was affected early and may have a role in pathogenesis (development) of disease (data not shown).

Distribution of Phosphorylation Sites

When all phosphorylated peptides derived from normal and MS samples are considered, phosphorylation appears in the normal samples much more frequently than in the MS sam-

ples. We recognize that the signals for the phosphorylated peptides were low and even lower for diphosphorylated peptides. However, the signals were sufficiently clear so the distinction was unambiguous. The comparisons of all peptides are collected in Table III. It is clear that normal samples have higher intensities than MS samples, and in several peptides only the normal samples were phosphorylated, *e.g.* RPSQR (5–9), HGSK (10–13), RGSGK (54–58), TAHYGSLPQK (66–75), NIVTPR (92–97), and FSWGAEGQR (114–122).

Some phosphorylated peptides, RGSGK (54–58) and GVDAQGTLSKIFK (143–155), were found only in undigested forms. It is assumed that the phosphate group on serine forms a strong interaction with an adjacent basic group on arginine or lysine. It is also possible that steric hindrance from the phosphate group prevents trypsin from digesting these peptides. This interpretation is consistent with earlier studies in which it was demonstrated that a phosphoseryl residue two positions C-terminal to a lysyl or arginyl residue was shown to inhibit tryptic digestion (29, 30). Resistance to proteinase digestion of phosphorylated bovine spinal MBP has been reported, whereas the unphosphorylated protein was readily digested (31). In the present study, the phosphoseryl peptide FSWGAEGQR was observed in our mass spectra, suggesting that tryptic cleavage between arginine 113 and phenylalanine 114 had occurred even though serine 115 was phosphorylated. We believe that the presence of the phenyl group might interfere with the interaction between arginine 113 and phosphoserine 115.

In peptide YLATASTMDHAR (14–25), phosphorylation always appeared with methionine oxidation. The amino acid phosphorylated, whether residue 17 (threonine), residue 19 (serine), or residue 20 (threonine) has not been established. However, the short distance from phosphorylated residue to the methionine residue (residue 21) may induce oxidation of the methionine. In peptide SGSPMAR (residues 163–169), we have not confirmed which serine (163 or 165) was phosphorylated in the monophosphorylated peptide. In an earlier study, Martenson *et al.* (32) demonstrated that the same peptide from rabbit MBP was phosphorylated on serine 165. Serine 165 has been identified also in bovine MBP by Kassis *et al.* (33) by the use of synthetic peptides with alanine substitutions for serine 163 and 165 in their Yak 1p kinase-treated bovine MBP. These earlier data support the view that serine 165 was phosphorylated in our samples.

Although there are two possible sites of phosphorylation on peptide GFDAQGTLSKIFK (residues 143–155), only one site was observed in our mass spectra. We assume it to be serine 151 because in earlier Edman degradation studies of a similar peptide from bovine MBP (GHDAQGTLSK) serine 151 was phosphorylated by protein kinase C (34).

In summary, the exact site of phosphorylation was not unambiguously assigned for all peptides. This should not detract from the major conclusion of this study that the tryptic peptides from six charge isomers of MBP were phosphorylated in the normal material but much less or not phosphorylated at all in the MS material. Because the method of extraction employed solvents and acid extraction, we did not use phosphatase inhibitors. Because most of the MS samples were chronic, long-standing cases, the possibility that phosphatases could arise from invading inflammatory cells was unlikely. Although the significance of this observation is not clear at this time, the studies on the transgenic mouse system (see below) support the view that the hypophosphorylated state has an important role in pathogenesis of disease.

Decreased Phosphorylation in a Demyelinating Mouse Model

Because the MBPs isolated for the studies reported above were from human brain obtained at autopsy, we used fresh brain from an animal model to confirm that the MBP changes were not due to post-mortem autolysis. Thr^{98} (human sequence) was a major phosphorylation site (Table III). To determine the extent of phosphorylation at this site in normal and transgenic mice, MBP was isolated by immunoprecipitation from brain homogenates and fractionated by SDS-PAGE from which Western blots were prepared. The blots were reacted with a monoclonal antibody (P12) that recognizes only phosphorylated threonine in the TPPPSQGK sequence. The amount of phosphorylated Thr was decreased in transgenic (ND4) mice at 3 and 10 months of age (Fig. 1*A*). We then fractionated MBP isolated from 6-month-old normal and transgenic mice on CM52 cation exchange columns to obtain several of the charge isomers. C-1 was not phosphorylated in either normal or transgenic mice; C-2 was phosphorylated in the normal but not in the transgenic mice; C-3, C-4, and C-5 were phosphorylated in both but the extent of phosphorylation was decreased in the transgenic mice in all cases (Fig. 1*B*). Therefore decreased phosphorylation was observed in this demyelinating model.

GSK phosphorylates MBP at three sites in bovine MBP (35). They are NIVTp^{94}PR, Tp^{97}PPPSQGK, and LGGRDSp^{160}R. The major site is Thr^{97} (bovine sequence). Using an antibody that recognizes all three subunits of GSK (α, β, γ), we demonstrated that all three subunits were decreased in brain homogenates prepared from transgenic mice when compared with normal. The density of each band corresponding to α, β, and γ subunits of GSK were summed and plotted as a function of age (Fig. 1*C*). GSK protein was reduced in the transgenic mice compared with normal, suggesting that the decreased phosphorylation of MBP by this kinase (Fig. 1, *A* and *B*) was the consequence of decreased amount of enzyme. With an antibody that recognized phosphoserine in proteins, we demonstrated decreased amount of phosphoserine containing MBP (expressed as phosphoserine reactivity (b)/MBP reactivity (a)) at 3 and 8 months in transgenic mice (data not shown). Therefore, hypophosphorylation of MBP was found in MBP isolated from fresh brains of transgenic mice and rep-

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TABLE III

Phosphorylation sites on MBP charge isomers from normal and MS white matter (relative intensities)

Relative intensity = (intensity of phosphorylated peptide)/(intensity of nonphosphorylated peptide). *, phosphorylated residue. †, numbers in brackets indicate number of samples used.

Component	Phosphorylation Sites										
	^{5}RPS*QR^{9}	^{10}HGS*K^{13}	^{14}YLAT*AS*T*MDHAR^{25}	^{55}GS*GK^{58}	^{66}TAHYGS*LPQK^{75}	^{92}NIVT*PR^{97}	^{98}T*PPPSQGK^{105}	^{114}FS*WGAEGQR^{122}	^{143}GVDAQGT*LS*K^{152}	^{163}S*GS*PMAR^{169}
C-1 (N)	0 (3)	0 (3)	0.52 (3)†	0.11 (1)	0 (2)	0.042 (1)	0 (2)	0 (2)	0.037 (1)	
C-1 (MS)	0 (3)	0 (3)	0.18 (1)	0 (3)	0 (2)	0 (1)	0 (2)	0 (2)	0 (1)	
C-2 (N)	0 (2)	0 (3)	0.69 (2)	0.091 (1)	0 (2)	0 (3)	0 (3)	0 (3)	0.044 (1)	0 (3)
C-2 (MS)	0 (2)	0 (2)	2.8 (1)	0 (3)	0 (2)	0 (3)	0 (3)	0 (3)	0 (1)	0.68 (1)
C-3 (N)	0 (5)	0 (5)	1.2 (3)	0.19 (1)	0 (3)	0 (5)	0.09 (3)	0.16 (2)	0.024 (1)	0.683 (2)
C-3 (MS)	0 (3)	0 (3)	0 (4)	0 (3)	0 (3)	0 (4)	0 (4)	0 (2)	0.036 (1)	
C-4 (N)	0.043 (3)	0 (3)	1.24 (10)	0.102 (3)	0 (4)	0 (3)	0.15 (13)	0.055 (13)	0.050 (7)	0.062 (8)
C-4 (MS)	0 (3)	0.45 (4)	0	0 (3)	0 (6)	0 (4)	0.075 (1)	0 (10)	0.015 (1)	0.053 (2)
C-5 (N)	0.032 (6)	0.031 (2)	0.83 (6)	0.069 (1)	0.009 (1)		0.211 (7)	0.18 (7)	0.046 (4)	0.506 (5)
C-5 (MS)	0 (6)	0	0.30 (3)	0 (6)	0		0.048 (4)	0	0.043 (1)	0.009 (1)
C-8 (N)	0.035	0	0	0	0	0	0.214	0.100	0	0.451
C-8 (MS)	0	0	0	0	0	0	0	0	0	0

FIG. 1. *A*, Western blot of MBP isolated from normal and transgenic mice of 3 and 10 months of age. Primary antibody was P12, a monoclonal antibody, which recognizes threonine in TPPPSQGK in MBP. The 18.5- and 14-kDa isoforms of MBP are identified. *B*, Western blot of charge isomers of mouse MBP. *N*, normal mouse; ND4, transgenic mouse. Antibody was P12. *C*, GSK protein levels obtained from densitometric scanning of Western blot of mouse brain homogenates. □, normal mice; ○, transgenic mice from 2 to 10 months of age. Antibody was reactive with all subunits of GSK (α, β, γ).

resented a generalized phenomenon possibly related to demyelination.

Deamidation

The conversion of asparagine and glutamine in proteins to the corresponding carboxylic acid occurs spontaneously (36,

TABLE IV
Deamidation of glutamine in MBP charge isomers from normal and MS brain (relative intensities)
Relative intensity = (intensity of modified glutamine)/(intensity of unmodified glutamine). *n*, number of samples.

Component	*n*	Deamidated Glutamine				
		Gln 8	Gln 81	Gln 103	Gln 121	Gln 147
C1 (N)	1	0	0	0.087	0.14	0
C-1 (MS)	1	0	0	0.066	0.41	0.073
C-2 (N)	1	0.021	0	0.38	0.44	0.201
C-2 (MS)	1	0	0	0.37	0.62	0.08
C-3 (N)	2	0	0.03	1.08	0.37	0.11
C-3 (MS)	2	0	0.25	0.35	0.26	0.6
C-4 (N)	4	0.058 ± 0.03	0.075 ± 0.05	0.49 ± 0.28	0.11 ± 0.03	0.20 ± 0.06
C-4 (MS)	7	0.04 ± 0.005	0.16 ± 0.08	0.37 ± 0.07	0.11 ± 0.03	0.12 ± 0.06
C-5 (N)	4	0.042 ± 0.03	0.09 ± 0.02	1.12 ± 0.15	0.11 ± 0.05	0.17 ± 0.02
C-5 (MS)	7	0.011 ± 0.006	0.10 ± 0.03	0.24 ± 0.1	0	0.08 ± 0.02
C-8 (N)	3	0.03		1.27	0.05	0.07
C-8 (MS)	2	0.04		0.13	0.03	0.11

37), *e.g.* an asparagine to aspartic acid conversion in long-lived proteins such as α and β-crystallin from lens (37) has been reported with aging. MBP is also a slowly turning over protein, with a half-life estimated to be 40–100 days in the mouse (38).

Little deamidation was observed in Gln 8 and 81 of C-1, C-2, and C-3 from both normal and MS samples (Table IV). Gln 103, 121, and 147 were deamidated in both normal and MS samples. Partial deamidation was reported earlier on residues 103 and 147 in C-2 (7). Components C-4 and C-5 were deamidated at all positions. The differences in ratios between normal and MS samples at some positions was quite large, *e.g.* at Gln 8 in C-5 a 4-fold difference, a 5-fold difference at Gln 103, and a 2-fold difference at Gln 109.

If the decreased phosphorylation observed in the MS samples (Table III) was due to degradative processes, it would be expected that deamidation, which is also a degradative process, would be increased in MS. With few exceptions the MS samples were less deamidated than the corresponding normal samples, supporting our view that the lower extent of phosphorylation of the MS samples (Table III) is unlikely to be artifactual and is probably related to pathogenesis.

Deimination of Arginyl Residues

Citrulline in proteins arises by the deimination of peptide-bound arginine residues by the action of peptidyl arginine deiminase. In the present report, we have extended the earlier studies (9, 10) so that instead of measuring the average number of citrullinyl residues in C-8 we have examined each arginine-containing peptide in all components (C-1 → C-8) isolated from both normal and MS white matter by mass spectrometry (Table V). Components C-1, C-2, and C-3 from both normal and MS tissues were only slightly deiminated. In C-1, only R54 in the MS sample was found to have significant deimination. In C-2N, deimination was found at R54 and R162, whereas in C-2 MS, R49, R54, and R162 were deiminated. In C-3 from both normal and MS samples, R54 and R162 were deiminated. In C-4, R25, R54, and R162 were deiminated to a greater extent in the MS samples than the normals. C-5 was similar to C-4, showing greater deimination in the MS samples. The C-8 component from normal tissue showed increased deimination when compared with the other components, as reported earlier (9). Deimination was found at R43, R49, R54, R65, R97, R122, R130, and R162. Of the two MS samples, C-8 (MS-1) was deiminated at R49, R65, R97, R130, and R162 but the extent of deimination was relatively small compared with the other C-8 samples. These data were consistent with a very mild form of MS in an 85-year-old woman. The other MS sample, C-8 (MS-2), showed extensive deimination throughout, reflecting a more aggressive course in a 46-year-old woman and was more representative of progressive MS. As suggested by us earlier, the extent of deimination correlated with severity of disease (10). Whereas we reported total moles of citrulline per mole MBP in our earlier studies, the present studies confirmed what we suspected, *i.e.* partial deimination at several residues was common.

DISCUSSION

Proteins are encoded by the nucleotide sequence in the DNA; the message is transcribed and then translated on the ribosomes. However, the protein encoded by the nucleotide sequence rarely represents the functional molecule. Instead, each protein is modified by one or more of several hundred post-translational modifications, *e.g.* N-terminal blocking, disulphide bonds, glycosylation, etc. Several hundred different modifications are known. However, the role played by these post-translational modifications is poorly understood at this time (39). Even less well understood is the role of post-translational processing in the pathogenesis of disease. We have attempted to address this issue in demyelinating disease by defining the modifications on MBP in normal and diseased tissue as a first step in achieving an understanding of the role of these modifications.

Mass spectrometry is a powerful tool by which to assess the nature of the modifications on peptides generated by tryptic digestion, *e.g.* a mass difference of 1 Da can be re-

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TABLE V

Deiminated arginine residues in MBP from normal and MS tissue relative intensities

Relative intensity = Ratio of Intensity of (modified arginine)/(unmodified arginine). *n*, number of samples.

Charge Isomer	n	R5	R25	R31	R43	R49	R54	R65	R97	R122	R130	R162
C-1 (N)	3	0.013 ± 0.011	0.00	0.00	0.003 ± 0.004	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C-1 (MS)	2	0.016	0.010	0.00	0.00	0.659 ± 0.932	0.00	0.00	0.023 ± 0.023	0.00	0.005 ± 0.007	0.00
C-2 (N)	3	0.010 ± 0.009	0.019 ± 0.018	0.00	0.00	0.015 ± 0.025	0.303 ± 0.305	0.008 ± 0.013	0.010 ± 0.008	0.00	0.022 ± 0.021	0.214 ± 0.188
C-2 (MS)	2	0.033	0.050 ± 0.071	0.00	0.00	0.132	0.106	0.008	0.034	0.00	0.041	0.259
C-3 (N)	3	0.016 ± 0.015	0.017 ± 0.014	0.00	0.00	0.033 ± 0.016	0.12 ± 0.207	0.005 ± 0.005	0.006 ± 0.010	0.00	0.018 ± 0.020	0.295 ± 0.481
C-3 (MS)	2	0.040	0.055	0.00	0.00	0.085	0.100	0.012	0.024	0.00	0.043	0.554
C-4 (N)	16	0.025 ± 0.021	0.115 ± 0.120	0.00	0.008 ± 0.011	0.066 ± 0.049	0.395 ± 0.546	0.008 ± 0.006	0.016 ± 0.012	0.00	0.030 ± 0.024	0.295 ± 0.461
C-4 (MS)	7	0.032 ± 0.019	0.176 ± 0.071	0.00	0.005 ± 0.009	0.068 ± 0.027	1.089 ± 0.857	0.16 ± 0.010	0.020 ± 0.007	0.007 ± 0.009	0.043 ± 0.019	0.554 ± 0.543
C-5 (N)	8	0.033 ± 0.032	0.114 ± 0.141	0.00	0.010 ± 0.014	0.077 ± 0.064	0.542 ± 0.587	0.008 ± 0.009	0.018 ± 0.012	0.000	0.038 ± 0.033	0.285 ± 0.064
C-5 MS	6	0.049 ± 0.060	0.308 ± 0.099	0.00	0.022 ± 0.012	0.118 ± 0.038	1.453 ± 0.9344	0.027 ± 0.014	0.026 ± 0.009	0.013 ± 0.015	0.085 ± 0.042	0.633 ± 0.440
C-8 (N)	3	0.086 ± 0.060	0.81 ± 0.281	0.031 ± 0.022	0.238 ± 0.133	1.450 ± 0.823	1.156 ± 2.33	1.977 ± 2.33	1.121 ± 0.628	0.243 ± 0.153	1.438 ± 0.747	1.776 ± 0.038
C-8 (MS-1)	1	0.027	0.00	0.00	0.0	0.003	0.318	0.338	0.188	0.00	0.222	0.217
C-8 (MS-2)	1	0.182	0.200	0.029	0.115	2.127	1.091	2.667	1.125	0.186	1.250	1.955

solved readily. However, it is difficult to obtain accurate quantitation of each modified peptide. To overcome this difficulty, we calculated the ratio of the intensity of the modified peak relative to unmodified peak. In this way we were able to obtain a reasonable assessment of the extent of modification in each tryptic peptide. The data is much stronger in those cases where the tryptic peptide was totally unmodified because the sensitivity of the instrumentation is high enough to detect even a small amount of the modified peptide. This was especially true in the case of phosphorylation, where we found a total absence of phosphorylation in tryptic peptides from MS samples compared with corresponding peptides from normal samples. In those cases where both peptides from normal and MS samples were modified, considerable variation in the relative amounts was observed, resulting in large standard deviations. This was due to the recognized heterogeneity of the disease (40) making it impossible to obtain a number of samples at a defined stage of disease. Recognizing these limitations of our methodology we were, nevertheless, able to obtain trends that we believe are significant. Some of these are described below.

In a comparison of MBPs from normal and MS white matter, we found that MBP from MS tissue was less cationic (less positive charge) than MBP from normal tissue (9), which we attributed to deimination of arginyl residues in part at least (10). With the conversion of each arginyl residue to citrulline, one positive charge is lost. These results led us to carry out a more extensive investigation into the number and nature of other post-translational modifications of MBP from normal and MS tissue. Six charge isomers were isolated, and the modifications in various peptides were documented by mass spectrometry. In addition to the deimination of arginyl residues, we selected two major modifications, methylation on arginine 107 and phosphorylation, because these two modifications are postulated to act in concert in signaling events involving the interferon receptor (17, 18).

The methylation of arginyl residues (both mono and dimethylation) was lower in C-1, C-2, and C-3 of the MS samples compared with normals but the intensities were low, so these data are difficult to interpret. They were much higher in C-4 and C-5 of MS samples and unchanged in C-8. When the total methylation of all components was determined, an overall increase in the MS samples was observed. Monomethylation was 1.14 ± 0.54 in the normal but 1.44 ± 0.98 in the MS samples. Dimethylation was 2.21 ± 1.06 in the normal and 3.21 ± 2.74 in the MS samples. Because methylation of MBP has been reported to be essential for the formation of compact myelin (16), the increased methylation in MS probably reflects attempts at remyelination, which fails in the demyelinating environment. Alternatively, the apparent increase in methylation may reflect the increased resistance of MBP imparted on it by methylation, *e.g.* we reported that the rate of deimination of MBP by peptidyl arginine deiminase was 2-fold increased in the absence of methylation, suggesting methyl-

ation had a protective effect on MBP (41). Therefore the apparent increase in methylation in the MS samples may reflect the greater resistance to proteolysis by methylation, imparting a longer half-life to the protein.

Proteolipid protein (PLP) is the other major protein of myelin accounting for about 50% of the total protein. In contrast to MBP, which is a basic protein, PLP is very hydrophobic, containing more than 60% apolar or hydrophobic amino acids. It is post-translationally modified with palmitate and stearate. When we compared the amounts of these two fatty acids on PLP isolated from our DM20 transgenic mouse, both were increased 6-fold over PLP isolated from normal mouse brain. Of considerable interest, we also found that the N-terminal glycine, which was not blocked in PLP from normal brain, was methylated in the PLP from the transgenic mouse brain (42). This unusual N-terminal modification suggests that the increased methylation of MBP reported in this manuscript may be more generalized than originally thought, involving other myelin proteins.

In contrast to methylation, phosphorylation was dramatically reduced in the MS samples. Of the 10 phosphorylation sites studied in each of the six charge isomers, *i.e.* a total of 60 sites, only 4 sites in the MS samples showed the same amount or increased phosphorylation compared with normal. The animal studies in which the amount of phosphothreonine in the TPPPSQGK sequence was reduced during demyelination was probably due to the decreased GSK in brain (Fig. 1*C*). With an antibody that detects phosphoserine in a variety of peptides, we demonstrated that the hypophosphorylation was a general defect. These data combined with the deamidation data support the view that the low level of phosphorylation of MBP in MS brain may have pathogenic significance and is not simply due to post-mortem degradative processes.

Although we do not have an explanation for this state of hypophosphorylation at this time, studies in Alzheimer's disease demonstrated that uncoupling of mitochondrial oxidative phosphorylation resulting in decreased amounts of ATP-activated protein phosphatases and inactivated MBP kinases (43). Our above-mentioned data on GSK and phosphoserine kinases, which are decreased in our transgenic mouse brain, may explain the hypophosphorylated state of MBP in this model. Studies of other kinases, such as protein kinase C, protein kinase A, and tyrosine kinase, are underway to determine if these are affected also possibly by the result of uncoupling oxidative-phosphorylation, although this remains to be determined.

In an earlier study (8), we demonstrated that phosphorylation of C-1 → C-4 by protein kinase C induced large changes in β-structure of all charge isomers stabilized by salt bridges between phosphate groups and arginyl residues. In the MS samples, deimination of arginyl residues would preclude the formation of β-structure by these interactions, resulting in less structure, which would increase the susceptibility to proteases. Significantly highly deiminated C-8 samples contained few phosphorylated peptides (Tables III and V).

In summary, our data suggest that demyelinating disease may represent a new disease category tentatively referred to as "post-translational diseases." The genetic contribution, in the form of a number of genes of small effect, may be manifest through specific pathways involving enzymes responsible for post-translational processes. Whereas some modifications, especially those that do not affect the tertiary structure of MBP, may be tolerated in small amounts, the presence of several modifications acting in concert may induce disease. The heterogeneity in pathological and clinical course, a hallmark of MS, may reflect the nature, site, and extent of the modifications, *e.g.* in Marburg's variant, a fulminating form of MS, almost 100% of the MBP was of the citrullinated variety (10), compared with 45–50% in chronic MS and 20% in normal brain. Because this MBP (Marburg's) induces vesicle fragmentation *in vitro* (13), a similar effect *in vivo* may result in extensive vesiculation of myelin. In other studies, we found that deimination of protein-bound arginyl residues renders MBP more susceptible to digestion by cathepsin D, a myelin-associated protease (44). Recent studies in two different areas have shown that post-translational modification of self antigens creates neo-self antigens that give rise to specific T cell responses. Because these neo-antigens are foreign to the immune system, the sensitized T cells escape tolerance. The failure to define an auto-antigen in MS may be related to the failure to detect neo-self antigens (45). The data presented in our study suggests that possible self-antigens may be modified peptides. Furthermore, the extent and nature of post-translational modifications may be related to the extent of demyelination and disease heterogeneity in MS (40) as suggested by our studies of citrullinated MBP (10).

Acetylation of histones represent important regulatory events in transcriptional regulation. Phosphorylation at Ser^{10} of histone H3 facilitates acetylation of Lys^{14}. Loss of Ser^{10} by mutation results in a complete loss of acetylation at Lys^{9} (46). A model is suggested by these authors in which the type and extent of modification at specific sites facilitates or antagonizes association of regulatory proteins with chromatin. The above-mentioned studies strongly suggest that the site and extent of modification affects modifications at other sites, *i.e.* a concerted effect. The enzymes involved in these modifications represent potential therapeutic targets as we have demonstrated recently with peptidylarginine deiminase and paclitaxel (Taxol) (47).

* This work was supported by the Multiple Sclerosis Society of Canada (Grant F77911, to M. A. M.), National Multiple Sclerosis Society (Grant RG3167-A-1, to R. Z.), and the National Science Foundation (Grant DBI-998722, to D. M. L.).

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