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Studies of Posttranslational Modifications in Spiny Dogfish Myelin Basic Protein

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The objective of this investigation was to determine whether nonmammalian myelin basic protein contained charge isomers resulting from extensive posttranslational modifications as seen in mammalian MBP. Four charge isomer components from dogfish MBP have been isolated. These forms arise by phosphorylation and deamidation modifications. Components C1, C2 and C3 have been characterized. We are currently characterizing component C8. Dogfish MBP is less cationic than mammalian MBP and has about 50% lower mobility on a basic pH gel electrophoresis relative to human and to bovine MBP. The mammalian component C1, which is unmodified, is modified in the dogfish by phosphorylation. The reduced electrophoretic mobility is largely attributable to the charge reduction resulting from phosphorylation in serine 72, 83, and 120 or 121 in C1, and C3. In component C2, two or three phosphate groups were distributed among residues 134, 138 and 139. It was found that dogfish amino acid residue 30 was a lysine residue and not a glutamate residue as reported in the literature.

KEY WORDS: Dogfish myelin basic protein; charge isomers; post-translational modification; mass spectrometry.

INTRODUCTION

The roles of integral and peripheral proteins present in the myelin membrane are poorly understood with respect to their function in the organization and maintenance of myelin integrity. The major peripheral protein known as myelin basic protein (MBP) is believed to play a major role in assembly, maintenance of membrane integrity, and biological function. Mammalian MBP's from the central nervous system are conserved with respect to primary structures, and exhibit 45% identity to MBP from cartilaginous fishes. Jawless fishes, lamprey, and hagfish have nonmyelinated fibers and lack MBP's (1,2). Mammalian MBP's undergo extensive posttranslational modifications. The reason for these modifications is yet unknown. These posttranslational modifications result in charge microheterogeneity attributed to the attachment of phosphate groups, loss of C-terminal arginine residues (3), methylation of arginine residue 106, yielding a monomethyl and dimethyl derivatives (4), deamidation of a glutamine residue (5,6), conversion of arginine residues in com-

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Abbreviations: MBP, myelin basic protein; CE, capillary electrophoresis; HPLC, high performance liquid chromatography; LC, liquid chromatography; TOF, time of flight; TIE, total ion electropherogram; MS, Mass spectroscopy; MALDI, Matrix assisted laser desorption ionization; CMC, carboxy methylcellulose; PMSF, phenylmethanesulfonic acid.

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ponents C6 and C8 to citrulline by the enzyme peptidyl arginine deiminase (7), and the potential loss of one or two C-terminal arginine residues (4,6).

The present study on the MBP from an early non mammalian vertebrate was undertaken so that an assessment of primary structure conservation and a comparison of posttranslational modifications with mammalian MBP's could be made. Neither the extent of posttranslational modification nor the number of charge isomers in non-mammalian MBP's has been extensively investigated. Tai et al. (8) were unable to separate charge isomers from the MBP of the gummy shark, Mustelus antarticus, and suggested that this MBP is not posttranslationally modified. Conflicting reports exist as to the spiny dogfish MBP phosphorylation state (9,10).

The MBP gene consists of seven exons and leads to the expression of MBP of molecular weights of 14, 17.5, 18.5, and 21.5 kDa in mouse, and human MBP's (11–13). The 18.5 kDa isoform contains of 170 amino acids in human MBP and contains 169 amino acid residues in bovine MBP. This 18.5 kDa form of human MBP results from the reading of exon 1 and exons 3 through 7. Spiny dogfish MBP which contains 155 amino acid residues, lacks the information encoded in exon 2. The expressed protein having 141 amino acid residues is missing the information from exons 2 and 5 (10). The primary structure of dogfish MBP has been reported (10), as have the sequences of the human (14,15) and bovine MBP's (16). Although there is a significant overlap in the human and bovine MBP sequences, the dogfish MBP sequence has a reduced overlap level with very little homology in the serine and threonine residues when compared with the sequence of human and bovine MBP, Table I.

A major difference in the sequence of MBP from higher animals, is the change of the residues surrounding the tri-proline sequence starting at residue 98 in the bovine sequence. The bovine sequence of RTPPP is modified to KKAPV in the dogfish. In this modification the threonine residue in bovine and human MBP charge isomers C3 to C6 is phosphorylated, possibly by MAP kinase (17), and has been suggested to be part of the signal transduction ligand for SH3. An equivalent phosphorylation site at this location in the dogfish sequence is not possible.

We have previously reported on the application of capillary electrophoresis-mass spectrometry for the determination of posttranslational modifications in myelin basic proteins (18,19). This study reports the first extensive assessment of charge isomers and posttranslational modifications in a non-mammalian MBP. The role of the MBP's in the assembly and maintenance of the myelin membrane also needs to be elucidated. In human MBP in the early stages of development the amount of component C8 is high and the amount of component C1 is low (20). When component C8 is present in large amounts the compaction of the membrane into a tight spiral is not very extensive (20). With age, and as component C1 begins to accumulate, compaction becomes more evident. Literature reports of myelin morphology from some non mammalian species reveal regions of loose compaction even in adult animals. Electron microscopic studies of myelin from the shark Scyllium stallare (21) and the toad (22) show variations in compactness that may be related to the extent of various posttranslational modifications. The results of the present study are consistent with such a hypothesis for the assembly and stability of the myelin membrane.

EXPERIMENTAL PROCEDURE

Isolation and Purification of Dogfish MBP. Adult dogfish brains and spinal cords were obtained from freshly killed specimens at the Marine Biological Laboratory, (Woods Hole, MA). The tissues were immediately frozen and kept frozen at −80°C until used for the preparation of MBP.

The procedure for the isolation and purification of the MBP followed the method of Fannon and Moscarello with slight modifications (23). Final purification was obtained by chromatography on a CMC column according to the procedure of Deibler and Martenson (4). The charge isomer fractions were collected and dialyzed against distilled water and lyophilized. Each fraction (C1,C2,C3 and C8) was subjected to mass spectral and amino acid analysis as previously described (18,19).

The amino acid analysis compositions of the MBP fractions was achieved on a Water's Pico Tag Amino Acid Analyzer after prior hydrolysis at 100°C in 5.7N HCl for 24 hrs under nitrogen.

The procedure of Towbin, et al (24) was used for Western blot analyses.

Sample Preparation for On-Line Sheathless CE/IT/reTOF MS. A quantity of each charge isomer, approximately 20μg, of MBP was treated with trypsin (in a ratio of protein to trypsin approximately 50:1) at 37°C overnight, in an ammonium bicarbonate buffer, pH 8.2. The digest was then dried in vacuo and dissolved in deionized water to give a peptide concentration of ca. 1×10^{-5} M.

The MBP digests were separated using a capillary electrophoresis apparatus employing a polybrene coated capillary column as described by prepared Li et al. (25). The resolved peptides fed directly into the mass spectrometer apparatus as previously described (25,26). Data analysis was performed using custom 2D false color image (27). The m/z spectrum served as one axis, and TIE elution time as the second axis. The m/z intensity served as the image color (28). To increase the signal to noise ratio the cursor on this image map (or on the mass spectra and TIE plot) was used to display the corresponding individual m/z spectrum on the x-axis and additionally to integrate multiple spectra over an elution time range. This integration capability allowed peaks of low intensity to be identified.

On-Line CE/MS/MS with an IT/re TOF System. Application of On-line CE/MS/MS with stored waveform inverse Fourier transform (SWIFT) capability for use with the ion trap reflectron time-of-flight mass spectrometer has been previously described (24). While MS/MS spectra can be acquired at sampling rates up to 8 Hz (19), this rate compromises the S/N of the spectrum. In the present studies 4 Hz was chosen as the best compromise between the speed of the MS/MS data acquisition and the quality of the mass spectra obtained.

MBP Dephosphorylation. Dephosphorylation of MBP tryptic peptides was achieved by incubating the tryptic protein digests in ammonium carbonate buffer, pH 8.0 at 37°C for two hours (29) with calf intestine alkaline phosphatase (Sigma Chemical Co. St. Louis, MO). The resulting digest was dried in vacuo, and reconstituted in 20μl of deionized H_2O. Tryptic peptides maps before and after dephosphorylation were established by on line LC/MS.

On-Line LC/oa TOF MF System. HPLC separations were carried out using a narrow bore 150 mm by 2.1 mm YMC C18 column (5mm, 300åA from Waters). The flow rate was set at 200 μl/min. The peptides were resolved with a linear gradient using 0.1% TFA in H_20 for reservoir A and 0.1% TFA in acetonitrile in reservoir B with a gradient from 0 to 80% buffer B in 40 min. The effluent was introduced directly into a Micromass LCT orthogonal acceleration TOF instrument. The ions generated from the electrospray source were sampled with a dual orthogonal z-spray cone, and transferred to the orthogonal time of flight analyzer. Typical instrumentation parameters were: voltage 3,500 V, sample cone 30 V, extraction cone 10 V, source temperature 100°C, desolvation temperature 300°C, MCP detector 2,700 V, and flight time of 75μsec which corresponds to m/z values from 0 to 3,800.

MALDI/TOF MS. Enzyme digested solutions were desalted and concentrated using C18 Zip-Tips (Millipore). The purified peptide solutions were then spotted onto the MALDI plate for subsequent MALDI-TOF MS analysis. These analyses were performed on a PerSeptive Voyager Biospectrometry Workstation equipped with delayed extraction technology, a one meter flight tube and a high current detector. The nitrogen laser provided 337 nm emission for desorption and ionization. MALDI/TOF MS was used to determine the masses of tryptic peptides using a slightly modified version of the two layer dried droplet method (30). The MALDI matrix α-cyano-4-hyrdroxycinnamic acid (Sigma Chemical Corp, St. Louis, MO) was prepared as a saturated solution in acetone containing 1% TFA. This solution was diluted 8 fold with an acetone solution containing 1% TFA and was then added to the sample droplet in a 1:2 (v/v) ratio. The mixed droplet was allowed to air dry before introduction into the MALDI/TOF instrument. All spectra are averages of 128 scans and were either calibrated internally or externally using the PerSeptive standard peptide mixture consisting of angiotensin I, ACTH (residues 1–17) ACTH (residues 18–39) and ACTH (residues 7–38).

RESULTS AND DISCUSSION

Isolated spiny dogfish MBP was resolved on a CM-52 column into four major peaks designated as C1, C2, C3, C8 and a minor peak, adjacent to C8, (Fig. 1). Charge isomer designations were assigned according to their elution profile. This system is the same one used to assign mammalian charge isomers. Whereas the last eluted fraction in mammalian MBP's is unmodified, the corresponding spiny dogfish C1 component is modified. Western blots confirmed that the spiny dogfish MBP components were recognized by bovine MBP antibody, thereby confirming that the isolated protein from dogfish brain and dogfish spinal cord belonged to the class of myelin basic proteins, (Fig. 2). The mobility of the protein on the electro phoretic gels was about 50% of the human or bovine MBP's, indicating that the dogfish MBP is much less cationic. This is similar to the observation that less cationic forms of MBP are a phenomenon observed in young animals in early stages of myelination. Also, spiny dogfish MBP contains seven less positive charges. Unfractionated dogfish MBP contains hydroxyproline and citrulline (Table II). These amino acid residues are most likely present in component C8 since they are not present in components C1, C2, or C3 of mammals.

The tryptic peptides for components C1, C2, C3 and the molecular weights that would be expected for an unmodified peptide from the dogfish MBP are presented in Table III. The peptide with the amino acid composition Acetyl-A-S-T-T-S-D-H-A-K has an average theoretical mass for $[M + H^+]$ of 1030.1. An experimental mass peak of 1030.6 Da was observed, indicating that this peptide is not phosphorylated. It differs from the N-terminal bovine and human sequences in that it lacks fifteen amino acid residues between residue 2 and residue 17 of human MBP. In the N-terminal bovine sequence of MBP, the Ser 7 is phosphorylated. The approximate analogous Ser residue in the Dogfish is not phosphorylated.

The dogfish tryptic peptide sequence T4, from residue 21–38 (Table I) has been reported to have the sequence DSGLLDQLGQ$_{30}$LFGQEGSR, with a $[M + H]^+$ of 1921.1 Da. In our results this mass was never observed using CE/MS, LC/MS and MALDI/MS. Instead, two strong peaks with m/z values of 1045.9 and 893.7 respectively were obtained by LC/MS and CE/MS analysis. Values for m/z that a peptide sequence of DSGLLDQLGK (theoretical average mass 1046.2), and LFGQEGSR (theoretical average mass 894.0) (Fig. 3). On the basis of these results, it was concluded that instead of a glutamine residue at this location as is reported in the literature, a lysine residue is present and the correct sequence is QRDSGLLDQLGK$_{30}$LFGQEGSR.

The theoretical average $[M + H]^+$ value for the peptide DSGLLDQLGQ$_{30}$LFGQEGSR is equal to 1921.1 Da. Replacement of the glutamine residue Q_{30} by a lysine residue K_{30} then should yield an additional trypsin cleavage site to give two peptides consisting of the sequences DSGLLDQLGK, and LFGQEGSR of

Table I. Comparison of Amino Acid Sequences for Human, Bovine and Dogfish MBP

	5 10 15 20 25	
Human	Acetyl A S Q K R P S Q R H G S K Y L A T A S T M D H A R	
Bovine	Acetyl A A Q K R P S Q R * * S_{10}K Y L A S_{15}A S T M D_{20}H A R	
Squalus	Acetyl A S * * * * * * * * * * * * A T T_5S D H A K_{10}	
	30 40 50	
Human	H * * G F L P R H R D T G I L D S I G R F F G G D R G	
Bovine	H * * G_{25}F L P R H_{30}R D T G I_{35}L D S L G_{40}R F F G S_{45}D R G	
Squalus	Q A G G A_{15}H S R Q R_{20}D S G L L D Q L G K_{30}L F G Q E * G	
	60 70	
Human	A P K * R G S G K D S H H P A * R * * * * * * T A H Y G	
Bovine	A P_{50}K * R G S G_{55}K D G H H_{60} A A * R * * * * * * T T_{65}H Y G	
Squalus	S R KV_{40}P E K G K_{45}E * * * P A T R_{50}S V L M A PT T H K_{60}A	
	80 90	
Human	S L P Q K S * H G R T Q D E N P V V H F F K N I V T P R	
Bovine	S L_{70}P Q K A Q_{75} H G R P Q_{80}D E N P V_{85}V H F F K_{90}N I V T P_{95}R	
Squalus	H * * Q G A * * R R Q T D_{70}D S * P V V H F F K N_{80}M M S P K	
	100 110 120	
Human	T P P P S Q G K G R * G * * L S L S R F S W G A E G Q R P	
Bovine	T P P P_{100}S Q G K G R * G * * L S L_{110}SR F S W G A E G Q_{120}K P	
Squalus	K A P V Q_{90} Q * K A K S G A S R A_{100}I TK F I W G T D G_{110}Q R A	
	130 140	
Human	G F G Y G * * G R A S D Y K S A H K G F K * G V * D A Q	
Bovine	G F G Y G * * G R A_{130}S D Y K S A H K G L_{140}K * G H * D A Q	
Squalus	* * H Y G A A G * * * S_{120}S K S * * K D G F R G R_{130}RD G S	
	150 160 170	
Human	G T L S K I F K L G G R D * * * * S R S G S P M A R R	
Bovine	G T L S_{150}K I F K L G G R D * * * *S_{160}R S G S_{164} P M A R R_{169}	
Squalus	G T L S S F_{140}FK M G K KG E * * * * * * G S_{150} P * A R R_{154}	

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

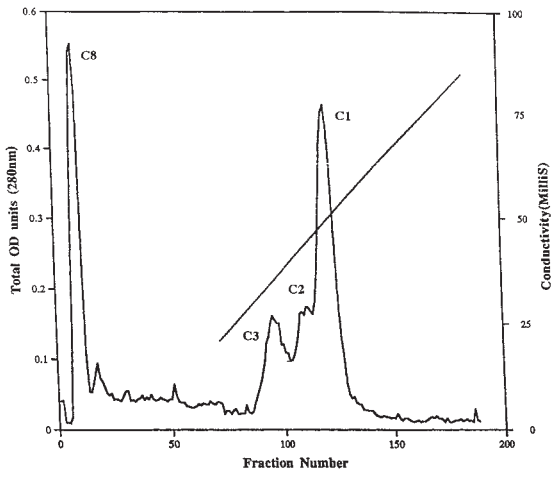

Fig. 1. Separation of spiny dogfish charge isomers on a carboxymethyl cellulose column. The protein was dissolved in a urea glycine buffer pH 9.6 and applied to a CM52 column equilibrated in a urea glycine buffer, pH 10.6 and eluted with a 0 to 0.2M NaCl gradient.

$[M + H]^+$ 1046.2 Da and 894.0 Da respectively as noted above. No mass values to support the presence of the uncleaved peptide containing glutamine were observed. This observation was found for each of the charge isomer components C1, C2, and C3.

The amino acid sequence for residues 80 to 85 in charge isomer components C1, C2, and C3 is NMMSPK. The mass peak in each component is consistent with the presence of a phosphate group attached to the serine residue. The theoretical average $[M + H]^+$ value for the unmodified NMMSPK peptide should be 707.9 Da and this mass value was obtained. In addition, if phosphorylation has occurred in some fraction of the molecules and if each methionine residue had been oxidized, a m/z value of 819.9 Da would have been predicted. A peak with m/z of 819.5 Da was present in the spectra and assigned to this peptide. After treatment of the peptide with alkaline phosphatase the peak of m/z equal to 819.5 Da disap-

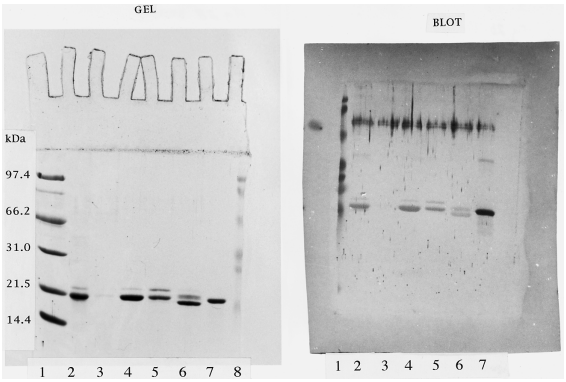

Fig. 2. SDS Slab gel of isolated charge isomers of Dogfish MBP
Lanes: 1, Molecular weight standard
2, unfractionated protein ≈ 2.5 μg.
3, Component C8 ≈ 2.5 μg.
4, component C1 ≈ 2.5 μg.
5, component C2 ≈ 2.5 μg.
6, component C3 ≈ 2.5 μg.
7, human MBP component C1 ≈ 1.25 μg.
8, prestained standard.
Western blots of Dogfish MBP charge isomers
1st antibody is rabbit antibovine MBP 1:300
2nd antibody is goat anti-rabbit horse radish peroxidase 1:5000
Lanes: 1, Molecular weight standard;
2, unfractionated protein
3, Component C8;
4, component C1;
5, component C2;
6, component C3;
7, human MBP component C1.

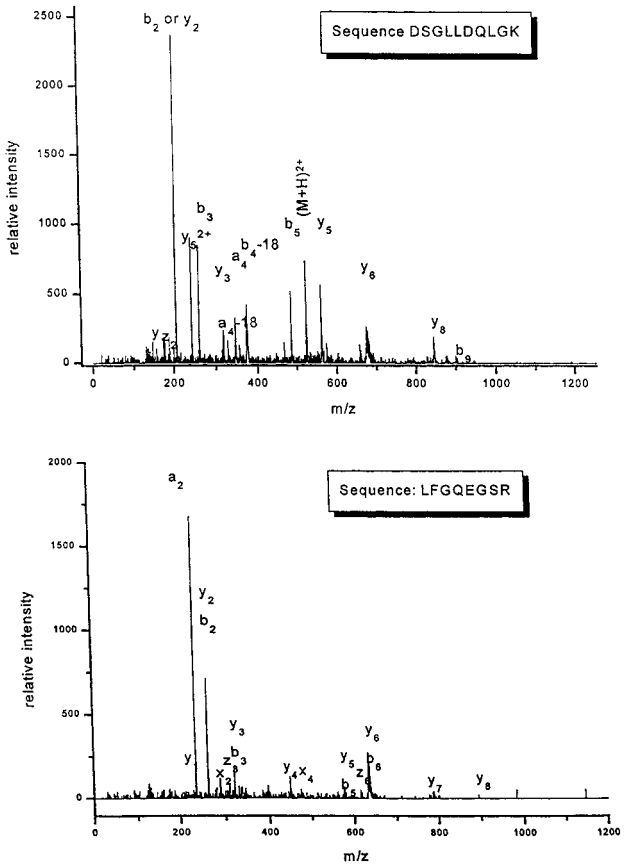

Fig. 3. CE/MS/MS spectra of the two peptides, residues 19–30 and 31–38. These spectra demonstrate the cleavage of the peptide 19–38 at a lysine residue thereby yielding two peptides. If a glutamine residue was at position 30 in the sequence only a single peptide of residues 19 to 38 would be present in the spectra.

peared and concomitantly a peak of m/z 740.3 Da appeared. This result indicates that the peptide has lost 79.2 Da and suggests the loss of a phosphate residue. However, the resulting peptide still exhibited a mass value larger than the value for the unmodified peptide. We attribute this increase to the oxidation of each of the methionine residues in the peptide. A second peptide of m/z 723.3 was also present. We hypothesize that this peak arises from a peptide lacking a phosphate residue and in which one of the methionine residues is oxidized since the theoretical m/z is 723.9 Da.

A third peak of m/z 835.4 was also obtained. This is interpreted as demonstrating that the peptide still retains a second lysine residue at the C-terminal end, NMMSPKK, with a theoretical m/z of 836.1 Da. If this peptide was phosphorylated the mass would increase by 80.0 Da. A peak with m/z of 915.5 Da was also found and was assigned to the peptide. Thus, in peptide sequence NMMSPKK, obtained from charge isomers C1, C2, and C3, a significant fraction of the peptide Ser residue is phosphorylated. Therefore, both the phosphorylated and unphosphorylated species of the peptide are present as in the whole protein mixture.

The amino acid sequence consisting of residues 113 to 122 is AHYGAAGSSK. This sequence is phosphorylated on components C1 and C3 but not on component C2. The theoretical m/z value for the unphosphorylated peptide is 949.0 Da. The theoretical m/z value for the phosphorylated form of the peptide in C1 and C3 is 1029.0 Da and the observed m/z value was 1028.4, suggesting that this sequence is phosphorylated.

The peptide comprised of amino acid residues 67–85 has the sequence RQTDDSPVVHFFKNMMSPK. The m/z value obtained from the MALDI experiment is 2424.3 and is consistent with the presence of two phosphate groups on this sequence for which the theoretical m/z value is 2425.6 Da. The C-terminal portion of this peptide was discussed above and shown

Table II. Average Number of Residues/100 Residues

	Dogfish	Bovine	Human	Alligator
Aspartic acid	7.47	6.5	6.5	5.9
Threonine	5.07	4.2	4.7	3.3
Serine	9.8	9.8	11.2	11.1
Glutamic acid	11.91	6.8	5.3	11.1
Profile	5.7	7.5	7.1	7.3
Glycine	14.81	17.5	15.3	17.1
Alanine	11.35	7.9	7.1	7.8
Valine	3.55	1.2	2.4	3.4
1/2 cystine	0.1			
Methionine	2.34	1.5	1.2	1.4
Isoleucine	1.64	1.1	2.4	1.5
Leucine	3.9	5.5	4.7	4.7
Tyrosine	1.01	2.4	2.4	1.9
Phenylalanine	3.4	4.5	5.3	2.4
Ornithine	0.14			
Lysine	8.96	6.1	7.1	6.7
Histidine	2.89	6.4	5.9	4.4
Arginine	5.35	10	11.2	4.9
Hyroxyproline	0.63			

to contain a phosphate group. The presence of a second phosphate on a serine residue immediately adjacent to a proline residue is not unusual. Serine groups are often located on a protein surface and accessible to kinase enzymes residues are often exposed on the protein surface and accessible to kinase enzymes.

Lastly, the peptide DGSGTLSSFFKMGK, consisting of amino acid residues 132–145 of component C2, contains two or three phosphate groups. No additional mass was found that could be assigned to any other peptide. The modifications for components C1, C2, and C3 are summarized in Table IV.

The amino acid sequence of component C8 has not been elucidated for the dogfish. Cleavage of component C8 with CNBr yielded a peak with m/z of 5810.4 Da. In the absence of a phosphate group a value of 5682.9 would have been anticipated; if two phosphates were present a m/z value of 5842.8 would have been anticipated. The theoretical m/z value for the CNBr peptide shown below,

Acetyl-ASATTSDHAKQAGGAHSRQRDSGLLDQLGLKLFGQEGSRKVPEKGKEPATRSVLM

of residues 1–54 and containing two phosphate groups, is 5846.3 Da. The observed mass corresponds to a sequence having N-terminal residues 1–54 and two phosphorylated sites along with two deamidation sites plus four Arginine to citrulline modifications resulting in a mass of 5810–.

Analysis of a tryptic digest of component C8 gave a m/z value of 1190.5 Da a value that matched the theoretical m/z value of 1191.0 Da calculated for amino acid residues 1–10 with two phosphorylated sites. MALDI and LC/MS experiments of C8 tryptic digests yielded a peak with an m/z value of 1621.2 Da. This mass corresponds to the amino acid sequence T25 + 26 DGSGTLSSFFKMGK with two phosphorylated groups and has a calculated m/z value of 1621.6 Da. A m/z peak of m/z 1749.4 Da was also detected. This corresponds to the mass for peptide T25 + 26 + 27 with the amino acid sequence DGSGTLSSFFKMGKK containing two phosphorylated groups. An additional peak of m/z 1385.9 Da was observed which matched the T25 sequence that putatively contains three phosphate groups.

The presence of citrulline in component C8 was inferred from a peak of m/z 1564.6 obtained from a tryptic digest of C8. This is in good agreement with the mass value predicted from the amino acid sequence GTDGQRAHYGAAGSSK with one citrulline since the theoretical average $[M + H]^+$ value for the modified peptide with a m/z mass of 1564.6 Da. If the arginine residue contained in this sequence had not been converted into a citrulline residue, the peptide would be anticipated to undergo tryptic cleavage. The presence of the intact peptide, the presence of citrulline in the amino acid analysis of the protein, and the strong mass peak support our interpretation of the data for conversion of this arginine to citrulline in this peptide.

The above described results demonstrate unambiguously, for the first time, the presence of charge isomers in the dogfish MBP, and also, clearly demonstrate the presence of phosphorylated serine and threonine residues in dogfish MBP. These findings support the results by Spivac et al (10) and negates the report by Agrawal et al (9). Since the spiny dogfish (Squalus Acanthias) is a member of the shark family, the report that shark MBP does not contain charge isomers (8) must be considered too general. For this species of shark, charge isomers arising from posttranslational modification are definitely present. The significance of half the number of charge isomers in the dogfish relative to the number present in mammalian MBP remains to be fully explored. Three of the charge isomers are more extensively phosphorylated than their counterparts in the mammalian MBP's and the number of amino acid residues in the primary structure is significantly reduced.

In addition to resolving the controversy regarding the presence and number of phosphate groups in spiny dogfish MBP, the need also relates to the report by Martenson et al. (31) stating that the MBP's obtained from carp and shark did not induce experimental aller-

Table III. Tryptic Peptides for Components C1, C2, C3 and Molecular Weights for Unmodified Peptide from Dogfish MBP

Fragment	Start	End	Sequence	Theoretical mass	Charge	Theoretical m/z	Actual m/z
T1 (Acetyl)	1	10	ASATTSDHAK	1030.5	d	515.8	516.4
					s	1030.5	1030.6
T2	11	18	QAGGAHSR	783.39	d	392.2	385*
T2 + T3	11	20	QAGGAHSRQR	1067.54	d	534.2	526.27*
T3	19	20	QR	303.18	s	303.2	303.5
T4	21	38	DSGLLDQLGQLFGQEDSR	1920			Never found
T5	39	39	K	147.2	s	147.7	147.7
T5 + T6	39	43	KVPEK	600.4	d	300.7	301.1
T7 + T8	44	50	GKEPATR	758.4	d	379.7	379.7
T8	46	50	EPATR	573.6	d	287.3	286.8
T9	51	60	SVLMAPTTHK	1084.6	d	542.8	543.2
T10	61	66	AHQGAR	639.3	s	639.3	639
					d	320.2	321
T11	62	62	R	175.1	s	175.1	175.6
T12	68	79	QTDDSPVVHFFK	1419.7	d	710.8	711.2
					t	473.9	474.6
T13	80	85	NMMSPK	707.3	s	707.3	707.4
					d	354.2	354.6
T14	86	86	K	147.2	s	147.7	147.7
T14 + T1	86	92	KAPVQQK	798.5	d	399.8	400.2
T16 + T17	93	99	AKSGASR	676.4	s	675.2	675.2
T17	95	99	SGASR	477.2	s	477.2	477.5
					d	239.1	240.7
T18	100	103	AITK	432.3	s	432.2	432.2
					d	216.7	218.8
T19	104	112	FIWGTDGQR	1079.5	d	540.3	541
T20	113	122	AHYGAAGSSK	948.4	d	474.2	475.3
T21	123	124	SK	234.3	s	234.3	234
T22	125	128	DGFR	494.2	s	494.2	494.4
					d	247.6	249.1
T21 + T22	123	128	SKDGFR	709.4	d	355.2	355
T23	129	130	AK	232.3	s	232.3	233.9
T24	131	131	R	175.1	s	175.1	175.6
T25	132	142	DGSGTLSSFFK	1145.6	d	573.3	574.2
T26	143	145	MGK	335.2	s	335.2	335
					d	168.1	168
T27	144	144	K	147.2	s	147.2	147.7
T28	145	153	GEGSPAR	673.4	d	337.2	337.4
T27 + T28	144	153	KGEGSPAR	801.9	d	401.4	402.8
T29	154	154	R	175.1	s	175.1	175.6

* —16 amu difference indicates deamidation of residue Q11.

gic encephalomyelitis, EAE, in guinea pigs and Lewis rats. Spiny dogfish MBP has also been reported, by Agrawal et al. (32), not to induce EAE in guinea pigs. Mixtures of whole brain and spinal cord from turtles, snakes and frogs were found by Patterson (33) to be ineffective in inducing EAE. These observations are in contrast to the report by Levine and Wenk (34) who reported that the chicken, turtle, and frog but not carp MBP were effective in inducing EAE in Wistar rats. The question then arises as to whether the ability to induce EAE is largely influenced by the primary structure or by the posttranslational modifications which can influence the secondary and tertiary structure thereby affecting the antigenicity of the protein. The role played in human MBP by phosphorylation and the conversion of arginine to citrulline in the induction of Multiple Sclerosis has been discussed by Moscarello (35). In view of the proposed role of posttranslational modifications in the induction of EAE and Multiple Sclerosis it is important to document the presence of posttranslational modifications in the MBP from non-mammalian animals and to identify in-vivo modified sites in each of the charge isomers from the MBP of non-mammalian species.

Table IV. Summary of Phosphorylated Sites in Charge Isomers C1, C2, and C3

Serine 72 is phosphorylated in components C1, C2, and C3
Serine 83 is phosphorylated in components C1, C2, and C3
Serine 120 or 121 is phosphorylated in components C1 and C3 not C2
Serines 134, 138 and 139 contain two or three phosphate groups in component C2 only.

Our demonstration of the presence of a lysine residue in place of a glutamine residue generally found at this location in the primary structure of mammalian MBP is strongly supported by the mass spectral results. Tryptic cleavage of the peptide containing residues 19–38 into two peptides consisting of residues 19–30 and 31–38 can only be explained by the presence of a lysine residue and not a glutamine residue at residue 30 in the amino acid sequence. Since the codons for lysine and glutamine differ by only a single base, the misreading of a C instead of an A would explain this sequence error.

The loss of 17 Da in peptides T2 and T3 of components C1 and C2 is consistent with the formation of a pyroglutamate residue from glutamine at residue 11. The theoretical m/z value for T2, QAGGAHSR, is 783.8 Da. This m/z peak has never been observed in components C1, C2. A peak with $[M + H]^+$ of 766.6 is consistent with the presence of a pyroglutamine residue since the theoretical average $[M + H]^+$ for Q_{pyro}AGGAHSR is 766.8 indicating the presence of the pyroglutamic acid residue.

The absence of an unmodified charge isomer component can be considered within the framework of the hypothesis that the normal C1 component is essential for tight wrapping of the membrane around the axon (36). It follows from this hypothesis that it is reasonable to expect that the wrappings of the membrane in myelin sheaths with a deficiency or lack of unmodified MBP, will be less compact than for those wrappings where the amount of unmodified component is greater than the modified components.

This inference may be relevant to the demyelination observed in multiple sclerosis where the amount of MBP component C1 is reportedly reduced relative to component C8. This situation has been reported to prevail in human myelin from two children, a 2 month and a 2 year old (20). A possible rationale for the variation in charge isomers in Multiple Sclerosis is that the system recognizes it is defective and is attempting to generate new myelin membrane wrappings with the proper components of charge isomers C1 to C8.

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