CHARGE MICROHETEROGENEITY OF MYELIN BASIC PROTEIN IN MURINE BRAIN

by

Anthony Edward Palma

A Thesis submitted in conformity with the requirements for the Degree of Master of Science, Graduate Department of Clinical Biochemistry, in the University of Toronto

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ABSTRACT

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In murine brain, myelin basic protein represents a highly heterogeneous family of proteins consisting of several isoforms and charge isomers (or components). On alkaline-urea slab gels, murine MBP resolved into two major groups of isomers: the 18.5 (slow migrating group) and 14 kDa (fast migrating group) isoforms. Following isolation by CM-52 cation exchange chromatography, individual murine MBP components were characterized. Component 1 (C-1), the most cationic isomer, had a molecular weight of 14 140.38 ± 0.79 amu. C-2 consisted of two 14 kDa species: 14 136.37 ± 0.74 and 14 204.45 ± 0.70 amu. Species with molecular weights of 14 215.57 ± 0.94 and 18 413.57 ± 0.76 amu constituted C-3. The 14 and 18.5 kDa isoforms were also present in C-4, C-5 and C-8 (the least cationic isomer).

Over the course of development, C-8 levels decreased whereas levels corresponding to the most cationic isomers for both the 14 and 18.5 kDa isoforms increased. These findings suggested that an increase in the production of the most cationic isomers is necessary for maturation of the myelin membrane.

MBP isolated from the brains of natural mutant and transgenic mice was also characterized. The dysmyelinating shiverer mouse which carries a deletion of exons 3 to 7 in the MBP gene was unable to synthesize the major MBP isoforms. However, characterization of high-molecular weight proteins immunoreactive with anti-MBP antibodies suggested that golli-mbp may be present in shiverer brain. Studies on the transgenic shiverer mouse, which was generated by incorporation of a wild-type MBP transgene onto a shiverer background, demonstrated that (1) the 18.5 kDa isoform and corresponding isomers were the major MBP species and (2) charge microheterogeneity was similar to that of an early myelinating animal.
ACKNOWLEDGEMENTS

First off, I must thank Dr. Moscarello whose guidance and patience has made me realize the value of knowledge and life.

To Denise and Teresa.

Thanks for the laughter...even when nothing is being said.

To Baldwin, Laura, Phil and Il Sang.

After going through so much together, we have created a friendship that can never be broken.


To Pankaj,

Thanks "little bro." for being there every step of the way.

To Cecilia and Christina.

Thanks for putting up with me all these years. Love ya' guys.

To Michelle.

Your unending love, friendship and support has given me the strength to take on any challenge. Thank you.

Most importantly, I dedicate this thesis to my loving mother and father.
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LIST OF ABBREVIATIONS

C-1. C-2 etc. MBP component 1. 2. etc.
CM-52 carboxymethyl cellulose
ddH₂O double distilled water
DM-20 alternative splice product of the primary PLP transcript
ECL enhanced chemi luminescence
EDTA ethylenediaminetetraacetic acid
ESI-MS electrospray ionization mass spectrometry
golli-mbp gene that is expressed in the oligodendrocyte linkage and carries the MBP gene
HMW high molecular weight
HPLC high pressure liquid chromatography
IgG immunoglobulin G
IPL intraperiod line of myelin
kDa kiloDalton
mAb monoclonal antibody
MBP myelin basic protein
MDL major dense line of myelin
m/z mass over charge ratio
O.D. optical density
PC phosphatidylcholine
PITC phenylisothiocyanate
PLP proteolipid protein
PS phosphatidylserine
PTH phenythiohydantoin
SDS sodium dodecyl sulphate
SDS-PAGE sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SSC sodium citrate/sodium chloride
TBS Tris-HCl buffered saline
TEMED N,N,N',N'-tetramethylethylenediamine
CHAPTER 1

INTRODUCTION

The myelin sheath is a multilamellar structure wrapped around axons of the central and peripheral nervous systems (CNS and PNS, respectively). The specialized system originates from a continuous extension of the plasma membrane of myelin-producing glial cells: oligodendrocytes in the CNS and Schwann cells in the PNS. Cytoplasm-filled processes which range from 30 to 50 per cell in the CNS (Peters and Vaughn, 1970) extend from the oligodendroglial cell body or soma to form internodal myelin sheaths around adjacent axons (Figure 1.1.A). After deposition of a single layer of membrane, continuous enwrapment ensues. Cytoplasmic surfaces of the oligodendroglial plasma membrane come together to form the major dense line (MDL) whereas the apposition of the extracellular membrane surfaces give rise to the intraperiod line (IPL) (Figure 1.1.B). As determined by electron microscopy, an alternating array of electron dense lines corresponding to the MDL and IPL, with a periodicity of 10.5 to 11.5 nm (distance between two adjacent MDLs), characterizes the structure of mature myelin (Figure 1.1.C).

The high-resistance, low-capacitance sheath acts as an effective insulator and mainly functions as a facilitator for the transmission of nerve impulses along axons. Currents associated with the nerve impulses flow through the membrane at intervals where the sheath is interrupted by non-myelinated regions (nodes of Ranvier). Impulses undergo a process referred to as saltatory conduction, "jumping" of impulses from one node to the next. As a result, conduction velocity of the impulse is increased. Although myelin has been historically classified as a static insulator, the discovery of numerous enzymes within the multilamellar structure suggest myelin to also possess a dynamic function (Ledeen, 1992).
The process of myelin formation mainly consists of three stages: early myelination (from birth to post natal day 10), active myelination (day 10 to 25) and myelin maturation (≥ day 25). In the murine brain, the first stage involves proliferation of oligodendrocytes, cell enlargement, plasma membrane formation and histologically, mature myelin is relatively absent (Davison and Dobbing, 1968). The active myelination phase is characterized by increased amounts of membrane in which the maximum rate of deposition occurs at around post natal day 20 in rat brain (Norton and Poduslo, 1973a). From electron microscopic evidence, it was suggested that immature myelin is wound rather loosely around axons of the CNS while as myelin maturation proceeds, the degree of membrane compaction is increased (Peters et al, 1970).

The composition of myelin, characterized by high lipid and low protein levels, renders the sheath very distinct from other plasma membranes which generally have equal proportions of these membrane constituents. On a dry weight basis, a 2.3 to 1 ratio of lipid to protein was determined in myelin (see Table 1.1: Norton and Cammer, 1984). Myelin lipid content consists mainly of glycolipids, phospholipids and cholesterol. These structural elements in conjunction with the two major proteins, myelin basic proteins and proteolipid proteins, which together constitute approximately 85 % of the total myelin protein content, function in the structural organization of the myelin membrane.

Figure 1.1 (A) Schematic diagram of an oligodendrocyte and adjacent axons in the central nervous system (taken from Brophy et al, 1993) (B) Diagram of an oligodendrocyte enwrapping an axon with a single myelin membrane. Apposition of the cytoplasmic membrane surfaces and the extracellular membrane surfaces of the oligodendrocyte give rise to the major dense line and intraperiod line, respectively. (taken from Raine, 1984) (C) An electron micrograph of a myelinated axon. (taken from Stryer, 1988). Please see following page.
Table 1.1 Composition of central nervous system myelin and brain.

<table>
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<th>Substance</th>
<th>Myelin</th>
<th>White Matter</th>
<th>Whole Brain</th>
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<tr>
<td></td>
<td>Human</td>
<td>Bovine</td>
<td>Rat</td>
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<tr>
<td>Total protein(^b)</td>
<td>30.0</td>
<td>24.7</td>
<td>29.5</td>
</tr>
<tr>
<td>Total lipid(^b)</td>
<td>70.0</td>
<td>75.3</td>
<td>70.5</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>27.7</td>
<td>28.1</td>
<td>27.3</td>
</tr>
<tr>
<td>Total galactolipid</td>
<td>27.5</td>
<td>29.3</td>
<td>31.5</td>
</tr>
<tr>
<td>cerebroside</td>
<td>22.7</td>
<td>24.0</td>
<td>23.7</td>
</tr>
<tr>
<td>sulphatide</td>
<td>3.8</td>
<td>3.6</td>
<td>7.1</td>
</tr>
<tr>
<td>Total phospholipid</td>
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<td>46.0</td>
<td>44.0</td>
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<tr>
<td>phosphatidylethanolamine</td>
<td>15.6</td>
<td>17.4</td>
<td>16.7</td>
</tr>
<tr>
<td>phosphatidylcholine</td>
<td>11.2</td>
<td>10.9</td>
<td>11.3</td>
</tr>
<tr>
<td>phosphatidyserine</td>
<td>4.8</td>
<td>6.5</td>
<td>7.0</td>
</tr>
<tr>
<td>phosphatidyinositol</td>
<td>0.6</td>
<td>0.8</td>
<td>1.2</td>
</tr>
<tr>
<td>sphingomyelin</td>
<td>7.9</td>
<td>7.1</td>
<td>3.2</td>
</tr>
<tr>
<td>plasmalogen</td>
<td>12.3</td>
<td>14.1</td>
<td>14.1</td>
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(taken from Norton and Cammer, 1984)

a All values were taken from adult tissue.
b Protein and lipid values are in percentage dry weight; all others are in percentage total lipid weight.

**LIPIDS IN MYELIN**

The high concentration of lipid makes myelin more buoyant than other membranes, a property that facilitates its isolation by discontinuous sucrose gradient centrifugation (Norton and Poduslo, 1973a, 1973b; Zimmerman et al, 1975; Quarles, 1980; Pereyra and Braun, 1983; Cruz and Moscarello, 1985). In 1973, Norton and Poduslo (1973a) used this technique to isolate myelin in order to study the lipid composition in the myelin of developing rat brain. They were able to show the lipid content of the myelin isolated from brains of early myelinating animals to be different in
composition from that of mature animals (Norton and Poduslo, 1973a). In Table 1.2 (taken from Norton and Poduslo, 1973a), the mole ratios of lipids isolated from rat myelin during development reflect these changes. As the animal matures from 15 to 425 days, galactolipids, such as cerebrosides and sulphatides, increased from 39 to 53%. Phosphatidylcholine (PC) decreased from 32 to 20% over the same period. Total sterol and other lipid levels remained relatively constant although a slight decrease in desmesterol levels was reported (Smith et al. 1967; Norton and Poduslo, 1973a). By day 60 no further changes were observed suggesting that myelin had reached full maturity.

Table 1.2 Mole ratios of myelin lipids isolated from myelin of rat brain during development Taken from Norton and Poduslo (1973a).

<table>
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<tr>
<th>Age (days)</th>
<th>15</th>
<th>20</th>
<th>30</th>
<th>60</th>
<th>144</th>
<th>190</th>
<th>425</th>
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<tr>
<td>Sterol</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Total galactolipid (GL)</td>
<td>39</td>
<td>42</td>
<td>47</td>
<td>54</td>
<td>55</td>
<td>50</td>
<td>53</td>
</tr>
<tr>
<td>cerebroside</td>
<td>31</td>
<td>33</td>
<td>41</td>
<td>43</td>
<td>39</td>
<td>40</td>
<td>39</td>
</tr>
<tr>
<td>sulphatide</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>11</td>
<td>10</td>
<td>11</td>
<td>12</td>
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<tr>
<td>Total phospholipid (PL)</td>
<td>102</td>
<td>96</td>
<td>93</td>
<td>85</td>
<td>78</td>
<td>78</td>
<td>82</td>
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<td>ethanolaminephosphatides (PE)</td>
<td>40</td>
<td>37</td>
<td>36</td>
<td>33</td>
<td>29</td>
<td>32</td>
<td>34</td>
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<tr>
<td>phosphatidylcholine (PC)</td>
<td>32</td>
<td>29</td>
<td>25</td>
<td>22</td>
<td>19</td>
<td>18</td>
<td>20</td>
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<tr>
<td>sphingomyelin</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>phosphatidylinositol (PI)</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Phosphatidylserine (PS)</td>
<td>13</td>
<td>13</td>
<td>14</td>
<td>12</td>
<td>11</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>plasmalogen (Plas)</td>
<td>29</td>
<td>27</td>
<td>28</td>
<td>30</td>
<td>27</td>
<td>26</td>
<td>28</td>
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Localization of individual classes of lipid in the myelin has been difficult. However, it has been concluded that they are asymmetrically distributed across the bilayer. Cholesterol appears to be asymmetrically distributed with relatively more in the IPL (outer leaflet) of the bilayer than in the MDL in an approximate ratio of 2 to 1 as determined by X-ray diffraction studies (Caspar and Kirschner, 1971). Cerebrosides have been localized mostly to the outer leaflet of the bilayer (Linnington and Rumsby,
1980: Raine et al. 1981), i.e. at the IPL. The ethanolamine-containing plasmalogens were exclusively localized to the inner leaflet (Kirschner and Ganser, 1982), i.e. at the MDL. with acidic phospholipids (Brophy, 1992).

PROTEINS IN MYELIN

Myelin proteins, accounting for 30% of myelin on a dry weight basis (Norton and Poduslo, 1973b), are represented by four major families. The integral membrane proteins, myelin proteolipid proteins (PLP and DM-20), account for 50% of the total. Myelin basic proteins (MBPs), constituting 35% of myelin proteins, are the major peripheral membrane proteins. 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) and myelin associated glycoprotein (MAG) represent 4 and 1% of the total proteins, respectively. Enzymes associated with myelin constitute the remainder of the proteins (10%). The following sections will provide some background concerning the two major myelin proteins, PLP and MBP.

I. Proteolipid Proteins

The proteolipids, a family of organic-soluble intrinsic membrane proteins (Folch and Lees, 1951), account for approximately 50% of the total myelin protein in the CNS (Eng et al., 1968; Norton and Poduslo, 1973a). The proteolipid proteins consist of two species, PLP and DM-20. PLP is encoded by the PLP gene that has been mapped to the X-chromosome (Willard and Riordan, 1985). DM-20 arises from the translation of alternatively spliced products of the primary PLP transcript (see below for more information). Expression of the PLP gene is oligodendrocyte-specific and is tightly regulated during brain development (Lees and Brostoff, 1984; Nave and Milner, 1989).
The synthesis of PLP and DM-20 occurs on membrane-bound ribosomes which is expected for the synthesis of integral membrane proteins (Colman et al. 1982).

Figure 1.2 PLP gene structure and its gene products (adapted from Campagnoni. 1988)

PLP

PLP is a 276 amino acid polypeptide with an apparent molecular weight of 30 000 Da (Lees et al. 1983; Stoffel et al. 1983). The structure of the 17 kb PLP gene, which contains 7 exons (Figure 1.2), is similar between mouse and human (Diehl et al. 1986; Macklin et al. 1987a). Transcription of the PLP gene gives rise to three PLP mRNAs (3.2, 2.4 and 1.6 kb) which arise from three different polyadenylation signals in the 3'-noncoding region of exon 7 (Milner et al. 1985; Gardinier et al. 1986; Macklin et al. 1987a). Alternative splicing of these transcripts gives rise to mRNA species corresponding to the smaller proteolipid. DM-20 (see below; Nave et al. 1987a; Simons et al. 1987).
The amino acid composition of PLP (19 basic residues and 25 acidic residues) accounts for an isoelectric point greater than 9 (Draper et al., 1978). The hydrophobic nature of this integral membrane protein is primarily due to an abundance of apolar residues which account for 60% of the total amino acid composition (Lees and Brostoff, 1984). Covalently linked fatty acyl chains which account for 2% by weight contribute further to the hydrophobic properties of PLP (Gagnon et al., 1971; Stoffyn and Folch, 1971).

PLP appears to have some structural role in the myelin sheath. In the studies by Duncan et al. (1987) on the myelin-deficient rat, loosely wrapped myelin displayed a defective IPL which is formed by the apposition of the extracellular membrane surfaces. From immunocytochemical studies, the absence of PLP was demonstrated suggesting a role for PLP in IPL formation (Duncan et al., 1987). The role of PLP in IPL formation has been further supported by studies on transgenic "knock-out" mice in which the expression of the PLP gene was disrupted (Boison and Stoffel, 1994). In these mice, the PLP gene carried a deletion in exon 3, analogous to DM-20, and a neo cassette in reverse orientation in intron 3. It is the antisense integration of the neo cassette that disrupts or "knocks-out" the expression of the PLP gene. From electron microscopic studies, the myelin in the brains of these "knock-out" mice lacked an IPL suggesting a potential role for PLP in IPL formation (Boison and Stoffel, 1994). However, a follow-up study on the same PLP-null mutant demonstrated that spinal cord-specific myelin revealed compact myelin with few regions of defective MDL and IPL as determined by electron microscopy (Rosenbluth et al., 1996). This study suggested that PLP may not be necessary for MDL or IPL formation but may function in the stabilization of the compact myelin structure.

Although PLP may have a structural role, detection of ion fluxes and conductivity following the incorporation of PLP into reconstituted lipid membrane systems suggested that PLP may function as an ion channel (Ting-Beall et al., 1979; Lin
and Lees. 1982; Helynck et al. 1983; de Cozar et al. 1987). However, the significance of PLP in vivo as an ion channel has not yet been demonstrated.

**DM-20**

The smaller proteolipid protein, DM-20, first identified by Agrawai et al (1972), has an apparent molecular weight of 21 000 Da. The DM-20 transcript is an alternatively spliced product of the primary PLP transcript (Figure 1.2), resulting from the excision of exon 3b of the primary PLP transcript (Nave et al. 1987a; Simons et al. 1987). Cloning of the DM-20 cDNA confirmed that DM-20 is related to PLP through alternative splicing events and that the DM-20 transcript bears a 105 kb deletion in its coding region (Macklin et al. 1987a). The absence of exon 3b which encodes for a highly hydrophilic domain (Val-116 to Lys-150) increases the hydrophobicity of DM-20 in comparison to PLP.

**Developmental Expression**

The relative proportions of PLP and DM-20 expression differ throughout development. Transcripts of DM-20 were more abundant than those of PLP in murine embryos (Ikenaka et al. 1992; Timsit et al. 1992), in bovine fetuses (Van Dorsselaer et al. 1988) and human fetal spinal cord (Kronquist et al. 1987). At all post-natal ages, PLP transcript levels were higher than that of DM-20 and with development, a significant increase in the PLP:DM-20 mRNA ratio was observed (Levine et al. 1990).

Before the onset of myelination, DM-20 was more abundant than PLP in very young animals (Gardinier and Macklin. 1988). PLP was the prominent proteolipid species during later stages of CNS myelination (Lees and Brostoff, 1984). Further studies are required to understand the requirement for the more hydrophobic proteolipid, DM-20, during early stages of myelination and a need for the more polar protein, PLP, during later stages.
PLP/DM20 Mouse Models

Dysmyelinating mutants serve as tools for studying the roles of protein and lipid in myelin development and structure. Dysmyelination, a term used to describe the absence of myelin formation, has been reportedly found in rodents carrying lesions in either the PLP or MBP gene (Hogan and Greenfield, 1984). Although dysmyelinating mutants have been studied extensively, the roles of myelin constituents remain unclear. In the following sections, a brief summary of some dysmyelinating PLP-mutants will be provided.

The X-chromosome-linked jimpy mutation in mouse results in severe dysmyelination which is accompanied by tremors, seizures, premature death by 3 to 4 weeks of age (Sidman et al. 1964) and prominent oligodendroglial cell death (Knapp et al. 1986). In the jimpy mouse, the genetic lesion has been described as a base change (A to G) in the 3'-end of intron 4 of the PLP gene (Nave et al. 1987b; Macklin et al. 1987b). This mutation leads to the aberrant splicing of 74 nucleotides which includes exon 5 of the PLP gene (Nave et al. 1987b; Macklin et al. 1987b; Hudson et al., 1987). Consequently, the deletion introduces a frameshift into the jimpy PLP gene which encodes a protein with a truncated C-terminal domain enriched in cysteinyl residues (Nave et al. 1986). From immunohistochemical localization studies, PLP in jimpy mouse brains has been detected in the rough endoplasmic reticulum (RER) of jimpy oligodendrocytes but not in the Golgi complex (Roussel et al. 1987) suggesting that transport of PLP from the RER to the Golgi was inhibited. If PLP was inhibited in jimpy oligodendrocytes, a consequential accumulation of PLP in the RER may be deleterious to the cell and may be the event leading to cell death in jimpy brain.

In 1995, Schneider et al (1995) attempted to rescue the jimpy mouse from dysmyelination by introducing a wild-type PLP transgene into its genome. Unfortunately, the rescue attempt was unsuccessful. The transgenic mice developed tremors, seizures and premature death by 3 to 4 weeks. From electron microscopic
studies. dysmyelination was prominent in the brains of these animals although some myelin sheaths were rarely found. However, oligodendroglial cell death in transgenic brain was reduced approximately 50% to that found in jimpy brain. From immunocytochemical studies, PLP was localized to existing myelin sheaths suggesting that the expression of the PLP transgene, having undergone normal synthesis, transport and assembly into myelin, was not sufficient to eliminate cell death. Thus, in this study, the existing cell death was attributed to the expression of the truncated jimpy PLP although no evidence was provided.

The myelin synthesis deficient jimpy (jp-msd) mouse is allelic to jimpy. Severe dysmyelination in these mice is caused by a mutation in the PLP gene defined by an amino acid substitution (Ala-242 to Val) (Gencic and Hudson, 1990). Decreased levels of mature oligodendrocytes in the jp-msd mutant suggested that dysmyelination resulted from oligodendrocyte death (Wolf et al. 1983).

The rumpshaker mutation, an amino acid substitution (Ile-186 to Thr) in PLP (Schneider et al. 1992), gives rise to a dysmyelinating phenotype similar to jimpy. However, the dysmyelination was not caused by oligodendrocyte cell death (Knapp et al. 1986). Although myelin deficient, the oligodendrocytes appear normal in the rumpshaker mice (Fanarraga et al. 1992). Therefore, certain mutations in the PLP gene can lead to dysmyelination in the absence of premature oligodendrocyte death suggesting that dysmyelination in the jimpy mouse may be due to other factors apart from oligodendrocyte cell death.

**PLP and DM-20 transgenic mouse models**

Transgenic mice carrying the whole PLP genome (Readhead et al. 1994; Kagawa et al. 1994), PLP cDNA (Nadon et al. 1994) or DM-20 cDNA (Mastronardi et al. 1993; Nadon et al. 1994; Simons-Johnson et al. 1995) were produced to study the function of PLP gene products. Introduction of the whole PLP genome into mice resulted in a
dysmyelinating phenotype (Readhead et al., 1994; Kagawa et al., 1994). The incorporation of both PLP and DM-20 cDNAs into the genome of jimpy mice did not eliminate jimpy-like symptoms (tremors, seizures and premature death by day 21) and was not sufficient for normal myelin formation (ie. defective IPLs) (Nadon et al., 1994).

In our institution, transgenic animals were generated by incorporating a human DM-20 cDNA into the genome of normal mice. Lines carrying 2 to 70 copies of the DM-20 transgene were produced (Simons-Johnson et al., 1995). The most interesting transgenic line, referred to as the ND4 mouse, carried 70 copies and has been well-characterized by Mastronardi et al. (1993). Following a 3 month period of normal growth, the ND4 mouse developed tremors and seizures. Death occurred by 8 to 10 months. Morphological analysis demonstrated normally compacted myelin up to 3 months. Spontaneous breakdown of the membrane, termed demyelination, ensued. The demyelination was characterized by the following criteria: myelin loss, astrogliosis, lymphocytic infiltration. myelin breakdown accompanied by sparing of axons and myelin debris contained within astrocytes.

For a number of years, the experimental allergic encephalomyelitis or EAE mouse has been the model for the study of demyelination (Alvord et al., 1992). In the EAE mouse, demyelination is induced following the introduction of antigen. Because demyelination in the ND4 mouse does not require the introduction of antigen, the ND4 mouse may be used as a better tool for studying the role of myelin protein and lipid before the onset and during demyelination.

Characterization of PLPs and MBPs in the ND4 mouse was performed in order to understand how they were affected by the transgene (Mastronardi et al., 1996). By Western immunoblot analysis, a 5-fold increase in DM-20 was detected in compact myelin isolated from ND4 mouse brain (10 to 15 months) in comparison to age-matched controls (Mastronardi et al., 1996). A similar increase in DM-20 transcript levels (4.4-fold) was determined following Northern blot analysis of total brain RNA (Simons-
Johnson et al, 1995). The amount of PLP in myelin isolated from ND4 mouse brain (10 to 15 months) was 1/2 of that in normal (Mastronardi et al. 1996).

The effects of DM-20 transgene levels on MBP were not evident at the transcript and translation levels. The pattern of total MBP mRNA expression during development in the ND4 brain was similar to normal. Translation of these transcripts gave rise to the major MBP isoforms (14, 17, 18.5 and 21.5 kDa: see section on MBP isoforms for more information). However, aberrant patterns of MBP charge microheterogeneity, a product of post-translational modification (see section on MBP isomers), was demonstrated by alkaline-urea gel electrophoresis. In general, the overall cationic nature of the MBP isolated from ND4 mouse brains was altered. With increasing age (2, 5 and 10 months), an increase in less cationic forms of MBP compared to normal was observed suggesting that a decrease in the cationicity of MBP was occurring. The ability of this MBP isolated from the ND4 mouse brain to promote aggregation of vesicles composed of acidic lipids was less effective than normal MBP, a reflection of the decreased positive charge (Mastronardi et al, 1996). A similar change (ie. a shift to less cationic MBP) was reported in victims of multiple sclerosis, a human demyelinating disease (Moscarello et al. 1994), suggesting that the mechanisms of demyelination in the ND4 was similar in this respect at least to that found in the human disease.

II. Myelin Basic Proteins

Myelin basic proteins (MBPs), originally known as the basic proteins of myelin (Kies, 1965), the encephalitogenic or the A1 proteins (Eylar et al, 1971), are peripheral membrane proteins. The highly cationic nature of MBP results from the presence of a large number of basic residues which constitute 18 % of the total amino acid composition, yielding an isoelectric point of more than 10.5 (12 lysines and 19 arginines in the 170 amino acids of 18.5 kDa human MBP) (Carnegie, 1971). The primary sequence of MBP is highly conserved among species (Figure 1.3; Stoner, 1990).
Figure 1.3 Primary sequences of 18.5 kDa human MBP along with other MBP sequences from nine other species (taken from Stoner, 1990).
Accounting for 35% of the total proteins, MBP has been suggested to function in the formation of the MDL (apposition of the cytoplasmic membrane surfaces). This postulated role of MBP was supported by immunolocalization of MBP to the cytoplasmic spaces of myelin as determined by electron microscopy (Herndon et al. 1973; Omlin et al. 1982). In addition, the absence of the MDL in the myelin of shiverer mutant mice (Privat et al. 1979) was related to the absence of MBP (Roach et al. 1985) suggesting that MBP was involved in MDL formation.

However, Dickinson et al. (1970) localized MBP indirectly to the IPL after observing alterations in the IPL in isolated myelin, using electron microscopy, before and after post-mortem loss of MBP. In addition, Mendell and Whitaker (1978) also localized basic protein to the IPL by immunocytochemistry. Finally, a recent study demonstrated that a less cationic form of MBP characterized by the presence of citrullinyl residues (see MBP isomers) localized to the IPL (McLaurin et al. 1993) suggesting that MBP is found in both the MDL and IPL.

**Isoforms of MBP**

In murine brain, MBP is represented by several isoforms (14, 17, 18.5 and 21.5 kDa) that are encoded by four MBP mRNA species (de Ferra et al. 1985; Takahashi et al. 1985) (Figure 1.4). The MBP transcripts are encoded by a single 30 kb MBP gene (consisting of 7 exons) (de Ferra et al. 1985; Takahashi et al. 1985) which has been mapped to chromosome 18 in both mouse (Roach et al. 1985) and human (Saxe et al. 1985; Sparkes et al. 1987).

In human, differential splicing of exons 2 and/or 5 resulted in the 17, 18.5, 20.5 and 21.5 kDa isoforms (Kamholz et al. 1986; Roth et al. 1987). In mouse, alternative splicing of exons 2, 5 and/or 6 results in five variants (14, 17.22, 17.26, 18.5 and 21.5 kDa) (de Ferra et al, 1985; Takahashi et al, 1985). The major MBP isoform in adult mice is the 14 kDa isoform representing approximately 70% of the MBP in 60 day old mouse
brains (Barbarese et al. 1978). The transcript encoding the 14 kDa isoform lacks both exons 2 and 6. Approximately 20% of the MBP isoform pool is represented by the 18.5 kDa species: exon 2 is spliced out. The 21.5 kDa which constitutes 2% of the total isoform population contains all seven exons. The 17 kDa isoforms, which are indistinguishable on SDS-PAGE, together represent 7% of the total MBP. One 17 kDa isoform is encoded by the MBP transcript lacking both exons 2 and 5 while in the other, exon 6 was spliced out.

Figure 1.4  The MBP gene and its gene products (adapted from Campagnoni. 1988).
In addition to the major MBP isoforms (14 to 21.5 kDa), MBP-related proteins possessing molecular weights of 34, 30, 29 and 26 kDa were detected by in vitro translation of mRNA isolated from mouse brain (Carson et al., 1983). By Western immunoblot analysis, these molecules were detected in brain homogenates of various ages (Carson et al., 1983). The 34 and 30 kDa MBP-related proteins were detected from birth to day 5 while the 29 and 26 kDa polypeptides appeared from day 25 to 60. Minor isoforms that utilize novel exons (exons 0, 1a and 5a) have also been reported (Kitamura et al., 1990; Aruga et al., 1991). Recently, reverse transcription-PCR amplification of transcripts isolated from mouse brain have shown that novel MBP isoforms primarily characterized by the splicing of exon 5 were predominantly expressed during the embryonic stage (Nakajima et al., 1993). The novel isoforms, encoded by the classic 7 exon-MBP gene, were proposed to have apparent molecular weights of 20 (minus exon 5), 15.6 (minus exons 5 and 6) and 12-13 kDa (minus exons 2, 5 and 6).

Recently, the MBP gene was shown to be part of a larger gene complex referred to as the golli-MBP gene which is 105 kb in length (Campagnoni et al., 1993). The name golli-mbp is an acronym for the gene expressed in the oligodendrocyte lineage in the brain and that it encompasses the MBP gene. The golli-MBP gene consists of 11 exons in which the final 7 exons correspond to the classic MBP gene (Figure 1.5). Three novel transcripts isolated from post-natal mouse brains were identified by Northern blot. The major mRNA species was represented by BG21 (5.1 kb) whereas the minor forms migrated as 2.6 (J37) and 2.3 kb (TP8) transcripts (Figure 1.5, Campagnoni et al., 1993). The 3 leading exons of the golli-MBP gene were common for each of the golli transcripts. The J37 species lacked exons 4, 6, 9 and 10 of the golli primary transcript;
Figure 1.5  The Golli-MBP gene carrying the classical 7 exon-MBP gene and its gene products. (adapted from Campagnoni et al, 1993)
the latter 3 exons corresponded to exons 2, 5 and 6 of the classic MBP gene, respectively. Exons 4 and 6 to 11 (the latter corresponding to exons 2 to 7 of the MBP gene) were missing in the BG21 transcript. The MBP sequences in the BG21 and J37 transcripts were in-frame following alternative splicing of the primary golli-MBP transcript. (Figure 1.6: Campagnoni et al. 1993). The intact MBP sequences were confirmed by positive immunoreactivity of recombinant BG21 and J37 with a golli-specific antibody and an antibody reactive with bovine MBP (Landry et al. 1996). In 14 and 28 day mouse brains, the major reactive species was the 31 kDa BG21 golli protein as determined by Western blot analysis of brain homogenates (Landry et al. 1996). However, the 35 kDa J37 golli protein, which is reactive with the anti-ovine MBP antibody, was not detected. Interestingly, the BG21 golli protein which lacks exons 2 to 7 of the classic MBP gene was present in postnatal day 16 shiverer brain (Landry et al. 1996). the mouse mutant that carries a MBP gene bearing a deletion of exons 3 to 7 (Roach et al. 1985). The oligodendrocyte-specific expression of golli-mbp suggests that these novel species may function in the differentiation of oligodendrocytes (Campagnoni et al. 1993). Recent evidence has shown that golli transcripts and proteins localize also to neuronal structures in the brain as determined by in situ hybridization and immunocytochemistry (Landry et al. 1996). However, the role of the golli proteins in neuronal axons and dendrites remains unknown.

**Developmental Expression of MBP**

The relative proportions of MBP isoforms changed throughout myelogenesis. Expression levels of exon 2-containing MBP isoforms (17 and 21.5 kDa) decreased from postnatal day 15 to 60 in murine brains whereas those of the major MBP isoforms, 14 and 18.5 kDa, increased (Barbarese et al. 1978). The higher levels of 17/21.5 kDa isoforms during early stages of myelination suggest that these isoforms may be required for myelin formation. Increased expression levels of the 14 and 18.5 kDa isoforms
Figure 1.6  Predicted protein sequences of golli-mbp isoforms

Note that the J37 and BG21 isoforms contain MBP protein sequences (labelled as \textit{MBP antigenic sequences.}) (taken from Campagnoni et al, 1993).
during myelin maturation are suggestive that these isoforms may be involved in membrane compaction.

Developmental regulation of MBP expression occurs also at the transcriptional level. Using probes that recognized all MBP mRNA species, two groups reported that MBP transcripts were first detected by post natal day 4 (a period of early myelination), reached maximum levels by day 18 (active myelination phase) and decreased to constant levels (25% of the maximum) in adulthood (Zeller et al. 1984; Roth et al. 1987). Unfortunately, developmental expression patterns of individual MBP mRNA species are not available, a necessary prerequisite in studying the influence of changes in the relative proportions of individual MBP transcripts during development.

Cellular distribution

Developmental changes in the distribution of MBP transcripts in the oligodendrocyte have been reported. From in situ hybridization studies, MBP transcripts were localized to oligodendrocyte cell bodies during early development (Trapp et al. 1987; Verity and Campagnoni, 1988). During later stages of myelination, regions of developing myelin sheaths revealed an increasing concentration of MBP mRNA (Trapp et al. 1987; Verity and Campagnoni, 1988; Jordan et al. 1989). In the report by Trapp et al (1987), the cellular distribution of MBP transcripts was studied in tissue sections of developing rat nervous system by in situ hybridization. In 10 day old animals, the MBP mRNA was concentrated in oligodendroglial cell bodies rather than in the cytoplasmic extensions whereas by day 35, the distribution of the transcripts was significantly increased in the processes, developing myelin sheaths. From the studies by Trapp et al (1987), in concert with the early findings of free polysomes containing MBP mRNA enriched in myelin-containing fractions (Colman et al. 1982), it was suggested that MBP mRNA was transported to and translated at sites near myelin.
Cellular distribution of MBP isoforms has also been demonstrated. In 1991, Allinquant et al (1991) determined the distribution pattern of MBP by immunofluorescence and confocal microscopy following MBP cDNA transfection into MBP-deficient shiverer oligodendrocytes. The nuclear- and cytoplasm-associated polypeptides were represented by the 17/21.5 kDa isoforms (lacking exon 2) while the exon 2-containing 14/18.5 kDa isoforms were distributed along the plasma membrane. In a follow-up study, Hardy et al (1996) demonstrated that in normal oligodendrocytes the four MBP isoforms localized to all three regions (nucleus, cytoplasm and plasma membrane) suggesting that the specific distribution patterns of isoforms observed by Allinquant et al (1991) may be influenced by factors present in the MBP-deficient shiverer oligodendrocytes. Furthermore, developmental studies on MBP cellular distribution have shown that all MBP isoforms have been detected in the nuclear and cytoplasmic regions before and during myelination (Hardy et al., 1996) suggesting that (1) translation of MBP mRNA is not restricted to the myelin sheath as suggested earlier by Colman et al (1982) and Trapp et al (1987) and (2) nuclear entry of MBP isoforms may be governed by MBP peptide sequences (ie. exon 2) or by other factors in the oligodendrocyte.

Isomers of MBP

MBP isolated from human or bovine white matter migrates as a single 18.5 kDa band on SDS-polyacrylamide gels. However, alkaline-urea gel electrophoresis at pH 10.6 can be used to further resolve the MBP into a series of components or charge isomers (Figure 1.7A) (Martenson and Gaitonde, 1969). Individual MBP components, in which adjacent components differ by a single net positive charge, are isolated using cation exchange carboxymethyl cellulose (CM-52) chromatography (Figure 1.7B). Characterization of MBP components has shown that the charge differences resulted from post-translational modifications of which about 20 are known (Figure 1.8).
Modifications of MBP identified so far include: acetylation (Carnegie, 1969; Hashim and Eylar, 1969) or heterogeneous acylation of the N-terminal alanyl residue (Moscarello et al. 1992); methylation of arginine-107 (Baldwin and Carnegie, 1971); loss of C-terminal arginine (Deibler et al. 1975); phosphorylation of seryl and threonyl residues (Deibler et al., 1975; Chou et al. 1976; Ulmer, 1988; Ramwani and Moscarello, 1990; Cosentino et al. 1994); deamidation of glutamyl or asparagyl residues (Chou et al. 1976); methionine oxidation (Cheifetz et al. 1984); GTP binding (Chan et al. 1988); deimination of arginyl residues (Lamensa and Moscarello, 1993); ADP-ribosylation (Boulias and Moscarello, 1994).

The overall net positive charge varies for individual MBP components. Component 1 or C-1, the most abundant isomer in adult brain (Moscarello et al. 1994), migrates the furthest distance on alkaline-urea gels (Figure 1.7A) indicating that C-1 is the most cationic isomer. Because of its high net positive charge, C-1 requires the highest salt concentration for elution from the CM-52 column (Figure 1.7B). Less cationic isomers (C-2, C-3, C-4, etc.) migrate with decreasing distances on alkaline-urea gels, respectively. (Figure 1.7A) and require decreasing salt concentrations, respectively, for elution from CM-52 columns (Figure 1.7B).

Defined as the least modified isomer, C-1 modifications consist of either an acetylation (Carnegie, 1969; Hashim and Eylar, 1969) or heterogeneous acylation of the N-terminus (Moscarello et al. 1992) and a mono- or dimethylation of arginine-107 (Baldwin and Carnegie, 1971). A single net charge difference on the less cationic C-2, relative to C-1, has been attributed to an additional modification; deamidation of either glutamine 103 or 147 (Chou et al, 1976). Modifications such as the phosphorylation of either Thr-98 or Ser-168 or deamidation of two glutamyl residues render C-3 less cationic than C-1 by two net positive charges (Chou et al. 1976).

C-8, the least cationic isomer, migrates the shortest distance on alkaline-urea gels (Figure 1.7A). It is important to note that C-8 does not refer to the eighth MBP
Figure 1.7  (A) Alkaline-urea gel electrophoresis of unfractionated human MBP (18.5 kDa). (B) Carboxymethyl-cellulose (CM-52) chromatography of human MBP. Unbound material contains MBP labelled as C-8A and C-8B. More cationic isomers (C-4 to C-1) were eluted from the column using an increasing salt concentration (0 to 0.2M NaCl). Protein from the column was monitored by recording O.D. at 280 nm. Salt gradient was monitored by measuring conductivity.
Figure 1.8  Post-translational modifications of MBP
component but is defined as the only component(s) that does(do) not bind to the CM-52 column (Figure 1.7.B) ie. the unbound fraction in Figure 1.7B which consists of two C-8 peaks. C-8A and C-8B. The presence of citrulline residues at positions 25, 31, 122, 130, 159 and 170 (Wood and Moscarello, 1989) is characteristic of C-8. The citrulline residues arise from the deimination of arginyl residues, a process that is catalyzed by peptidyl arginine deiminase (Lamensa and Moscarello, 1993).

**Effects of post-translational modifications on MBP**

Phosphorylation

Ever since the first discovery of protein kinase activity in myelin (Johnson et al. 1971), a number of protein kinases have been found in myelin which use MBP as a substrate: cAMP-dependent kinase, protein kinase C, calmodulin-dependent protein kinase etc. (Ulmer, 1988). Several residues in MBP have been reported to undergo phosphorylation ie. Thr-98 and Ser-165 (Chou et al. 1976), Ser-115 (Turner et al. 1982), Ser-102 (Schulz et al. 1988), Ser-7 and 12 (Ramwani and Moscarello, 1990) etc. Because phosphorylation can occur on several sites, this modification can lead to the production of several MBP charge isomers.

Recently, MBP has been shown to act as an excellent substrate to a mitogen-activated protein (MAP) kinase (Erickson et al. 1990). From mass spectrometric studies, the site of phosphorylation was identified as Thr-97 in the bovine MBP primary sequence. Thr-Pro-Arg-Thr-97-Pro-Pro-Pro. Phosphorylation at Thr-97 (Thr-98 in human MBP) has been reported earlier in vivo (Chou et al., 1976; Martenson et al., 1983). Using a monoclonal antibody reactive with phosphorylated Thr-98 (Yon et al., 1995), MBP carrying a phosphorylated Thr-98 has been localized to the major dense line
of myelin as determined by immunogold electron microscopy (Yon et al. 1996). Because phosphorylated Thr-97 has been suggested by Stoner (1984) to stabilize β-structure in MBP (see section on Model of MBP), the localization of phosphorylated MBP suggests that highly ordered MBP structure, induced by phosphorylation, may facilitate interactions with lipid at the MDL.

Deimination

Deimination of arginyl residues producing citrullinyl residues is a unique modification of MBP that has been detected in the least cationic isomer, C-8. Sequence analysis of human C-8 has shown that citrullinyl residues occur at position 25, 31, 122, 130, 159, and 170 (Wood and Moscarello, 1989). The decrease in net positive charge, attributed to the deiminated residues, has been shown to alter the ability of C-8 to interact with lipids in reconstituted membrane systems. C-8 was the least effective isomer in promoting the aggregation of vesicles containing acidic lipid (92 % PC, 7.8 % PS). The aggregation of vesicles consisting of PC and PLP was strongly promoted by C-8 but not by the more cationic counterparts (C-1, C-2, C-3 etc.). These studies, in conjunction with the immunolocalization of C-8 to the intraperiod line, a region poor in acidic phospholipids (McLaurin et al. 1993), suggested that deimination decreased the ability of MBP to interact with anionic phospholipids electrostatically.

The deiminated species of MBP, C-8, is detected early during myelination (Moscarello et al. 1994). In two year old children, the population of MBP is enriched in C-8 whereas 20 % of the MBP is represented by C-8 in the adult (Moscarello et al. 1994). In the same report, MBP isolated from the white matter of victims of multiple sclerosis was less cationic (ie. 45 % of the MBP was C-8) than that isolated from normal individuals and from individuals suffering from other neurological diseases (Moscarello et al. 1994). Recently, a study of the MBP isolated from the white matter of a victim suffering from a malignant case of multiple sclerosis (Marburg's disease) has shown that
the C-8, in which 18 of 19 arginyl residues were deiminated, was the prominent isomer (Wood et al. 1996). In the ND4 demyelinating mouse model (mentioned in earlier sections), a decrease in MBP cationicity was associated with disease progression (Mastronardi et al. 1996). From the above studies, the overall decrease in MBP cationicity (due to increased C-8 levels) appears to be important in the pathogenesis of demyelination.

N-terminal acylation

The N-terminal alanyl residue was originally reported to be blocked by an acetyl group (Carnegie. 1969: Hashim and Eylar. 1969). Recently, the N-terminal cyanogen bromide fragment of MBPs C-1 and C-8 (residues 1-21) was subjected to fast atom bombardment and electrospray ionization mass spectrometry (Moscarello et al. 1992). From these studies, it was shown that the N-terminus was not only acetylated but also acylated with carbon chain lengths ranging from 4 to 10. The 4- and 6-carbon acyl chains represented the major species. The same report confirmed the presence of heterogeneous acylation of the N-terminal alanyl residue by gas chromatography/ mass spectrometry following the derivatization of the fatty acids as pentafluorobenzyl esters (Moscarello et al. 1992).

Acylation of the N-terminal MBP peptide has been shown to alter hydrophobicity, secondary structure and post-translational modifications (Cosentino et al. 1994). An increase in the acyl chain length appears to increase the randomness of the peptide's secondary structure as determined by circular dichroism. ADP-ribosylation of arginine-9 with cholera toxin was most effective with a hexylated peptide. Phosphorylation of serine-12 was detected only when the peptide was blocked. The mole per mole ratio of phosphate to peptide increased in proportion to increasing chain length of the N-terminal acyl group.
Two other CNS proteins bearing a heterogeneously acylated N-terminus have been reported: retinal recoverin, a calcium ion-binding protein (Dizhoor et al., 1992), and the α-subunit of the G-protein rod transducin (Neubert et al., 1992; Kokame et al., 1992). The fatty acyl moieties consisted of a lauroyl (12:0) and three myristoyl fatty acids of varying degrees of saturation (14:0, 14:1 and 14:2). Both of these proteins are involved in the cGMP synthesis and hydrolysis in the retinal photo-transduction system. It had been suggested that the heterogeneous acylation influences GTPase activity and ADP-ribosylation of transducin α-subunit (Kokame et al., 1992) and binding of recoverin to photoreceptor membranes (Dizhoor et al., 1993).

Involvement of MBP in signal transduction

The presence of a number of enzymes associated with myelin suggested that the sheath functions not only as a static insulator but also as a dynamic membrane structure (Ledeen, 1992). The enzymes may be responsible for the signal transduction cascades (communication between the oligodendrocyte and myelin sheath) that may regulate the compaction of the myelin membrane during development. The involvement of MBP in a putative signal transduction cascade has been suggested by the ability of MBP to act as a G-protein by binding GTP. G-proteins have been found to bind GTP via glutaminyl residues (Dever et al., 1987) as well as being ADP-ribosylated on arginyl residues by cholera toxin (Van Dop et al., 1984; Tsai et al., 1988). GTP binding to human MBP has been shown to occur at Gln-3 (Chan et al., 1988). ADP-ribosylation of C-1 by cholera toxin has been identified at arginyl residues 9 and 54 (Boulias and Moscarello, 1994). Recently, an ADP-ribosyl transferase that was capable of ADP-ribosylating MBP was isolated from bovine brain (Yoon, 1996). The potential for MBP to be phosphorylated by a number of kinases (see section on phosphorylation) is another indication that MBP may be involved in a signal transduction cascade. Recently, the phosphorylation site on Thr-97 of MBP (Chou et al., 1976; Martenson et al., 1983) has been identified as a MAP-
kinase site (Erickson et al. 1990). Because MAP kinases are members of signal transduction cascades, phosphorylation at Thr-98 further supports the involvement of MBP in a signalling pathway.

Methylation

Methylation of MBP was first reported by Baldwin and Carnegie (1971) by primary sequence analysis. It was shown that monomethylation or dimethylation occurred at the N- and/or N'-positions on the guanidino group of arginine-107 in human MBP. The overall charge of the molecule was not affected by this modification. An MBP-specific protein methylase I was found to be the enzyme responsible for the methylation of the arginyl residue in the sequence Gly-Lys-Gly-Arg107-Gly-Leu (Ghosh et al. 1990). It has been suggested that the methyl group contributes to the hydrophobic properties of MBP and, thereby, mediates lipid-MBP interactions (Young et al. 1987). Intramolecular interactions of MBP have been suggested to stabilize secondary structure of MBP by interaction of the methylated arginyl residue with the Pro-97 (Stoner, 1984).

MBP-Lipid Interactions

Earlier studies have demonstrated the affinity of MBP for anionic lipids (e.g. PS) (Palmer and Dawson, 1969). In 1973, Demel et al (1973) reported that interaction of MBP existed with cerebroside sulphate, acidic lipids (i.e. PS) and some interaction with PC. The interaction of MBP with acidic lipids was diminished upon the addition of 1 M NaCl suggesting that the interactions were electrostatic (Demel et al, 1973). From differential scanning calorimetric studies, MBP was shown to cause lipid phase separation in a mixture of acidic and neutral lipids by preferentially binding to the polar head groups of acidic lipids (Boggs et al, 1977).

There have been strong suggestions that portions of MBP interact hydrophobically with lipids. The ability of MBP to induce leakage of glucose (Gould and
London, 1972) and sodium (Papahadjopoulos et al. 1975) trapped inside vesicles and to
decrease lipid phase transition temperatures (Papahadjopoulos et al. 1975: Boggs and
Moscarello, 1978) has been interpreted as being the consequence of the partial
penetration into and/or deformation of the acyl chain packing within the bilayer. Thus,
MBP interacts with lipid bilayers in two manners: electrostatically through its basic
residues and hydrophobically via stretches of apolar residues (Boggs et al. 1982).

Since electrostatic interactions are involved in protein-lipid interactions, changes
in net positive charge on MBP by post-translational modifications (ie. phosphorylation,
deimination, deamidation etc.) will affect the ability of MBP to interact with the
phospholipids. The effectiveness of MBP to promote aggregation of unilamellar acidic-
lipid vesicles was dependent upon a proportional increase in cationicity (C-1 > C-2 > C-4
> C-8) (Brady et al. 1981: Cheifetz and Moscarello, 1985: Wood and Moscarello,
1989). The most cationic isomer, C-1, induced the formation of "crystalline"
multilayers composed of phosphatidylglycerol with repeat distances similar to myelin as
determined by liquid X-ray diffraction (Brady et al. 1985). C-2 was less effective. C-3
was only 40% as effective as C-1. C-8 was unable to organize the bilayer. MBP isolated
from white matter of multiple sclerosis victims, which consists of the less cationic
isomers (Moscarello et al. 1986), was less effective than that of normal in the promotion
of acid lipid-vesicle aggregation and organization of multilayers (Brady et al. 1981;
Moscarello et al. 1986). Aggregation of vesicles composed of only the zwitterionic lipid,
PC, was most effective with the least cationic component (ie. C-8 > C-3 > C-2 > C-1)
(Sridhara et al. 1984: Moscarello et al. 1986). The above-mentioned studies suggest that
the manner by which MBP interacts with lipids is dependent upon the net positive charge
on the protein.
**MBP Mouse Models**

As mentioned earlier, a dysmyelinating phenotype in mice has been associated with a lesion in either the PLP or MBP gene. An earlier section focused on the effects of a mutated PLP gene on myelination in mutant mice. Lesions in the MBP gene and its influence on myelination will be presented in the following section.

The *shiverer* mouse carries a mutation in the MBP gene which decreases the amounts of myelin produced in the CNS (Biddle et al. 1973). The genetic lesion is represented by a large deletion of exons 3 to 7 in the MBP gene (Roach et al. 1985). Mice homozygous for the *shiverer* mutation develop tremors by post natal day 14, seizures by day 30 and premature death by days 50 to 100 (Cheroff et al. 1981). At the ultrastructural level, the small amounts of myelin in the *shiverer* brains were loosely compacted and lacked an MDL (Cheroff et al. 1981). Negative immunohistochemical staining of *shiverer* myelin with antibodies reactive with MBP (Dupouey et al. 1979; Privat et al. 1979), along with the studies by Cheroff et al. (1981), suggested that MBP was important for the formation of the MDL. The expression of MBP was very low at the protein level (Cammer and Zimmerman, 1983). Reports by Roach et al. (1983) revealed MBP protein and transcript levels to be below 1% of normal levels.

The expression patterns of MBP in *shiverer* myelin and brain was studied by Western blot analysis (Barbarese et al. 1983). Mice homozygous for the *shiverer* mutation were not able to synthesize any of the four major MBP isoforms (14, 17, 18.5 and 21.5 kDa) which were present in the normal and heterozygous *shiverer* mice. However, high molecular weight proteins (ranging from 25 to 100 kDa) reactive with anti-14 kDa MBP antibody were present in *shiverer* brain homogenate but not in myelin (Barbarese et al. 1983) which suggested that MBP-related proteins were being synthesized but not incorporated into myelin. These reactive species may account for the low levels of MBP that were detected by Cammer and Zimmerman (1983), Roach et al. (1983) and Dupouey et al. (1979). One of these high molecular weight species (31 kDa)
was reactive with an antibody against the golli-mbp (BG21) protein which carries exon 1 of the classical MBP gene (Landry et al., 1996). Although golli-mbp proteins have been shown to react with both anti-golli-mbp and anti-bovine MBP antibodies (Landry et al., 1996), recognition of the 31 kDa shiverer species by an anti-MBP antibody was not demonstrated.

Another dysmyelinating model is represented by the myelin deficient (mld) mouse which is allelic to the shiverer. The mutation consists of a duplication of the MBP gene in which the upstream copy carries an inversion of the segment encompassing exons 3 to 7 (Akovitz et al., 1987; Popko et al., 1988; Okano et al., 1988). The downstream copy is intact. In comparison to shiverer mice, the severity of dysmyelination in the mld is considerably less which allows for an increased lifespan (approximately 9 months) (Shen et al., 1985). From immunohistochemical studies, compact myelin sheaths were rare and contained very little MBP in postnatal 30 day mld mice (Matthieu et al., 1984).

In an attempt to rescue the shiverer mouse from dysmyelination, Readhead et al. (1987) introduced a wild-type MBP transgene, carrying all introns and exons, into the germ-line of shiverer mice by microinjection into fertilized eggs. Shiverer mice heterozygous for the transgene displayed slight shivering whereas the shivering phenotype was eliminated in homozygous mice. The developmental expression of total RNA encoded by the MBP transgene was identical to normal suggesting that the transgene carried the essential sequences for normal developmental regulation of the MBP gene. MBP transcript and protein levels in mice homozygous for the transgene were 25% of normal levels. These levels of MBP appeared to be sufficient for compaction of the myelin membrane and formation of the MDL as determined by electron microscopy. From SDS-PAGE analysis, the MBP isolated from the transgenic shiverer mouse brain contained proteins corresponding to the four major isoforms (14, 17, 18.5 and 21.5 kDa) by Coomasie blue staining. Unfortunately, a Western blot of these MBPs which would have confirmed the presence of the major isoforms was not
provided. Studies involving the characterization of the MBP at the protein level in the transgenic shiverer mice will be presented in this thesis, which are at variance with the above-mentioned studies.

In another attempt to rescue the shiverer mouse from dysmyelination, Kimura et al (1989) incorporated murine MBP cDNA encoding the 14 kDa isoform into the genome of homozygous shiverer mice. Phenotypically, the behaviour of the transgenic mice seemed normal. Tonic convulsions were absent in transgenic mice which lived for 7 months. As determined by electron microscopy, compact myelin containing a MDL was seen suggesting that a single isoform was sufficient for compaction although the necessity for other isoforms cannot be ruled out. Immunohistochemical staining of myelin confirmed the presence of MBP. However, characterization of MBP in the 14 kDa MBP transgenic shiverer mouse was not reported.

A study involving the correlation of myelination with MBP expression was performed by Shine et al (1992). From morphological analysis and Northern blot analysis of the following mouse models (normal, shiverer, shiverer-myelin deficient and Readhead's transgenic shiverer mouse (1987)), Shine et al (1992) suggested that the extent of myelination paralleled an increase in MBP transcript levels. Normal levels of myelin were produced when MBP transcript levels were at or above 50%. Unfortunately, this study did not measure the expression levels of MBP isoforms in these mice which would have addressed questions concerning whether certain isoforms are required for different stages of myelination.

**Structural Models of MBP**

Two models based on theoretical analysis of the secondary structure of MBP have been proposed. Based on analysis of the amino acid sequence of MBP, the Martenson (1981) model consisted of five domains with the potential of forming β-strands and some regions containing α-helical structure. In 1984, Stoner proposed a model for MBP
(Figure 1.9.A and B) in which five hydrophobic sequences with β-structure potential were organized into anti-parallel β-sheets (arrows. Figure 1.9.B) with a Greek-key type topology along with two α-helical domains (labelled α-1 and α-2. Figure 1.9.B). This model was successfully tested against standard predictive algorithms, against available data on β-sheet amino acid composition and against interstrand nearest-neighbour pair correlations.

The Stoner model (1984) incorporated a “hairpin loop” in the structure of MBP which consisted of a triproline sequence (residues 100 to 102) (Figure 1.9A) originally suggested by Eylar et al (1971). Stoner (1984) suggested that the hairpin loop structure was stabilized by the interaction of phosphorylated Thr-97 with Lys-105 and Arg-107. The hairpin loop was suggested to be further stabilized by the hydrophobic interaction of the methylated Arg-107 with Pro-97. From the Stoner model, the cationic side chains of basic residues (Arg-43, Arg-114, Lys-13, Lys-92, Lys-153, Lys-156) were positioned on the faces of the β-sheets to facilitate the interaction of MBP with the polar head groups of myelin phospholipids.

The predictive algorithms applied to the elucidation of bovine and human MBP structure were used to determine the structural features of murine MBP isoforms (Figure 1.9.C). The structure of the alternatively spliced isoforms of murine MBP demonstrated anti-parallel β-sheets which were oriented in a Greek-key type manner (Stoner. 1990). The presence of exon 2 in the 17 and 21.5 kDa isoforms provides an additional loop (dark arrows in Figure 1.9.C). Deletion of exon 6 in the 14 and 17 kDa isoforms removes one of the β-strands (Figure 1.9.C).

Stoner (1984) suggested that phosphorylation may stabilize β-structure of MBP. From circular dichroism studies, phosphorylation of individual MBP charge isomers (see section on MBP isomers) increased β-structure (35 to 40%) (Ramwani et al. 1989). Removal of the phosphate groups with acid phosphatase had very little effect on β-structure (Ramwani et al, 1989). A phosphorylation site (Ser-7), that was not available
to acid phosphatase was suggested to be involved in promoting β-structure (Ramwani et al. 1989).

The β-sheet formation can also be influenced by protein-lipid interactions. From circular dichroism studies, the binding of MBP to lipid vesicle composed of negatively charged phospholipids induced β-structure formation (Keniry and Smith, 1979). In a study of MBP conformation in solution and in lipid vesicles by Fourier-transform infrared spectroscopy, β-structure was detected in lipid-bound MBP (Surewicz et al., 1987).

Recently, a new model for MBP based on electron microscopic data (Beniac et al. 1997) and molecular modelling computer programs (Ridsdale et al. 1997) has been proposed. In the study by Beniac et al. (1997), electron microscopic images of purified bovine MBP C-1, adsorbed onto a lipid monolayer consisting of anionic lipids phosphatidylserine and monosialoganglioside (Gm1), demonstrated "C"-shaped particles with diameter of approximately 11 nm. Several 2-dimensional scans of the "C"-shaped particle in different orientations were compiled and used to perform 3-dimensional reconstructions of the protein. The reconstructed protein had the following dimensions: 5.5 nm (outer radius), 3.0 nm (inner radius), 15 nm (overall circumference) and 4.7 nm (height).

The structure of the new model (Ridsdale et al. 1997) was based on the dimensions of the electron microscopical reconstruction (Beniac et al. 1997) and on the conservation of the "β-sheet backbone", presented earlier by Martenson (1981) and Stoner (1984). Using molecular modelling software, Ridsdale et al. (1997) obtained atomic coordinates for MBP and localized certain sites of post-translational modification. Interestingly, the triproline region (residues 99 to 101) was exposed on the back side of the model while several sites of post-translational modification (ie. phosphorylation sites on Ser-7 and Thr-98; arginyl residues) were exposed on the outer surface. From this
model, the localization study suggests that certain residues are positioned in order to facilitate interactions between MBP and lipids in the myelin membrane.

Figure 1.9 Structural Models of MBP

(A) Stoner Model: Amino acid composition of human 18.5 kDa MBP and alignment of the β-strands (in boxed area). (B) Ribbon representation of the Stoner model for MBP. Arrows represent β-strands. Spirals represent α-helices. (C) Schematic diagram of murine isoforms in ribbon representation of the Stoner model. Numbers on ribbon structures correspond to exons of MBP. (A and B taken from Stoner, 1984; C taken from Stoner, 1990). Please see following pages.
THESIS OUTLINE

Myelin basic protein represents a family of proteins consisting of several isoforms and charge isomers. The roles of these particular MBP species in murine myelinogenesis is unclear mainly because very little is known about murine MBP. In order to gain a better understanding of the biological significance of MBP, more information concerning MBP is needed.

Our first study involved the isolation and characterization of MBP components from normal mouse brains. Two techniques (preparative CM-52 cation exchange chromatography and alkaline-urea gel electrophoresis) were scaled-down to accommodate for the small amounts of material available from a single mouse brain. Characterization of the individual components enabled us to deduce possible post-translational modifications which may be responsible for the charge microheterogeneity. The following developmental study was performed to determine whether certain MBP charge isomers were preferentially produced during certain stages of myelinogenesis in normal mouse brain. In our final study, we used the information obtained from the above-mentioned studies to interpret the effects of the genetic perturbations on MBP in the brains of the dysmyelinating shiverer, transgenic shiverer and the demyelinating ND4 transgenic mice.
CHAPTER 2
MATERIALS AND METHODS

Materials

Normal CD-1 and demyelinating ND4 mice, housed in the University of Toronto Banting Animal Facility, were used in this study. Shiverer mouse brains were supplied by the laboratory of Dr. R.A. Lazzarini, Mt. Sinai Hospital, New York (courtesy of Dr. F. Mastronardi). Transgenic shiverer brains were gifts from the laboratory of Dr. C. Readhead, UCLA, California.

Chloroform, methanol, acetone, sulfuric acid, dialysis tubing were purchased from Fisher. Acrylamide and Bis-acrylamide were purchased from Pharmacia. The following were used for detection in Western blot analysis: N,N-diethyl-p-phenylene diamine and 4-chloro-1-naphthol from Sigma; Enhanced Chemi Luminescence (ECL) kit purchased from Amersham.

The following materials were used for the column chromatographic studies: carboxy-methyl cellulose (CM-52) resin (Whatman); glycine (Bio-rad); sodium chloride (Fisher); G-25 Sephadex resin (Pharmacia); formic acid (BDH); HPLC and a reverse-phase HPLC column (C2-18) by LKB; trifluoroacetic acid and HPLC-grade acetonitrile (Caledon).

The antibodies used in this study are as follows: a polyclonal rabbit anti-bovine MBP antibody (1:300); a monoclonal clone 22 antibody which recognizes residues 83-88, found in all isoforms of MBP (a gift of N. Groome, Oxford, UK); a monoclonal clone 26 antibody which recognizes residues 69-74, found in all isoforms (a gift of N. Groome, Oxford, UK); a monoclonal antibody which recognizes only the 18.5 and 21.5 kDa isoforms (a gift of Dr. Al-Sabbagh, Autoimmune Inc., Boston). Polyclonal and monoclonal primary antibodies were probed with appropriate secondary antibodies; goat anti-rabbit IgG or goat anti-mouse IgG antibodies, both conjugated to horse radish peroxidase (Biorad), respectively.
Methods

Isolation of MBP from Murine Brain

Adult CD-1 mice (3-6 months) were sacrificed by cervical dislocation. The brains were removed immediately, frozen in liquid nitrogen and stored at -70°C until use. The isolation of acid-soluble brain protein (ie. MBP) was performed using the method described by Fannon and Moscarello (1991). Briefly, brains were homogenized in 2:1 (v/v) chloroform:methanol (10 mL per brain) for delipidation. After stirring overnight at 4°C, the homogenate was filtered through Whatman No. 1 filter paper. The residue was washed with cold 2:1 chloroform:methanol which was followed by a wash with cold acetone. The residue was homogenized in 0.2 N H2SO4 (2 mL per brain) and mixed overnight at 4°C to extract the acid-soluble protein (ie. MBP). The homogenate was centrifuged at 9 000 rpm for 20 minutes in a JA-20 rotor (Beckman) at 4°C. The supernatant was collected and dialyzed against ddH2O overnight to remove the acid. The contents of the dialysis tubing was filtered through Whatman No. 1 filter paper to remove insoluble material, lyophilized and stored for future use.

Isolation of MBP charge isomers

Following isolation from mouse brain (see above), MBP was fractionated on CM52 columns using a scaled down version of the method described by Chou et al. (1976) for bovine brain. For this chromatography, the MBP was solubilized in 500 μL of an 80 mM sodium glycinate buffer (pH 9.5), containing 6 M urea, and dialyzed against the same buffer for 2 hours. The retentate (8 OD280 units) was then applied to a mini-CM 52 column (0.8cm X 10 cm) equilibrated in an 80 mM sodium glycinate buffer (pH 10.5), containing 6 M urea. Bound material was eluted with a linear sodium chloride gradient (0 to 0.75 M) in an 80 mM sodium glycinate buffer (pH 10.5), containing 6M urea. The optical density at 280 nm and the conductivity of each fraction
were recorded as measures of the amount of eluted protein and salt gradient, respectively. Total optical units were calculated using the following equation.

\[
\text{optical density of fraction at 280 nm} \times \text{(volume of fraction (mL))} = \text{total optical density units}
\]

Fractionation required 5 to 6 hours. Whereas with human MBP the salt gradient was 0 to 0.2M, a higher salt concentration was required for the mouse since the latter is more cationic.

Pooled factions corresponding to individual components were desalted on a Sephadex G-25 gel filtration column equilibrated in 7% formic acid. Following the removal of acid by rotary evaporation, the components were dissolved in ddH₂O and lyophilized.

**SDS-PAGE**

SDS-PAGE was performed by following the procedure described by Laemmli (1970). Protein samples were solubilized in 2x sample buffer (2% SDS, 20% glycerol, 0.125mM Tris-HCl (pH 6.8), 10% β-mercaptoethanol, 0.01% bromophenol blue) and separated on 12.5% SDS-polyacrylamide gels. The 5% stacking gel consisted of 670 uL acrylamide-bis (30%:0.8%). 1.25 mL 0.5M Tris-HCl (pH 6.8), 50 uL 10% SDS, 25 uL 10% APS, 3.0 mL ddH₂O and 5 uL TEMED. The 12.5% separating gel consisted of 4.2 mL acrylamide-bis (30%:0.8%). 2.50 mL 1.5 M Tris-HCl (pH 8.8), 100 uL 10% SDS, 50 uL 10% ammonium persulphate, 3.2 mL ddH₂O and 10 uL TEMED. The gels were electrophoresed in the mini-gel Biorad apparatus at 170 V for 45 minutes at 4°C. Protein bands were visualized by staining with 0.25% Coomassie brilliant blue in 10% acetic acid and 50% methanol followed by destaining with 7% acetic acid and 20% methanol.

**Western Blot**

Following SDS-PAGE, protein samples were electrophoretically transferred to nitrocellulose membrane at 300 mA for 60 minutes in Western transfer buffer (25 mM
Figure 2.1: Flowsheet for Isolation and Purification of MBP Components Isolated from Whole Mouse Brain.

**ISOLATION OF MBP COMPONENTS**

Whole Mouse Brain

1. Delipidation using 2:1 (v/v) chloroform/methanol

2. Wash residue with acetone

3. Acid-extraction of basic proteins with 0.2 NH$_2$SO$_4$ from residue

4. Acid-insoluble material

5. Acid-soluble material

6. Dialysis for the removal of acid

7. Lyophilize retentate

8. Fractionation of MBP components using mini-CM52 chromatography (0.8 x 10.2 cm)

**PURIFICATION OF COMPONENTS**

1. MBP components

2. Desalting with a G-25 Sephadex column equilibrated in 7% formic acid

3. Rotary evaporation for the removal of formic acid

4. Lyophilization
Tris-HCl (pH 8.0), 192 mM glycine, 20% methanol) by the method of Towbin et al. (1979). The membrane was incubated in 5% blocking solution (5% (w/v) Carnation non-fat milk in 20 mM Tris-HCl buffered saline (TBS), pH 7.5) for 1 hour. Following blocking, the membranes were incubated with the primary antibody (see Materials section) overnight at 4 °C, washed 3x with TBS (pH 7.5) and incubated with appropriate secondary antibodies conjugated to horseradish peroxidase for 1 hour. Antibody binding was detected using either (1) 500 µL of solution A (2% 4-chloro-1-naphthol, 1.25% N.N-diethyl-p-phenylene diamine in methanol) in 25 mL 0.1 M citrate buffer containing 3 µL of 30% hydrogen peroxide or (2) an Enhanced Chemi Luminescence detection kit by Amersham.

Mini-Alkaline-Urea Slab Gel Electrophoresis

For the mini-alkaline-urea slab gel system, gels were cast in Novex cassettes (10 x 9 x 0.15 cm) with a resolving gel of 10% acrylamide in 8 M urea and a 5 mm stacking gel, consisting of 4% acrylamide in 8 M urea. For the resolving and stacking gels, 0.4 M sodium glycinate buffer (pH 10.6), and 0.2 M sodium glycinate buffer (pH 9.6) were used, respectively. Electrophoresis was carried out for 8 hours following 1 hour pre-electrophoresis in the Novex mini-X-cell II system with reversed polarity at 17 mA. The running buffer was mixed and the wells flushed hourly, to maintain adequate buffering capacity. The samples (5 - 40µg) were added to an equal volume of 8 M urea, vortexed and loaded onto the gels. Proteins were visualized by staining the gel for 10 to 15 minutes in 0.5% Amido Black in 7% acetic acid followed by destaining in 7% acetic acid.

Analysis of the murine MBP charge isomers during myelin development was determined by measuring the band intensities with an NIH (National Institute of Health) 1.52 computer program. The percentages of charge isomers were calculated as follows:

\[
\frac{(\text{band intensity of desired band}) \times 100}{\text{(total intensity of the material on the gel)}} = \% \text{ charge isomer}
\]
Western Immunoblotting of Alkaline-Urea Slab Gels

Following separation on the mini-alkaline-urea slab gel, proteins were transferred onto nitrocellulose membrane in 0.7% acetic acid by electrophoresis for 1 hour at 300 to 400 mA. The procedure for detecting immunoreactive material was similar to that for Western blot analysis (Towbin et al. 1979).

Reverse-Phase High Pressure Liquid Chromatography

Reverse-phase HPLC was used to remove any traces of salt from MBP components from the mini CM-52 column. MBP-containing samples were injected into an HPLC (LKB) equipped with an C2-18 reverse-phase column (Pharmacia). The column was equilibrated with 0.05 % trifluoroacetic acid (TFA). The sample was loaded onto the column at a flow rate of 0.5mL per minute. A linear gradient from 0 to 60 % acetonitrile was applied at the same flow rate. Gradient times were altered accordingly. Protein elution was monitored by optical density readings at 226 nm. Selected HPLC fractions were diluted with double-distilled H2O and lyophilized for the removal of TFA and acetonitrile. The lyophilized material was stored at -20 °C for future use. A chromatogram of C-1 is shown in Figure 2.2.

Electrospray-Ionization Mass Spectrometry

Mass spectrometry by the electrospray ionization method was performed on a SCIEX instrument (Thornhill, Ontario, Canada), atmospheric pressure ionization (API) III triple quadrupole mass spectrometer. Approximately 2 μL of each sample was injected into the carrier solvent (5% acetic acid) at a flow rate of 20 μL/min using a Rheodyne 7.25 injector. The skimmer orifice voltage was set at 8 eV and the mass range was scanned from m/z 400 to m/z 2000 with 0.2 amu steps. Data were acquired under multi-channel analysis (MCA) mode. The observed multiply charged peaks were deconvoluted by the hyper mass method.
Figure 2.2: Purification of Murine MBP Component C-1 by Reverse-phase HPLC

C-1 was loaded onto a reverse-phase C2-18 column equilibrated in Solvent A (0.05 % TFA). A linear gradient from 0 to 100% B (Solvent A plus 35 % acetonitrile) was applied for 30 minutes. The protein was monitored by measuring the optical densities at 226 nm.
Prior to injection into the mass spectrometer, each of the charge isomers was applied to a reverse phase HPLC column to remove traces of metal ions which interfered with the mass spectrometry. Each sample was dissolved in 0.05% trifluoroacetic acid and run on a Pharmacia C:2/C:18 Pep-@ column using a 50 minute linear gradient from 0 to 60% acetonitrile. Discrete fractions of material absorbing at 226 nm were pooled, lyophilized and prepared for mass spectrometry.

**Liposomal Preparation**

Liposomes consisting of 92.2 mol % PC: 7.8 mol % PS were prepared by the method of Wood and Moscarello, (1989). Egg phosphatidylcholine (egg PC) and bovine brain phosphatidylserine (PS) were purchased from Avanti (Birmingham, AL) as dry powders. These phospholipids were dissolved separately in chloroform at a concentration of 5 mg/mL and stored at -20°C under nitrogen. Aliquots of the dissolved lipid (92.2 mol % PC and 7.8 mol % PS) were transferred to acid-washed tubes. Nitrogen gas was used to evaporate the chloroform, traces of which were eliminated by lyophilization for 30 minutes. The dried lipids were suspended in 1 mL of 10mM HEPES buffer (pH 7.4) containing 100 mM NaCl, 1 mM EDTA and 0.01% sodium azide. The suspension was clarified by sonication at 20-25°C. The volume of the lipid vesicles was increased to 5 mL with the above-mentioned buffer. For the removal of large multi-layered vesicles, the vesicles were centrifuged at 40 000 rpm for 1 hour. Each aggregation assay required 0.32 μmol of total phospholipids. To a 500 μL phospholipid sample was added 0 to 40 μg of protein. After a 10 minute incubation at room temperature, the turbidity of the samples was measured at 450 nm.

**Southern Dot Blot**

Southern dot blot was used to identify ND4 mice carrying the transgene, human DM-20 cDNA. Total genomic DNA was extracted from mouse tail clips. Tails were minced and digested overnight (55-60°C) with 350 μg proteinase K (Boehringer...
Mannheim) in proteinase K buffer (50mM Tris-HCL (pH 8.0), 100 mM EDTA, 100 mM NaCl and 1% SDS). The mixture was treated with 200 ug RNAse A for 2 hr at 37°C. This was followed by two extractions with phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) and once with chloroform/isoamyl alcohol (24:1 v/v). Genomic DNA was precipitated with isopropanol. The precipitated DNA was washed with 70 and 95% ethanol, dried and redissolved in TE buffer (10mM Tris-HCL (pH 7.5), 1 mM EDTA). An aliquot containing 15 ug genomic DNA was dried down, treated with 100 uL denaturing solution (0.4M NaOH, 10 mM EDTA) and heated to 100°C for 10 minutes. Cold neutralizing solution (2M ammonium acetate, pH 7.0) was added to the DNA samples before application to nitrocellulose membrane. Prior to sample application, nitrocellulose membrane was soaked in 6X-sodium citrate/sodium chloride (SSC). After assembly of the Bio-Dot SF apparatus, the membrane was rehydrated with 500 uL TE. After rehydration, samples were loaded and loading wells were washed with 2X SSC. After sample application, the membrane was rinsed in 2X-SSC, air-dried and baked for 1 hour at 80 °C. The membrane was incubated in pre-hybridization buffer (5x SSC, 0.1%(w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, 0.1% (w/v) blocking reagent) at 65 °C for 1 hour. The probe consisting of the entire human DM-20 cDNA was labelled with α[^32P]dCTP by the random priming method (Feinberg and Vogelstein, 1983) using the Prime-a-Gene labeling system (Promega) to a specific activity of ~ 10^9 cpm/ug. This probe was added to the pre-hybridization solution and allowed to hybridize overnight at 65°C. Filters were washed at high stringency in 0.1x SSC, 0.1% SDS at 65 °C, dried and exposed to Kodak XAR-5 film.

Quantitation of protein

Protein determinations were done by using the procedure by Peterson (1977), a modification of the method described by Lowry et al. (1951). Briefly, 100 uL of 0.15% deoxycholate was added to a 1 mL protein solution. The solution was vortexed and
incubated at RT for 10 minutes. Following the addition of 100 uL of 72 % trichloroacetic acid, the mixture was centrifuged in a Micro-Hettich benchtop centrifuge for 10 minutes at 4°C. The supernatant was aspirated and the pellet was resuspended in 400 uL ddH2O. To the resuspended sample, 400 uL of solution A (0.1% copper sulphate, 0.2% potassium tartrate, 10% sodium bicarbonate, 10% SDS, 0.8 N NaOH and ddH2O) was added. This mixture was incubated at RT for 10 minutes. The mixture was treated with 200 uL of reagent B (Folin's reagent and ddH2O (1:5 v/v)) and incubated at RT for 30 minutes. Absorbances were measured at 750 nm. Serial dilutions of bovine serum albumin (0 to 80 ug) were used to construct a standard curve.

Amino acid analysis

HPLC-purified protein samples were hydrolyzed in 6 M HCl containing 0.1% phenol in vapour phase for 24 hours at 110 °C. The free amino acids were derivatized with phenylisothiocyanate (PITC) and analyzed on a Waters Pico-Tag amino acid analyzer using a reverse-phase HPLC column. The PITC-derivatized amino acids were monitored at 254 nm and quantitated on a Digital PC data microprocessor by comparison to the profiles of amino acid standards (Beckman).

N-terminal Protein Sequencing

Protein samples were electrophoresed on a 12.5 % SDS-polyacrylamide gel as described before. Before transfer, Immobilon P membrane was pre-wetted in 100 % methanol and then with transfer buffer (100 mL of 100mM CAPS (pH 11.0), 100 mL methanol and 800 mL ddH2O). Transfer of protein from the SDS-polyacrylamide gel onto Immobilon membrane was performed for 20 minutes at 300 mA. After transfer, membrane was rinsed with ddH2O for 5 minutes. For an additional 5 minutes, the membrane was stained with 0.1 % Coomassie blue R250 in 50 % methanol. Destaining
was performed using a solution containing 50 % methanol and 10 % acetic acid. Destaining was followed by drying of the membrane.

After drying, certain bands were cut out from the membrane and submitted for automated sequence analysis. Automated sequencing was carried out on a Porton Instruments' (Tarzana, CA) Gas-Phase Micro-Sequencer model PI 2090E with on-line phenylthiohydantoin (PTH) amino acid analysis. Sequencing was performed by Edman degradation using phenylisothiocyanate (PITC), with triethylamine (TEA), as a coupling agent, and trifluoroacetic acid (TFA) as the cleaving agent. PTH amino acid analysis was carried out using an HPLC equipped with a Hypersil ODS column, a reverse-phase HPLC column. Detection of the PTH amino acid residues was monitored by measuring the optical densities at 269 nm.
CHAPTER 3

ISOLATION AND CHARACTERIZATION OF MURINE MBP COMPONENTS

Introduction

Although vast amounts of information concerning human and bovine MBP are readily available, very little is known about murine MBP. The main difficulty lies in the small amounts of material obtained from a single mouse brain. In order to overcome this obstacle, two methods were adapted for use with the small amounts of MBP available. The first was a scaled-down version of the preparative CM-52 chromatographic system commonly used to isolate MBP charge isomers. The second was an alkaline-urea slab gel technique which required up to five times less material than the conventional tube gel system. From these gels, Western blots were readily obtained. In this study, these techniques were essential for the isolation and characterization of MBP charge isomers from normal mouse brain.

Results

Charge Microheterogeneity of Murine MBP

Following isolation from normal (CD-1) mouse brain as described in Methods, MBP was subjected to alkaline-urea gel electrophoresis to assess charge microheterogeneity at pH 10.6. An Amido-black stained gel is shown in Figure 3.1.A. Mouse MBP resolved into a pattern consisting of several bands (Lane M) that was more complex that that of human MBP (Lane H). Within this complex pattern of mouse MBP isomers, two distinct populations of isomers can be distinguished (Lane M). One group of isomers was more cationic than human MBP (bands below the marker, Lane M); the most cationic member of this group migrated a distance that was two-fold greater than
that of human MBP. The second series of isomers (bands above the marker) was relatively less cationic than the other group. Migration of the less cationic population of isomers was similar to human MBP suggesting that these isomers may represent the 18.5 kDa isoform of murine MBP.

Western blots of the material in Figure 3.1.A are shown in Figures 3.1.B and C. A monoclonal antibody (Clone 22) reactive with residues 83 to 88 in MBP, a sequence conserved in all MBP isoforms (Figure 3.1.B), and a monoclonal antibody that recognizes only the 18.5 and 21.5 kDa isoforms of MBP (Figure 3.1.C) were used. Immunoreactivity of all murine MBP components with clone 22 was demonstrated (Lane M; Figure 3.1.B). The intensities of the bands were high for the more cationic group of murine isomers and low for the less cationic isomers. Strong reactivity of human MBP with clone 22 was shown (Lane H). In Figure 3.1.C, the more cationic isomers of murine MBP were not detected by the anti-18.5/21.5 kDa MBP antibody. However, the antibody was strongly reactive with both the slow migrating group of murine MBP isomers (Lane M) and human MBP (Lane H) suggesting the former to be 18.5 kDa. These findings suggest that the less cationic population of murine MBP isomers is represented by the 18.5 kDa isoform whereas the population of the more cationic isomers is 14 kDa.

By using primary sequence data of mouse and human MBP (see Introduction) and pK_a values of amino acids (Stryer, 1988), we were able to calculate the overall net positive charges of unmodified 14 and 18.5 kDa isoforms at pH 10.6 (Table 3.1). At pH 10.6 (at which the alkaline-urea gel is run at), arginine (pK_a of side chain = 12.0) and 20% of total lysine (pK_a = 10.0) residues were positively charged. Aspartic acid (pK_a = 4.4), glutamic acid (pK_a = 4.4) and 80% of tyrosine (pK_a = 10.0) residues carried a negative charge. Unmodified 14 kDa murine MBP carried an overall net positive charge of + 8.8 at pH 10.6 whereas that of the 18.5 kDa isoform was + 5.6 (Table 3.1). The charge difference between the 14 and 18.5 kDa MBP isoforms of mouse corroborates
Figure 3.1  Alkaline-urea gel electrophoresis and Western blot of Murine MBP

(A) Murine MBP (M, 25 ug) was electrophoresed on a 10% acrylamide alkaline-urea slab gel (pH 10.6) with human MBP (H, 8 ug) as a control. Western blots are shown in B and C. The antibodies used were (B) monoclonal clone 22 antibody that recognizes all MBP isoforms and (C) a monoclonal antibody that is specific for the 18.5 and 21.5 kDa isoforms only.
with the migration patterns of the corresponding isomers in Figure 3.1 i.e. the isomer of 14 kDa migrates further and is more cationic than that of 18.5 kDa. The overall net charge on unmodified human MBP was +7.2 at pH 10.6 which makes it more cationic than mouse MBP 18.5 kDa but less than that of the 14 kDa. The charge differences are reflected in their respective migration patterns seen in Figure 3.1.

Table 3.1  Charge of Unmodified MBP Isoforms at pH 10.6

<table>
<thead>
<tr>
<th>Residue</th>
<th>Arg</th>
<th>Lys</th>
<th>Asp</th>
<th>Glu</th>
<th>Tyr</th>
<th>Net charge at pH 10.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoform</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 kDa</td>
<td>+17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+1.4</td>
<td>-7</td>
<td>-1</td>
<td>-1.6</td>
<td>+8.8</td>
</tr>
<tr>
<td>(mouse)</td>
<td>(17)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(7)</td>
<td>(7)</td>
<td>(1)</td>
<td>(2)</td>
<td></td>
</tr>
<tr>
<td>18.5 kDa</td>
<td>+18</td>
<td>+2.6</td>
<td>-9</td>
<td>-2</td>
<td>-4</td>
<td>+5.6</td>
</tr>
<tr>
<td>(mouse)</td>
<td>(18)</td>
<td>(13)</td>
<td>(9)</td>
<td>(2)</td>
<td>(5)</td>
<td></td>
</tr>
<tr>
<td>18.5 kDa</td>
<td>+19</td>
<td>+2.4</td>
<td>-9</td>
<td>-2</td>
<td>-3.2</td>
<td>+7.2</td>
</tr>
<tr>
<td>(human)</td>
<td>(19)</td>
<td>(12)</td>
<td>(9)</td>
<td>(2)</td>
<td>(4)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The values which correspond to the overall net positive charge of the respective amino acids at pH 10.6 were calculated by referring to pK<sub>a</sub> values taken from Stryer (1988).

<sup>b</sup> The numbers in brackets correspond to the total number of specified residues in the primary sequence of MBP (See Introduction).
Isolation and Characterization of MBP Components

Fractionation of MBP Components by Column Chromatography

MBP isolated from normal mouse brains (3 to 6 months) was fractionated on a mini-CM-52 cation exchange chromatographic column as described in Methods. The fractionation procedure resolved the MBP into several peaks. A large peak corresponding to the unbound fraction (containing C-8) came off the column in fractions 5 to 13. Following the application of the salt gradient, components (C-5 to C-1) eluted with increasing concentrations of salt, respectively (Figure 3.2). The material under each peak was pooled and desalted using a 25 mL G-25 Sephadex column that was equilibrated in 7% formic acid. A chromatogram for the desalting of C-1 is shown in Figure 3.3. The exclusion of C-1 (the first peak in Figure 3.3) from the column was monitored by measuring optical densities at 280 nm. Conductivity measurements were used to monitor the exclusion of salt from the column (the second peak in Figure 3.3). The G-25 column fractions containing the desalted components were pooled and the formic acid was removed by rotary evaporation. Following rotary evaporation, the desalted component was dissolved in ddH2O and lyophilized (see Methods). The characterization of the lyophilized components will be provided in the following sections.
Figure 3.2 Mini-CM52 Cation Exchange Chromatography of MBP Isolated from whole adult mouse brain.

Unbound material was washed off the column as described in Methods. Bound material was eluted from the column using a 0 to 0.75M NaCl gradient.
Mobility Properties of MBP on Electrophoretic Gels

Individual components were electrophoresed on an SDS-polyacrylamide gel (Figure 3.4.A). The unfractionated MBP sample (Lane 2), which corresponds to the starting material loaded onto the mini-CM-52 column, consisted of several acid-soluble proteins. Most of these proteins, along with C-8 MBP, did not bind to the mini-CM-52 column as indicated by the SDS-polyacrylamide gel (Lane 3). The intensely stained bands in lanes 7 and 8 which correspond to C-2 and C-1, respectively, were both the 14 kDa isoform although a weak 17 kDa band was visualized. The 18.5 kDa isoform first appeared in C-3 (Lane 6). In comparison to the 14 kDa isoform, relative proportions of the 18.5 kDa isoform increased from C-3 to C-5 (lanes 6 to 4, respectively). Faint bands...
Figure 3.4  Characterization of Murine MBP Components by SDS-PAGE (A) and alkaline-urea gel electrophoresis (B).

The lane assignments for the SDS-PAGE (A) were the following: lane 1 molecular weight markers; lane 2, acid-soluble material loaded onto the CM-52 column; lane 3, unbound material; lanes 4 to 8, C-5 to C-1, respectively; lane 9 human MBP. Lanes 1 to 9 in the alkaline-urea gel (B) correspond to lanes 2 to 9 in (A), respectively.
corresponding to the 21.5 kDa isoforms were weakly stained in C-3, C-4 and C-5 (Lanes 6, 5 and 4, respectively). A 15-16 kDa band in C-5 (Lane 4) and lower-molecular-weight (LMW) bands (below 14 kDa) were also detected. The identity of these proteins as either MBP isoforms or degradation products remains unknown.

The alkaline-urea slab gel of the isolated components is shown in Figure 3.4.B. The unfractionated material (Lane 1) resolved into a multitude of bands corresponding to the isomers of the 14 (fast migrating) and 18.5 kDa (slow migrating) isoforms. The isoform representing both C-1 and C-2 (Lanes 7 and 8, respectively) was 14 kDa. C-3 and C-4 (Lanes 5 and 4, respectively) consisted of the 14 and 18.5 kDa isoforms. C-5 (Lane 3) was enriched in the 18.5 kDa isoform. The unbound fraction from the mini-CM-52 column (Lane 2) consisted of several isomers of MBP referred to as C-8 although other material is present (see Figure 3.5.A., Lane 3). From the unbound fraction of human MBP, several isomers have been isolated (Boulias et al. 1995).

Characterization of MBP Components by Western Immunoblot Analysis.

Following SDS-PAGE, the murine MBP components were transferred onto nitrocellulose membranes and probed using the following antibodies: a polyclonal antibody against bovine MBP (18.5 kDa) reactive against all isoforms (Figure 3.5.A) and a monoclonal (P12) antibody that recognizes phosphorylated Thr-98 in human MBP (18.5 kDa). a conserved MAP kinase site (Yon et al, 1995) (Figure 3.5.B). In Figure 3.5.A, C-1 (lane 8) and C-2 (lane 7) consisted of the 14 kDa species. The 18.5 kDa isoform was first detected in C-3 (lane 6). The proportion of the 18.5 kDa isoform increased, relative to the 14 kDa, in components C-3 to C-5 (lanes 6 to 4, respectively). In the unbound fraction (lane 3), the ratio of 18.5 to 14 kDa MBP constituting C-8 was 3 to 2 as determined by scanning the blot and computing the band intensites with an NIH 1.52 computer program. Bands corresponding to the exon-2 containing isoforms (17 and 21.5 kDa) were detected also. However, the bands were too faint to study further. This
Figure 3.5 Western blot analysis of Murine MBP Components

Acid-soluble material and MBP components isolated from mouse brain were electrophoresed on SDS-PAGE and Western blotted onto nitrocellulose membrane. The membranes were probed with (A) a polyclonal rabbit anti-bovine MBP antibody and (B) a monoclonal P12 antibody that recognizes phosphorylated threonine-95 in mouse MBP (threonine-98 in human). Lane assignments for both (A) and (B) are as follows: lane 1, molecular weight markers; lane 2, acid-soluble material loaded onto the CM-52 column; lane 3, unbound material; lanes 4 to 8, C-5 to C-1, respectively; lane 9, human MBP.
finding suggested charge microheterogeneity to extend not only to the exon 2-lacking isoforms (14 and 18.5 kDa) but also to the exon 2-containing species (17 and 21.5 kDa).

In figure 3.5.B, the P12 monoclonal antibody, which detects MBP species carrying a phosphate group on Thr-98 (Thr-95 in mouse), reacted with all components except C-1 (14 kDa) (lane 8) and the 18.5 kDa isoform in C-3 (lane 6) suggesting that these isomers were not phosphorylated. The absence of a phosphate group in these particular isoforms was suggested by mass spectrometric findings (see next section).

Amino Acid Analysis of C-1

Desalted C-1 fractions were purified using reverse-phase HPLC and subjected to amino acid analysis. Table 3.2 summarizes the results of the analysis. Literature values for the amino acid composition for 14 kDa mouse MBP were taken from de Ferra et al. (1985). The amino acid composition of C-1 was very similar to literature values (Table 3.2). Tryptophan residues were not quantitated because they readily degrade under acid-hydrolysis conditions.

Molecular Weight Determination of MBP Components

In order to obtain molecular weight information from which possible post-translational modifications may be deduced, murine MBP components (C-1, C-2 and C-3) were subjected to electrospray-ionization mass spectrometry (ESI-MS). The mass spectra for C-1, C-2 and C-3 are shown in Figures 3.6.A, B and C, respectively. The mass spectra for C-1 and C-2 (Figures 3.6.A and B, respectively) demonstrated low background levels and narrow peak widths suggesting the samples to be homogeneous. The broader peaks representing C-3 (Figure 3.6.C) indicated that more than one component was present. Mass spectral peaks, representing different charged states of the molecule, were labelled with a number corresponding to the mass to charge (m/z) ratios of the charged molecule (i.e. C-1, C-2 or C-3). Each peak possessed its own net positive
Table 3.2: Amino acid analysis of HPLC-purified MBP C-1.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Murine MBP C-1 (14 kDa) (res/100)</th>
<th>14 kDa MBP (res/100)</th>
<th>measured</th>
<th>(taken from deFerra et al., 1985)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>7.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>5.92</td>
<td>5.56</td>
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<td>Ser</td>
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</tr>
<tr>
<td>Gly</td>
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<tr>
<td>His</td>
<td>5.27</td>
<td>5.56</td>
<td></td>
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</tr>
<tr>
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<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
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<td>13.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>6.88</td>
<td>7.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>5.00</td>
<td>4.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>8.24</td>
<td>7.93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td>1.54</td>
<td>1.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>1.55</td>
<td>2.38</td>
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<td></td>
</tr>
<tr>
<td>Met</td>
<td>1.03</td>
<td>1.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cys</td>
<td>0.06</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>2.06</td>
<td>2.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>5.00</td>
<td>4.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>4.70</td>
<td>4.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orn</td>
<td>0.16</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>6.84</td>
<td>5.56</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Values represent the mean of two events.
charge and differed from adjacent peaks by one net charge. From the m/z ratios, the molecular weights of C-1, C-2 and C-3 were determined. The molecular weights determined by mass spectrometry are shown in Table 3.3.

Mass spectrometric analysis showed that the molecular weight of C-1 was 14140.38 ± 0.79 amu (Table 3.3). This mass differs by 60 amu from the calculated molecular weight of 14080 for the unmodified protein obtained from the protein sequence. We postulated that C-1 was acetylated (42 amu) at the N-terminus and methylated at arginine-104 (14 amu). The remaining 4 amu were unaccounted for. Since

Figure 3.6 Mass Spectra of Murine Components C-1 (A), C-2 (B) and C-3 (C). An electrospray-ionization mass spectrometer (SCIEX) was used to obtain the spectra.
the error of the method is well below 4 amu (Table 3.3). Further studies are required to account for the 4 amu.

From the SDS-PAGE and alkaline-urea gels, C-2 migrated as a single 14 kDa component (Figures 3.4.A and B). However, molecular weight determinations by mass spectrometry showed that C-2 was represented by two species (C-2_L and C-2_H, respectively). 14 136.37 ± 0.74 (56 amu difference) and 14 204.45 ± 0.70 (124 amu difference) compared to the unmodified protein (Table 3.3). A C-2_L-to-C-2_H ratio of 3.3 to 1 was determined from the relative abundances obtained from the mass spectral data. The difference in mass between C-2_L and C-2_H was not sufficient to resolve the two proteins by SDS-PAGE (Figure 3.4.A), which showed a single sharp band.

For the lower molecular weight species (C-2_L), a mass difference of 56 amu from the computed mass of the unmodified protein (14 080) was observed. This mass difference can be accounted for readily by acetylation of the N-terminus (42 amu) and by monomethylation of an arginyl residue (14 amu). The higher molecular weight species of 14 204 ± 0.70 differed from the computed value by 124 amu. This difference in mass can be accounted for in several ways. A heterogeneous N-terminus consisting of acetyl, butyl and hexyl (98 amu) groups similar to the N-terminal heterogeneity observed for human C-1 (Moscarello et al. 1992) and dimethylated arginine (28 amu) would account for the mass (total = 126 amu). Alternatively, an N-terminal acetyl group (42 amu) and a phosphate (80 amu) could also account for the mass (total = 122 amu). In fact, a Western blot with a monoclonal antibody P12, which recognizes phosphorylated threonine-98 in the MAP kinase site of human MBP (Thr-95 in mouse) (Yon et al. 1995) showed some reactivity with C-2, although stronger activity with the other isomers was observed (Figure 3.5.B). The increase in net negative charge by the presence of the phosphate group must be compensated by 2 positive charges in order that this higher molecular weight species would migrate with the lower molecular weight species (not phosphorylated). An alternative explanation is that both types of modification are found
in C-2. The measured difference of 124 amu (Table 3.3) may be an average representation of weight corresponding to the two types of modification.

Mass spectrometry of C-3 showed two proteins of masses 14 215 ± 0.94 and 18 413.57 ± 0.76 (Table 3.3), in a ratio of 3:2. The presence of the 18.5 kDa isoform in the C-3 fraction was observed earlier by alkaline-urea gel electrophoresis (Figure 3.4.B). The difference in mass from the computed value of 14 080 for the unmodified protein was 135 amu which was accounted for by acetylation at the N-terminus, methylation and phosphorylation. Since the 14 kDa species was highly reactive with the P12 monoclonal antibody, it must be phosphorylated at the Thr-95 (Figure 3.5.B). The higher molecular weight species (18 413.57 ± 0.76) differs from the unmodified protein by 56 amu which can be accounted for by an acetyl and a methyl group. The lack of reactivity with P12 suggests it is not phosphorylated (Figure 3.5.B, lane 6), in agreement with the mass spectrometric data (Table 3.3).
Table 3.3 Mass Spectrometric Molecular Weight Determinations of MBP Charge Isomers isolated from Adult Murine Brain

<table>
<thead>
<tr>
<th>Component</th>
<th>Molecular Weight (amu)</th>
<th>Mass Difference (amu)</th>
<th>Suggested Post-translational modifications (amu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 kDa</td>
<td>14 080</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.5 kDa</td>
<td>18 357</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measured</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-1</td>
<td>14 140.38 ± 0.79</td>
<td>60</td>
<td>acetyl (42)+ methyl (14)</td>
</tr>
<tr>
<td>C-2</td>
<td>14 136.37 ± 0.74</td>
<td>56</td>
<td>acetyl (42) + methyl (14)</td>
</tr>
<tr>
<td></td>
<td>14 204.45 ± 0.70</td>
<td>124</td>
<td>acetyl (42)+ phosphate (80)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>or</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hexyl (98) + dimethylation (28)</td>
</tr>
<tr>
<td>C-3</td>
<td>14 215.57 ± 0.94</td>
<td>135</td>
<td>phosphate (80)+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>acetyl (42)+methyl (14)</td>
</tr>
<tr>
<td></td>
<td>18 413.57 ± 0.76</td>
<td>56</td>
<td>methyl (14) + acetyl (42)</td>
</tr>
</tbody>
</table>
Vesicle-aggregating properties of murine MBP components

In order to determine whether the cationic properties of MBP components have an effect on its ability to promote aggregation of vesicles (92.2 mol % PC and 7.8 mol % PS), increasing amounts of MBP components (C-1, C-2, C-3) were added to vesicle suspensions. The turbidity of the sample was measured at 450 nm after a 10 minute incubation at room temperature (Figure 3.7). Murine MBP C-1, the most cationic isomer, was most effective at inducing vesicle aggregation of acidic lipids: C-2 was less effective than C-1; C-3 possessed the least ability of the three. In general, an increase in cationicity of the murine MBP charge isomers is proportional to the ability of these isomers to induce vesicle aggregation in this system. A similar finding concerning the ability of human MBP components to promote vesicle aggregation was reported (most effective C-1 > C-2 > C-4 > C-8 least effective) (Wood and Moscarello, 1989)
Figure 3.7  Lipid vesicle-aggregation with murine components.

Vesicles composed of 92.2 mol % PC:7.8 mol % PS were prepared as described in Methods. Increasing amounts of murine components C-1, C-2 and C-3 and human MBP C-1 (H) were added to the vesicle suspension. Turbidity was measured at 450 nm after a 10 minute incubation at room temperature.
Summary

Murine MBP was resolved into two populations of charge isomers: the 18.5 and 14 kDa isoforms. Component 1 (C-1), the most cationic isomer of the 14 kDa, has a molecular weight of 14 140.38 ± 0.79. C-2 consisted of two 14 kDa species, 14 136.37 ± 0.74 and 14 204.45 ± 0.70. Two size variants, 14 215.57 ± 0.94 and 18 413.57 ± 0.76 (the most cationic 18.5 kDa isoform), constituted C-3. C-4, C-5 and C-8 (the least cationic isomer) consisted of both 14 and 18.5 kDa isoforms. The efficacy of components to promote aggregation of acidic lipid vesicles was proportional to an increase in overall positive charge (e.g., C-1 > C-2 > C-3) suggesting that the ability for MBP to interact with lipids is highly dependent on the net positive charge on the protein.
CHAPTER 4
MYELIN BASIC PROTEIN CHARGE MICROHETEROGENEITY IN THE DEVELOPING MOUSE BRAIN.

Introduction

In an earlier study by Barbearese et al (1978), it was shown that the expression of the four major MBP isoforms (14, 17, 18.5 and 21.5 kDa) was developmentally regulated in mouse brain (from post-natal day 15 to 60). The expression levels of the 17 and 21.5 kDa isoforms were highest during early development whereas the 14 and 18.5 kDa expression levels were highest during later stages. Changes in charge microheterogeneity of the isoforms were not addressed at this time. Because these isoforms, especially the 14 and 18.5 kDa, can be post-translationally modified, a number of MBP charge isomers (C-1 to C-8) were produced in adult mouse brain (see Chapter 3). Therefore, the following study was undertaken to (1) extend the studies of Barbearese et al (1978) by analyzing the MBP expression before post-natal day 15 (i.e. post-natal day 10) and (2) assess the charge microheterogeneity of MBP during development in mouse brain to ascertain when microheterogeneity first appears.

Results

MBP Expression in Normal Mouse Brain

The Major MBP Isoforms and Normal Development

MBP was isolated from mouse brains ranging in age from post-natal day 10 to 20 as described in Methods. Following isolation, the MBP was fractionated on SDS-polyacrylamide gels (Figure 4.1.A) and subsequently, was analyzed by Western blot (Figure 4.1.B) in order to determine the MBP expression patterns of the major isoforms.
In the Coomasie-stained SDS-polyacrylamide gel (Figure 4.1.A), the material isolated from the mouse brains (from post-natal day 10 to 20: lanes 2 to 8, respectively) consisted of several bands suggesting that the isolation of the major MBP isoforms (14, 17, 18.5 and 21.5 kDa) from brain was accompanied by the isolation of several acid-soluble brain proteins. The MBP isoforms were identified by Western blot using a polyclonal antibody reactive with bovine MBP (Figure 4.1.B). The detection of the four major MBP isoforms by post-natal days 10 and 14 (Lanes 2 and 3, respectively) suggested that MBP is being expressed at stages earlier than those reported by Barbarese et al (1978). At post-natal day 10 (Lane 2), the immunoreactivity of the 18.5 kDa band was greater than that of the 14 kDa isoform. The reactivity of the 21.5 and 17 kDa isoforms indicated that small amounts of these isoforms were present at post-natal day 10 (Lane 2). The 14 and 18.5 kDa isoforms continued to be expressed during active myelination from post-natal day 14 to 20 (Lanes 3 to 8, respectively) whereas the expression of both the 17 and 21.5 kDa reached maximal levels by post-natal days 16 and 17 (Lanes 5 and 6, respectively) and remained constant thereafter. A high-molecular weight (HMW) band (~30 kDa) at post-natal days 15 to 18 (Lanes 4 to 7) was also detected. Whether the HMW protein represents an aggregate of MBP or possesses sequence identity with MBP is unknown.

In Figure 4.2, a Western blot similar to that in Figure 4.1.B was incubated with a monoclonal antibody (P12) that recognizes MBP phosphorylated at Thr-98 in human or Thr-95 in mouse. This antibody will allow us to ascertain when phosphorylation at Thr-95 in mouse first appears. At post-natal day 10 (lane 2), the phosphorylated species of the 18.5 kDa was relatively higher than that of the 14 kDa. By post-natal day 14 (lane 3), all isoforms were phosphorylated at Thr-95. This finding does not mean that all isomers of murine MBP are phosphorylated as well (i.e., murine MBP C-1 of 14 and 18.5 kDa are not phosphorylated. Chapter 3). In Figure 5.7 (see Chapter 5), P12 reactivity was absent in the acid-soluble material isolated from 4 day old normal mouse brain suggesting that
Figure 4.1  SDS-PAGE (A) and Western immunoblot (B) of acid-soluble material isolated from normal mouse brain.

The lane assignments for both (A) and (B) were the following: lane 1, molecular weight standards; lanes 2 to 8, acid-soluble material isolated from post-natal days 10, 14, 15, 16, 17, 18 and 20 normal mouse brains, respectively; lane 9, human MBP (18.5 kDa). Rabbit anti-bovine MBP antibody was used as primary antibody in (B).
Figure 4.2  MBP phosphorylation determined by Western blot analysis of acid-soluble material isolated from normal mouse brain.

Lane assignments are as follows: lane 1, molecular weight standards; lanes 2 to 8, acid-soluble material isolated from post-natal day 10, 14, 15, 16, 17, 18 and 20 normal mouse brains, respectively; lane 9, human MBP (18.5 kDa). Monoclonal P12 antibody reactive with phosphorylated Thr-95 in mouse was used as primary antibody.
phosphorylation of Thr-95 is an event that occurs during the active myelination and maturation phases.

**MBP Charge Microheterogeneity in Normal Development**

In order to determine the expression patterns of MBP charge microheterogeneity during myelogenesis, MBP was acid-extracted from mouse brains of various ages (4 days to 6 months) and fractionated on an alkaline-urea gel at pH 10.6 (Figure 4.2). The ages were chosen to correspond with certain stages of myelogenesis: early myelination (birth to 10 days), active myelination (10 to 25 days) and myelin maturation (25 days and onwards).

The presence of charge isomers over the course of murine myelogenesis is demonstrated in Figure 4.3. From post-natal days 4 to 14 (Lanes 1 to 3, respectively), the patterns of charge microheterogeneity suggested that modified forms of the 18.5 kDa isoforms were being generated as determined by mobility on this gel. The isomers corresponding to the 14 kDa isoforms were first detected by post-natal day 15 (Lane 4) and relative proportions increased thereafter. The increased production of the modified 14 kDa isoforms from post-natal day 16 to 180 (Lanes 5 to 10) was accompanied by a relative decrease in the levels of the less cationic components (18.5 kDa) suggesting that the overall positive charge of MBP in mouse brain was collectively increasing with development. The increased cationicity was reflected also in the alterations in the relative proportions of C-8, the least cationic isomer. C-8 levels were relatively high in the early myelinating animal (post-natal day 4 to 10, lanes 1 and 2, respectively). But during maturation, the relative proportions of C-8 decreased.
Figure 4.3  Alkaline-urea gel electrophoresis of acid-soluble material isolated from normal mouse brains.

Lanes 1 to 10 correspond to the following ages: post-natal days 4, 10, 14, 15, 16, 18, 20, 30, 60, 180, respectively.
In order to quantitate the changes in the least and most cationic charge isomers (C-8 and C-1, respectively) during myelin development, the alkaline-urea gel in Figure 4.3 was scanned and subsequently, the intensity of the corresponding bands on the gel were measured using the NIH 1.52 computer program from which the relative proportions of C-8 to C-1 were computed (Table 4.1). The proportions of C-8 decreased from 72.4 % at 4 days to 26.8 % at 180 days. The most cationic 18.5 kDa isomer, C-1 (18.5 kDa) (Table 4.1), increased approximately 6 % from 10 to 180 days (from 8.8 to 14.2 % of the total, respectively). Relative amounts of C-1 (14 kDa) were detected by day 16 and increased to 26.2% at 180 days. From 10 to 180 days, the C-8/C-1 ratio decreased to 0.72 from 8.7 at 10 days emphasizing the increased cationicity of MBP during development.
Table 4.1: Relative Proportions of MBP Charge Isomers During Myelinogenesis in Normal Mouse Brain

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>C-8 %a</th>
<th>C-1(18.5 kDa) %</th>
<th>C-1(14 kDa) %</th>
<th>C-8 1 (18.5 + 14 kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>72.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>76.4</td>
<td>8.8</td>
<td>-</td>
<td>8.7</td>
</tr>
<tr>
<td>11</td>
<td>55.8</td>
<td>12.6</td>
<td>-</td>
<td>4.4</td>
</tr>
<tr>
<td>14</td>
<td>65.8</td>
<td>9.0</td>
<td>-</td>
<td>7.3</td>
</tr>
<tr>
<td>15</td>
<td>58.4</td>
<td>8.5</td>
<td>-</td>
<td>6.9</td>
</tr>
<tr>
<td>16</td>
<td>38.3</td>
<td>13.0</td>
<td>9.2</td>
<td>1.7</td>
</tr>
<tr>
<td>17</td>
<td>44.2</td>
<td>11.4</td>
<td>5.7</td>
<td>2.6</td>
</tr>
<tr>
<td>18</td>
<td>54.0</td>
<td>11.9</td>
<td>6.6</td>
<td>2.9</td>
</tr>
<tr>
<td>20</td>
<td>37.1</td>
<td>13.3</td>
<td>11.1</td>
<td>1.5</td>
</tr>
<tr>
<td>180</td>
<td>26.8</td>
<td>14.2</td>
<td>26.2</td>
<td>0.7</td>
</tr>
</tbody>
</table>

a After measuring the band intensities on an alkaline-urea gel with an NIH computer program, the percentages of charge isomers were calculated as follows:

\[
\text{Percentage of charge isomer} = \frac{\text{band intensity of desired band}}{\text{total intensity of the material on the gel}} \times 100
\]
Summary

Alterations in MBP charge microheterogeneity in the developing mouse brain suggested that certain cationic species of MBP are required at different stages of myelinogenesis. During early stages of normal development, the population of MBP was enriched in the slow migrating isomers (i.e. isomers of the 18.5 kDa isoform). From post-natal day 15 to later ages (a period of active myelination and maturation), relative levels of the less cationic isomers (18.5 kDa) began to decrease once components corresponding to the 14 kDa were produced. The production of the more cationic isomers (14 kDa) continued to increase during the later stages of myelinogenesis suggesting that an increased MBP cationicity was required for myelin maturation. The increased cationicity in the MBP was reflected in the decreasing C-8/C-1 ratios during myelinogenesis suggesting that the low C-8 and high C-1 levels during later developmental stages may be important for stabilization of a mature compact myelin membrane.

In addition, phosphorylation of Thr-95 in murine MBP (as determined by P12 immunoreactivity) was identified as one post-translational modification responsible for the MBP charge microheterogeneity. Over the course of development, the MBP phosphorylated on Thr-95 was detected during stages of active myelination and maturation. Because P12 immunoreactivity was localized to the major dense line (MDL) of myelin (Yon et al. 1996), our studies suggest that phosphorylated species of MBP are contributing to the formation of the MDL as well as to the compaction of the myelin membrane.
CHAPTER 5
CHARACTERIZATION OF MBP IN NATURAL MUTANT AND TRANSGENIC MOUSE BRAIN

In the present study, we will attempt to demonstrate that the expression of MBP isoforms and the production of charge isomers are necessary for myelination in mouse brain. This will be done by characterizing the MBP isolated from brains of three different mice: the dysmyelinating shiverer mouse; the transgenic shiverer mouse; and the demyelinating ND4 mouse.

Briefly, the homozygous shiverer mouse, a mutant that carries a deletion of exons 3 to 7 in the 7 exon-MBP gene (Roach et al. 1985), bears a dysmyelinating phenotype and is characterized by the absence of mature MBP. In attempts to establish the association of the genetic lesion with dysmyelination, a transgenic shiverer mouse was produced by Readhead et al (1987) to carry a transgene that encoded for wild-type MBP (21.5 kDa). With both MBP transcript and protein levels at 25% of normal, these mice appeared to have been rescued from dysmyelination and were able to synthesize compact myelin although in small amounts (Readhead et al. 1987). In a follow-up study, the extent of myelination in the above-mentioned mouse models was shown to correlate with the expression levels of MBP transcripts (Shine et al. 1992). Unfortunately, MBP characterization at the protein level and its correlation with myelination in the above-mentioned studies were not done.

The ND4 mouse will be the third mouse model used in this study. Briefly, the ND4 mouse, which carries 70 copies of the human cDNA of the proteolipid DM20 on a normal background, has been shown to spontaneously demyelinate (Mastronardi et al. 1993). After 3 months of normal growth, the animal developed a wobbling gait, tremors and seizures and by 8 to 10 months, death followed. MBP transcript and protein levels were unchanged from normal although changes in the cationic properties of MBP have
been reported (Mastronardi et al., 1996). Because alterations in the cationicity of MBP in the ND4 mouse brain were similar to that in victims of the human demyelinating disease multiple sclerosis (Moscarello et al., 1994), it was necessary to study the role of MBP isomers during pathogenesis in the ND4 mouse.

**Results**

**Characterization of Acid-soluble protein from homozygous shiverer mouse brains**

Following isolation from each of five shiverer brains (See Methods), acid-soluble proteins were electrophoresed on an SDS-polyacrylamide gel and analyzed by Western blot (Figure 5.1 and 5.2, respectively). In Figure 5.1, the shiverer samples (Lane S) consisted of several acid-soluble brain proteins. Although several proteins were present in the normal sample (Lane N), the 14, 17, 18.5 and 21.5 kDa MBP isoforms were present as intensely stained bands. As expected, these bands were absent in the shiverer samples. In order to confirm the absence of the major MBP isoforms, the material used in Figure 5.1 was subjected to SDS-PAGE, transferred onto nitrocellulose membrane and probed with several anti-MBP antibodies. The Western blots are shown in Figure 5.2.

A polyclonal antibody reactive with MBP was used in Figure 5.2.A. The 14, 17, 18.5, 21.5 and 32 kDa isoforms in the normal sample (lane N) were reactive with the polyclonal antibody. In the case of the shiverer samples (lane S), faint bands representing a doublet (31 and 35 kDa, as indicated by an arrow) were detected. Reactivity with a polyclonal anti-MBP antibody suggests that some MBP-related material is present in the shiverer mouse. However, non-specific binding of the polyclonal antibody cannot be dismissed. In order to confirm the presence of these anti-MBP-reactive species, two separate monoclonal antibodies were used. Clone 26 (mAb 26)
Figure 5.1 SDS-PAGE of acid-soluble material isolated from homozygous shiverer mouse brain.

In the above figure, S represents the individual 2 month old shiverer samples. N corresponds to the 2 month old normal sample and H is the human MBP (18.5 kDa) marker. The molecular weight standards are in the far left lane.
Figure 5.2  Western blot analysis of acid-soluble protein isolated from homozygous
*shiverer* mouse brain.

For (A), (B) and (C), the following lane assignments were used: lane S, 2 month old
*shiverer* samples; lane N, 2 month old normal; lane H, human MBP (18.5 kDa).
Molecular weight standards are in the far left lane. The following antibodies were used:
(A) polyclonal rabbit anti-bovine MBP; (B) monoclonal antibody (mAb) reactive against
residues 69 to 74 (exon 3) of the human MBP sequence; and (C) monoclonal antibody (mAb 22)
reactive against residues 83 to 88 (exon 3) of the human MBP sequence.
recognizes residues 69 to 74 (YGSLPQ) in human MBP. The human sequence at residues 69 to 74 is 100% conserved with that in mouse and is situated in exon 3. The other monoclonal antibody, clone 22 (mAb 22), is reactive with residues 82 to 87 (DENPVV) of the human MBP sequence. This peptide sequence, which is also in exon 3, is conserved 100% between human and murine MBP.

The lesion in the shiverer mouse has been described as a deletion of exons 3 to 7 in the MBP gene (Roach et al. 1985). Because both mAb-26 and mAb 22 recognize exon 3 peptide sequences, we should expect neither of these antibodies to react. Interestingly, in Figures 5.2.B and C, the shiverer material contains high-molecular weight proteins that reacted with both monoclonal antibodies. The major band in the acid-soluble shiverer sample has an approximate molecular weight of 35 kDa. Similar reactivity was detected with the polyclonal antibody in Figure 5.2.A. Additional reactivity with the monoclonal antibodies was detected with proteins ranging from 40 to 100 kDa in all samples.

The above-mentioned Western blot analyses demonstrated that HMW proteins carrying MBP peptide sequences are being expressed in the shiverer mouse. The HMW doublet most likely represents isoforms of the golli-mbp gene (Campagnoni et al. 1993). BG21 (31 kDa) and J37 (35 kDa) (See Introduction for more information). Previous studies by Landry et al (1996) have demonstrated that the BG21 isoform was present in 16 day old shiverer mouse brain. Not only were we able to identify the BG21 isoform in shiverer brain but the use of anti-MBP exon 3 antibodies allowed us to demonstrate the presence of J37, the only golli-mbp isoform carrying exon 3 of the classic MBP gene although other exons are present (See Introduction). The unexpected presence of the J37 isoform suggests that the golli-mbp gene is not affected by the shiverer mutation.

The presence of charge microheterogeneity of the MBP-related species in shiverer brain was determined by alkaline-urea gel electrophoresis (Figure 5.3). Five individual
Figure 5.3 MBP Charge microheterogeneity in homozygous *shiverer* mouse brain.

Alkaline-urea gel electrophoresis of acid-soluble material isolated from 2 month old homozygous *shiverer* mouse brain (lane S), 2 month old normal mouse brain (lane N) and human MBP C-1 (lane H).
acid-soluble samples isolated from shiverer whole brains were electrophoresed on an alkaline-urea gel along with acid-soluble normal material (2 months old) and human MBP C-1 (Figure 5.3). The shiverer samples contained acid-soluble material that was basic enough to enter the gel (i.e. the faint band near the top of the gel). A Western blot (data not shown) performed on the same samples as in Figure 5.3 demonstrated that the shiverer acid-soluble material that migrated into the gel was not reactive with anti-MBP antibody.

N-terminal sequencing and amino acid analysis of the 35 kDa protein in shiverer brain

In order to determine whether the 35 kDa protein in shiverer brain represents an isoform of golli-mbp, the protein was subjected to N-terminal protein sequencing and amino acid analysis. Briefly, acid-soluble material isolated from homozygous shiverer brain was separated on an SDS-polyacrylamide gel containing 15 lanes. 30 μg of material was applied to each well. One lane was cut out and stained with Coomasie Blue. The rest of the gel was transferred onto Immobilon P membrane. Following the transfer, one lane on the membrane was subjected to Western blot analysis using a monoclonal antibody clone 26 which is specific for exon 3 MBP peptide sequences. Western blot analysis was used as a control to determine the migration distance of the immunoreactive 35 kDa protein. The remaining lanes containing the acid-soluble proteins on the membrane were stained with Coomasie Blue. Using the Western blot analyzed lane as a control, the 35 kDa protein band was cut out and subjected to protein sequencing.

An N-terminal protein sequence of the 35 kDa protein was not obtained suggesting that (1) the N-terminus was blocked or (2) not enough protein was available for sequencing (20 pmol to 5 nmol of sample is required). In order to determine whether sufficient amounts of protein were present, amino acid analysis of the same sample was performed. The amino acid composition is shown in Table 5.1. From the amino acid analysis, it was determined that approximately 6.2 nmol of protein was present in the
Table 5.1  Amino acid analysis of the 35 kDa protein, immunoreactive with anti-MBP exon 3 antibodies, from homozygous *shiverer* mouse brain following SDS-PAGE and transfer onto Immobilon P membrane.

<table>
<thead>
<tr>
<th>Residue</th>
<th>nmol</th>
<th>ng</th>
<th>res/100</th>
<th>golli-mbp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>0.53</td>
<td>61.09</td>
<td>8.52</td>
<td>12.0</td>
</tr>
<tr>
<td>Glx</td>
<td>0.99</td>
<td>127.28</td>
<td>15.83</td>
<td>9.3</td>
</tr>
<tr>
<td>Ser</td>
<td>0.47</td>
<td>41.23</td>
<td>7.60</td>
<td>10.8</td>
</tr>
<tr>
<td>Gly</td>
<td>0.77</td>
<td>44.14</td>
<td>12.41</td>
<td>10.5</td>
</tr>
<tr>
<td>His</td>
<td>0.14</td>
<td>18.57</td>
<td>2.17</td>
<td>4.8</td>
</tr>
<tr>
<td>Arg</td>
<td>0.32</td>
<td>50.24</td>
<td>5.17</td>
<td>9.3</td>
</tr>
<tr>
<td>Thr</td>
<td>0.31</td>
<td>31.51</td>
<td>5.00</td>
<td>6.8</td>
</tr>
<tr>
<td>Ala</td>
<td>0.57</td>
<td>40.30</td>
<td>9.10</td>
<td>8.1</td>
</tr>
<tr>
<td>Pro</td>
<td>0.33</td>
<td>31.62</td>
<td>5.23</td>
<td>6.8</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.17</td>
<td>27.68</td>
<td>2.72</td>
<td>0.8</td>
</tr>
<tr>
<td>Val</td>
<td>0.41</td>
<td>40.95</td>
<td>6.64</td>
<td>2.8</td>
</tr>
<tr>
<td>Met</td>
<td>0.05</td>
<td>6.97</td>
<td>0.85</td>
<td>1.6</td>
</tr>
<tr>
<td>Cys</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ile</td>
<td>0.23</td>
<td>25.56</td>
<td>3.63</td>
<td>2.8</td>
</tr>
<tr>
<td>Leu</td>
<td>0.47</td>
<td>53.22</td>
<td>7.55</td>
<td>4.0</td>
</tr>
<tr>
<td>Phe</td>
<td>0.16</td>
<td>23.10</td>
<td>2.52</td>
<td>3.2</td>
</tr>
<tr>
<td>Lys</td>
<td>0.30</td>
<td>38.38</td>
<td>4.81</td>
<td>6.4</td>
</tr>
</tbody>
</table>

**TOTALS** | 6.23 | 663.37 | 100.00 | 100.0 |

(Campagnoni et al. 1993)
sample suggesting that (1) sufficient amounts of protein were submitted for N-terminal sequencing and (2) the N-terminal residue of the protein was blocked. Furthermore, the amino acid composition of the analyzed sample drew some similarities with the predicted values of Campagnoni et al (1993) suggesting that the 35 kDa protein may correspond to the J37 isoform of golli-mbp.

**Characterization of MBP isolated from transgenic shiverer mice.**

Transgenic *shiverer* mouse brains (30 days old), homozygous for the wild-type MBP transgene, were obtained from the laboratory of Dr. C. Readhead. MBP was isolated from these mouse brains as described in Methods.

To confirm the presence of MBP isoforms in the transgenic *shiverer* mouse brains, MBP was electrophoresed on SDS-polyacrylamide gels and characterized by Western immunoblot analysis (Figure 5.4.A and B, respectively). Identification of the isoform composition of the MBP in the transgenic *shiverer* mouse was previously suggested by SDS-PAGE and Coomassie blue staining (Readhead et al, 1987). From this gel system, Readhead et al (1987) concluded that the 14, 17, 18.5 and 21.5 kDa isoforms were present in the transgenic mouse. However, a Western blot, which would have confirmed the presence of these isoforms, was not done.

In the SDS-polyacrylamide gel (Figure 5.4.A), the faint 14, 17, 18.5 and 21.5 kDa bands in the transgenic (lane T) mouse sample suggested that these isoforms were either absent or present in small amounts. As expected, intense bands corresponding to the major isoforms were present in the normal (Lane N) and absent in the *shiverer* (Lane S). Several bands corresponding to acid-soluble brain proteins were present in each of the samples.
Figure 5.4  Expression of MBP isoforms in transgenic shiverer mouse brain.

SDS-PAGE (A) and Western blot (B) of acid-soluble material isolated from 2 month old homozygous shiverer (lane S), 1 month old transgenic shiverer (lane T), 1 month old normal mouse brains (lane N) and human MBP (18.5 kDa). Lane M in (B) corresponds to murine MBP C-1 (14 kDa). Rabbit anti-bovine MBP antibody was used as primary antibody in (B).
Western blot analysis (Figure 5.4.B) was used to demonstrate the presence of translational products arising from the transgene. The samples in Figure 5.4.A were fractionated on SDS-PAGE, transferred onto nitrocellulose membrane and probed with a polyclonal anti-MBP antibody. The acid-soluble protein isolated from 30 day old normal brain (Lane N) consisted of the major MBP isoforms (14, 17, 18.5 and 21.5 kDa) and an immunoreactive species (doublet) at approximately 35 kDa. In lane S, the shiverer material consisted of MBP-reactive species that are represented by a single band at approximately 15 kDa and a doublet (31 and 35 kDa). Similar patterns of immunoreactivity were obtained earlier (Figure 5.2.A). In the transgenic mouse (Lane T), the presence of the 18.5 kDa isoform (not 14, 17 or 21.5 kDa) suggested that this MBP isoform was the only MBP synthesized in the presence of the transgene even though genomic DNA was used to generate the transgenic line.

In order to confirm the presence or absence of MBP isoforms in transgenic brain, enhanced chemiluminescence (ECL), a more sensitive technique in detecting immunoreactive material, was used in the following dilution study (Figure 5.5). Acid-soluble material isolated from 11 day normal brain (15, 7.5 and 3 ug: lanes 2, 3 and 4 respectively), 30 day transgenic shiverer brain (15, 30 and 75 ug: lanes 5, 6 and 7, respectively) and 30 day old normal brain (15, 7.5 and 3 ug: lanes 8, 9 and 10, respectively) were loaded onto a SDS-polyacrylamide gel and transferred onto nitrocellulose membrane which was probed with rabbit-anti-bovine MBP antibody. With increasing amounts of transgenic shiverer acid-soluble material, the intensity of the 18.5 and 15 kDa proteins increased. A faint band corresponding to the 21.5 kDa isoform was also detected. The 14 and 17 kDa isoforms were not detected even with longer exposure times.
Figure 5.5   Enhanced Chemi Luminescence (ECL) Detection of major MBP isoforms in acid-soluble material isolated from 30 day old transgenic *shiverer* mouse brain.

Acid-soluble material isolated from 11 day old normal brain (15, 7.5 and 3 ug; lanes 1 to 3, respectively). 30 day old transgenic *shiverer* brain (15, 30 and 75 ug; lanes 4 to 6, respectively), and 30 day old normal brain (15, 7.5 and 3 ug; lanes 7 to 9, respectively) were loaded onto an SDS-polyacrylamide gel, transferred onto nitrocellulose and incubated with rabbit anti-bovine MBP antibody. Lane 10 corresponds to human MBP (18.5 kDa). Exposure to film followed detection of MBP reactive material using ECL.
The relative proportions of the MBP isoforms in Figure 5.5 are shown in Table 5.2. In normal mouse brains, significant levels of the major isoforms were observed. In the 11 day old normal mouse brain, the MBP isoforms (14, 17, 18.5 and 21.5 kDa) were represented by the following percentages: 36.8, 18.3, 32.1 and 12.7 %, respectively. By day 30 in the normal mouse, these percentages changed to 41.2, 17.6, 26.9 and 14.2 %. In the 30 day old transgenic mouse brain, the 14 and 17 kDa isoforms were not detected. The major isoform was represented by the 18.5 kDa species at 73.0 %; 27.0 % of the MBP was 21.5 kDa.

Table 5.2 Relative proportions of the Major MBP isoforms in the acid-soluble fractions isolated from transgenic shiverer and normal mouse brains.a

<table>
<thead>
<tr>
<th>Type of mouse</th>
<th>% 21.5 kDa</th>
<th>% 18.5 kDa</th>
<th>% 17 kDa</th>
<th>% 14 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 day normal</td>
<td>12.8b</td>
<td>32.1</td>
<td>18.3</td>
<td>36.8</td>
</tr>
<tr>
<td>30 day normal</td>
<td>14.3</td>
<td>26.9</td>
<td>17.6</td>
<td>41.2</td>
</tr>
<tr>
<td>30 day transgenic shiverer</td>
<td>27.0</td>
<td>73.0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a Following a scan of Figure 5.5, values for the table were obtained by measuring the band intensities of 14, 17, 18.5 and 21.5 kDa isoforms. The percentages were computed by using the following equation.

\[ \text{measure intensity of chosen isoform} \times 100\% \]
\[ \text{sum of the intensities for the four major isoforms} \]

b. The values represent mean percentages of two events (range ± 2.0 %)
MBP Charge Microheterogeneity in the Transgenic shiverer mouse

In order to determine whether the transgenic shiverer mouse is capable of generating MBP charge isomers via post-translational modification, acid-soluble material isolated from 30 day old transgenic shiverer brain (Lane 3; Figure 5.6) was electrophoresed on alkaline-urea slab gels along with 4 and 30 day old normal material (Lanes 1 and 2, respectively). Previous studies in Chapter 3 have shown that adult mouse MBP is composed of two populations of charge isomers: the isomers of the 14 and 18.5 kDa isoforms. It was demonstrated that isomers of the 18.5 kDa isoform do not migrate as far as those of the 14 kDa isoform. This pattern of charge microheterogeneity was observed in the acid-soluble fraction of the 30 day old normal mouse (Lane 2). In the early myelinating animal (4 day old mouse: Lane 1), the existing isomers were represented by the 18.5 kDa isoform. The MBP charge microheterogeneity in the transgenic shiverer mouse (Lane 3) consisted of the 18.5 kDa isomers suggesting that the transgenic mouse possessed the machinery to splice the 21.5 kDa message and to post-translationally modify MBP. Interestingly, similar patterns of MBP charge microheterogeneity were observed between the transgenic mouse and 4 day old normal mouse suggesting that the MBP in the adult transgenic brain may be at a similar stage of development as that found in an early myelinating mouse.

The relative proportions of C-8 and C-1 in the acid-soluble material isolated from normal and transgenic brains are shown in Table 5.3. In the 4 day old normal mouse, C-8 represented 51.3% of the MBP population while C-1 of the 18.5 kDa constituted 5.7%. C-1 of the 14 kDa isoforms was not detected. By 30 days in normal brain, the C-8 levels dropped to 14.7%, the C-1 (18.5 kDa) increased to 15.2% and C-1 (14 kDa) expression levels represented 21.5% of the total. The increase in the expression of the C-1 (14 kDa) was reflected in the C-8/C-1 ratio. From 4 to 30 days in normal development, the C-8/C-1 ratio dropped from 9.0 to 0.40. In the 30 day old transgenic shiverer brain, C-8 levels
Figure 5.6 MBP Charge microheterogeneity in transgenic *shiverer* mouse brain.

Acid-soluble material isolated from 4 day old normal (lane 1), 30 day old normal (lane 2) and 30 day old transgenic *shiverer* mouse brains (lane 3) were electrophoresed on an alkaline-urea slab gel. Bands were visualized using the procedure outlined in the Methods section.
were high (53.8%) and C-1 (18.5 kDa) represented 3.6% of the total isomer population. The 14 kDa isoform was not detected.

Table 5.3  Relative Proportions of MBP Charge Isomers in Normal and Transgenic shiverer Brain.

| Type of mouse | C-8 %<sup>a</sup> | C-1 (18.5 kDa) % | C-1 (14 kDa) % | C-8  
|---------------|------------------|-----------------|----------------|--------
| 4 day normal  | 51.3             | 5.7             | -              | 9.0    |
| 30 day normal | 14.7             | 15.2            | 21.5           | 0.40   |
| 30 day transgenic shiverer | 53.8 | 3.6 | - | 14.9 |

<sup>a</sup> After measuring the band intensities on an alkaline-urea gel with an NIH computer program, the percentages of charge isomers were calculated as follows:

\[
\text{percent charge isomer} = \left( \frac{\text{band intensity of desired band}}{\text{total intensity of the material on the gel}} \right) \times 100
\]

Phosphorylation of Thr-95 MBP in the transgenic shiverer mouse brain

From earlier studies, phosphorylation on Thr-95 of mouse MBP is a modification that is found in murine brain as early as post-natal day 10 (Chapter 4. Figure 4.2). In order to determine whether such a modification is partially responsible for the MBP charge microheterogeneity observed in transgenic brain (Figure 5.6), acid-soluble material isolated from normal and transgenic brain was separated on an SDS-polyacrylamide gel, transferred onto nitrocellulose membrane and incubated with
Figure 5.7  MBP phosphorylation in transgenic *shiverer* brain as determined by Western blot.

Acid-soluble material isolated from transgenic and normal brains was separated by SDS-PAGE, transferred onto nitrocellulose membrane and incubated with monoclonal P12 antibody reactive with phosphorylated Thr-95 murine MBP. Lane 1, molecular weight standards: lane 2, post-natal day 4 normal; lane 3, 30 day old transgenic *shiverer*; lanes 4 to 6, post-natal days 11, 17 and 30 normal, respectively; lane 7, human MBP (18.5 kDa).
monoclonal (P12) antibody reactive with murine MBP species phosphorylated at Thr-95. The Western blot is shown in Figure 5.7. Phosphorylated MBP isoforms were absent at post-natal day 4 (lane 2) whereas at post-natal days 11, 17 and 30 (lanes 4, 5 and 6, respectively), all major isoforms were immunoreactive with the P12 antibody. In the 30 day old transgenic brain (lane 3), immunoreactivity with P12 antibody was absent. Because a similar finding was observed with the 4 day old normal mouse, the absence of this particular modification suggests that the MBP in the transgenic brain is at a stage similar to that of an early myelinating mouse.

MBP Expression in the Demyelinating ND4 Mouse (Normal Background)

Following the identification of ND4 transgenic mice by Southern dot blot analysis of DNA isolated from mouse tails (See Methods), acid-soluble material was isolated from whole brains of ND4 mice of varying ages (from 11 days to 9 months) as described in Methods. In Figure 5.8, the 14, 17, 18.5 and 21.5 kDa MBP isoforms were present as distinct bands from post-natal day 11 to 270 in ND4 development. A corresponding Western blot confirmed the presence of MBP (data not shown). These findings, which have been previously reported by Mastronardi et al (1996), suggested that the major MBP isoforms were normally expressed in the developing ND4 brain.

In order to assess the charge microheterogeneity of MBP in the ND4 mouse before onset of and during demyelination, the acid-soluble material was electrophoresed on an alkaline-urea gel at pH 10.6 (Figure 5.9). The MBP isomers during early stages of development (days 11 and 14; Lanes 1 and 2) consisted mainly of the least cationic species (mostly 18.5 kDa). From day 20 to 270 (Lanes 2 to 10; respectively), the expression of the more cationic MBP isomers (14 kDa) was detected. The C-8 expression levels were lowest by day 90 (3 months; Lane 7) and increased thereafter.
Figure 5.8  SDS-PAGE of acid-soluble material isolated from ND4 transgenic mouse brains.

The lane assignments are the following: lane 1, molecular weight standards; lanes 2 to 8, post-natal days 11, 14, 20, 60, 90, 180 and 210 to 270 ND4 transgenic, respectively; lane 10, 18 day old normal; lane 11, 180 day normal; and lane 12, human MBP (18.5 kDa).
Figure 5.9  Alkaline-urea gel electrophoresis of acid-soluble material isolated from ND4 mouse brains.

Lanes 1 to 10 correspond to the following ages: post-natal days 11, 14, 20, 24, 30, 60, 90, 120, 180 and 210 to 270.
Interestingly, the change in C-8 expression at 3 months coincided with the onset of demyelination in the ND4 mouse.

The relative proportions of the C-8 and C-1 isomers in the ND4 mouse brain are shown in Table 5.4. From post-natal day 11 to 60, the C-8 levels remained high (above 30%). Over the same period of growth in the ND4 mouse, C-1 levels for the 18.5 and 14 kDa isoforms decreased about 8% and 4%, respectively. C-8 levels were lowest (20%) by 90 days with C-1(18.5 kDa) and C-1(14 kDa) at 16% and 25%, respectively. After 3 months, demyelination in the ND4 mouse brain commenced. During this time, C-8 levels increased whereas C-1 levels for both 18.5 and 14 kDa isoforms decreased. High C-8/C-1 ratios were prominent during early development in ND4 mouse brain and also during demyelination (after 90 days) but were lowest on post-natal day 90.

Table 5.4 Relative Proportions of MBP Charge Isomers During Myelogenesis in the ND4 mouse

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>C-8 %</th>
<th>C-1(18.5 kDa) %</th>
<th>C-1(14 kDa) %</th>
<th>C-8 %</th>
<th>C-1 (18.5 + 14 kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>33.1</td>
<td>16.5</td>
<td>9.4</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>52.9</td>
<td>16.3</td>
<td>3.9</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>42.3</td>
<td>16.0</td>
<td>13.6</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>35.0</td>
<td>14.2</td>
<td>7.5</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>34.2</td>
<td>15.0</td>
<td>8.2</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>41.6</td>
<td>8.9</td>
<td>5.7</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>20.0</td>
<td>16</td>
<td>25</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>34.9</td>
<td>10.7</td>
<td>8.1</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>240-300</td>
<td>30.8</td>
<td>10.5</td>
<td>13.8</td>
<td>1.30</td>
<td></td>
</tr>
</tbody>
</table>

a After measuring the band intensities on an alkaline-urea gel with an NIH computer program, the percentages of charge isomers were calculated as follows:

\[
\left(\frac{\text{band intensity of desired band}}{\text{total intensity of the material on the gel}}\right) \times 100 = \% \text{ charge isomer}
\]
Summary

In our studies with the dysmyelinating *shiverer* mouse, we were able to demonstrate the absence of the major MBP isoforms (14, 17, 18.5 and 21.5 kDa), in agreement with earlier reports. However, we also identified HMW proteins immunoreactive with several anti-MBP antibodies in *shiverer* brain. It was postulated that these HMW proteins may represent isoforms of the golli-mbp complex (Campagnoni et al. 1993). Previous studies have demonstrated immunoreactivity of the two major golli-mbp isoforms (BG21 and J37, 31 and 35 kDa, respectively) with anti-golli-mbp and anti-MBP antibodies (Landry et al. 1996). The BG21 isoform has been detected in 16 day old *shiverer* brain homogenates (Landry et al., 1996) suggesting that golli-mbp was being expressed in this natural mutant. In our studies, we were able to detect the BG21 golli-mbp isoform (31 kDa). In light of the *shiverer* mutation (a deletion of exons 3 to 7 from the MBP gene), immunoreactivity of another HMW protein (~35 kDa) with anti-MBP-exon 3 antibodies was completely unexpected. Amino acid analysis of the 35 kDa protein demonstrated an amino acid composition similar to that of the J37 golli-mbp isoform (Campagnoni et al. 1993). If this protein is in fact the J37 golli-mbp isoform, the only golli-mbp isoform carrying exon 3 of the classical MBP gene (See Introduction), these findings would suggest that (1) the classical MBP exon 3 (exon 7 in golli-mbp) is intact in the golli-mbp gene. (2) the golli-mbp gene is not affected by the *shiverer* mutation and (3) the golli-mbp and MBP genes may be separate entities. Although further studies are required to confirm the identity of these HMW proteins, the presence of these proteins, which have been reported to be highly expressed during immature stages of development (Campagnoni et al. 1993), suggest that development in the adult *shiverer* brain may be arrested at an early stage.

In the transgenic *shiverer* mouse which synthesized some compact myelin, expression of a transgene on a *shiverer* background allowed for the synthesis of the 18.5 kDa isoform and corresponding isomers, although traces of the 21.5 kDa isoforms were
detected. In the 1 month old transgenic shiverer mouse, the MBP isomers were expressed in patterns similar to that of a 4 day old normal mouse (i.e. mostly isomers of the 18.5 kDa isoform). The absence of phosphorylation on Thr-95 was also common for both 30 day old transgenic and 4 day old normal mice. Because MBP carrying a phosphate group at this site has been localized to the major dense line (MDL) in myelin (Yon et al. 1996), the absence of this modified form in the transgenic shiverer brain suggests that there may be defects in the formation of the MDL although an intact MDL was demonstrated by electron microscopy (Readhead et al. 1987). Because both MBP charge microheterogeneity and the absence of phosphorylation of MBP on Thr-95 are similar between the transgenic shiverer and 4 day old normal mouse, it suggests that the MBP in the transgenic brain may be expressed in a developmental stage that reflects that of an immature animal.

The above studies on the transgenic (21.5 kDa) shiverer mouse suggest that the 18.5 kDa isoform may be involved in myelin compaction in transgenic shiverer brain although the involvement of other isoforms cannot be ruled out. A study by Kimura et al (1989) has shown that the introduction of a 14 kDa MBP transgene into a shiverer mouse can lead to the synthesis of compact myelin. Although the translational products of the 14 kDa MBP transgene were identified by immunohistochemistry on transgenic mouse brain slices. Western blot analysis of the MBP was not provided which would have given a more definitive answer concerning the identity of the MBP. Further studies on the shiverer, shiverer-myelin deficient, and transgenic mice have shown that the the extent of myelination depended on the MBP transcript levels (Shine et al. 1992). Unfortunately, the immunological identification of MBP isoforms, the production of charge isomers and their correlation with myelination were not provided by the study of Shine et al (1992).

In the ND4 mouse, the major MBP isoforms were expressed throughout development. In addition, charge microheterogeneity in the developing ND4 mouse brain appeared normal from post-natal day 11 to 3 months. High C-8/C-1 ratios,
resulting from the prominent levels of the 18.5 kDa isomers. were observed in young mice. Although isomers of the 14 kDa isoform appeared by day 20 (or earlier), the expression levels of the 18.5 kDa isomers remained relatively high up to 3 months. At 3 months, the low C-8/C-1 levels suggested that the myelin membrane in the ND4 mouse brain was mature and stable. However, alterations in the overall cationic properties of MBP followed. C-8 levels from 4 to 10 months were higher than at 3 months. The increase in the number of less cationic isomers was reflected in the increased C-8/C-1 ratios. These findings suggest that alterations in MBP charge microheterogeneity in ND4 mouse brain may be important for membrane destabilizational events leading to demyelination.
CHAPTER 6

DISCUSSION

The heterogeneous nature of MBP renders the study of MBP complicated. Like human MBP, the classical MBP gene in mouse brain consists of 7 exons. Alternative splicing of the MBP message gives rise to the synthesis of several isoforms. Murine isoforms of MBP, arising from the excision of exons 2, 5 and/or 6 from the seven exon transcriptional unit, are represented by the 14, 17, 18.5 and 21.5 kDa species in which the expression of these isoforms are developmentally regulated. During immature stages of myelination, the levels of the exon-2-containing 17 and 21.5 kDa isoforms are higher than at later developmental stages suggesting that these particular isoforms may be involved in myelin formation. The expression of exon-2 lacking isoforms (14 and 18.5 kDa) increased with development suggesting that myelin maturation and compaction may require the production of these isoforms. Size heterogeneity of MBP also includes high-molecular weight (HMW) proteins that possess immunoreactive epitopes common with MBP although the origin of some of these proteins is unknown. Some HMW proteins have been suggested to be products of the golli-mbp gene, an 11 exon gene that is believed to contain the classical MBP gene.

The heterogeneous representation of MBP is further complicated by the presence of post-translationally modified species of MBP. In human brain, MBP can undergo approximately 20 to 25 post-translational modifications, some of which are responsible for the charge microheterogeneity of MBP, the production of charge isomers. Individual charge isomers or components differ from one another on the basis of net positive charge. Human MBP component 1 (C-1) is defined as the most cationic and least modified isomer whereas the least cationic yet most modified isomer is defined as C-8. Alterations in the patterns of MBP charge microheterogeneity during development suggest that certain
isomers may be required for certain stages of myelination. Less cationic isomers (i.e. C-8) are highly produced during myelin formation whereas the more cationic isomers, especially C-1, are most abundant during maturation and compaction of the myelin membrane.

From extensive studies on the characterization of post-translational modifications on human MBP, it was suggested that certain modifications may be indicative of a more defined function for MBP. MBP species carrying a phosphorylated Thr-98 localized exclusively to the major dense line (MDL) whereas deiminated species of MBP (i.e. C-8) localized to the intraperiod line (IPL) suggesting that (1) such modified forms may be involved in the MDL or IPL formation, respectively or (2) the type of modification may dictate where a certain isomer is to be localized. Other modifications suggest that MBP may be involved in a signalling pathway. ADP-ribosylation and GTP binding render MBP a possible candidate as a G-protein. Phosphorylation of MBP at Thr-98 suggests that MBP may act as a substrate for MAP kinase, a well-known member of signalling cascades. Although alterations in the expression of isoforms and production of isomers during development may suggest possible functions for MBP, knowledge of post-translational modifications provides a better understanding of the role of MBP in myelinogenesis.

In recent years, mouse models have been used as tools for elucidating the roles of MBP and other myelin constituents in myelin formation and structure. Well-characterized dysmyelinating mouse models include the autosomal mutants which contain mutations in the MBP gene (shiverer, shiverer-myelin deficient) and the X-linked mutants which carry a defective PLP gene (jimpy, myelin synthesis deficient, rumpshaker). Although extensive studies on these dysmyelinating mutants have focused on establishing how genetic perturbations in these mice affect MBP expression, the role of MBP during myelinogenesis remains unclear mainly because very little is known about MBP in mouse brain.
In order to gain a better understanding of the role of MBP in murine myelinogenesis, more information about MBP is needed. Our first study involved the isolation and characterization of individual murine MBP charge isomers. In this study, the use of electrospray ionization mass spectrometry on isolated MBP components was instrumental in determining molecular weights from which possible post-translational modifications were deduced and how these modifications may be responsible for the production of these charge isomers. We also examined the production of MBP isomers in developing normal mouse brain to see whether certain components were preferentially generated during certain stages of myelinogenesis. These studies were followed by an examination of how genetic perturbations in mutant and transgenic mice affected MBP in correlation with myelination.

The initial study was undertaken to assess the protein modifications responsible for the charge isomers found in the mouse. Because small amounts of MBP were available from mouse brain, two techniques were adapted to accommodate for the small amounts. The first technique, the alkaline-urea tube gel system generally used to study charge microheterogeneity, was adapted to a slab gel system which would resolve small amounts of protein and several samples simultaneously. Our method using a high percentage resolving gel in conjunction with a stacking gel provided good reproducibility, sharp bands and from these gels, Western blot were readily obtained.

The next essential step was to scale down the CM-52 column chromatographic system for use with samples of about 10 OD units compared to about 100 OD units of bovine and human samples generally applied to the large columns. Because murine charge isomers are generally more cationic than those of human or bovine MBP, it was necessary to increase the sodium chloride concentration in the eluting buffer from 0.2 M to 0.75 M in order to obtain the resolution in Figure 3.2.

Murine MBP is not only heterogeneous in size (i.e. isoforms) but also in charge. Using the alkaline-urea slab gel, we were able to resolve murine MBP into several bands,
each of which corresponding to a single charged species. Because Western blots were readily obtained from these gels, we were able to demonstrate that the charge microheterogeneity was represented by two groups of isomers: the 14 and 18.5 kDa isoforms. However, the production of modified forms of the 17 and 21.5 kDa isoforms cannot be ruled out.

Following the isolation of these charge isomers, characterization studies provided some interesting observations. C-1 and C-2 were enriched in the 14 kDa isoform in agreement with the report of Fannon and Moscarello (1991) using the original CM-52 column with 110 O.D. units of starting material. Accurate molecular weights, from which possible post-translational modifications can be deduced, were obtained by electrospray-ionization mass spectrometry which was not available in the 1991 study. The postulated modifications on C-1 were an acetylation of the N-terminal alanine and a mono-methylation of an arginyl residue. Surprisingly, C-2 was represented by two proteins that differed by 65 amu. The lower molecular weight (LMW) species of C-2 has similar modifications to C-1 (acetylation, methylation). Because C-2 differed from C-1 by a single net charge (by analogy with human and bovine MBP), the modification responsible for the charge difference was postulated to be a deamidation. Assignment of possible post-translational modifications on the higher molecular weight (HMW) species of C-2 provided three possible scenarios. One set of modifications is to have an acetylation and phosphorylation producing a net charge difference of two with C-1. The phosphorylation was confirmed by reactivity with P12, a monoclonal antibody that reacts with MBP phosphorylated on Thr-98 in human MBP or Thr-95 in murine MBP. In order for the HMW C-2 to migrate with the LMW species, the net charge must be compensated by an additional positive charge which cannot be accounted for at this time. Another possibility is to have a heterogeneously acylated N-terminus (acetyl, butyl and hexyl), as suggested by the broad mass spectral peaks, and a dimethyated arginine accompanied by a single deamidation which may account for the charge difference with C-1. If both sets of modification took
The calculated mass difference between HMW C-2 and unmodified 14 kDa may be an average representation of the two modifications.

C-3 is the first isomer which contains both the 14 and 18.5 kDa isoforms. Again by analogy with human and bovine MBP C-3, the modifications were postulated to be a monomethylation and a phosphorylation. Phosphorylation was confirmed by P12 reactivity. The 18.5 kDa isoform, which represents the most cationic 18.5 kDa variant, was postulated to be monomethylated on an arginine and acetylated at the N-terminus, analogous to bovine and human MBP. The 18.5 kDa isoform did not react with the P12 antibody, a confirmation for the absence of phosphorylation.

Alterations in the cationicity of MBP appear to reflect certain stages in the developing brain. In the early myelinating animal (murine or human), the MBP is enriched in the less cationic isomers whereas the most cationic isomers predominate during maturation. In this respect, the developmental production of charge isomers in mouse brain is similar to that in human. In infantile brain, the least cationic isomer C-8 (18.5 kDa) is the only species present whereas the most cationic isomer (18.5 kDa) predominates during the adult phases of development (Moscarello et al. 1994). Because murine MBP is more complex than human MBP (i.e. more isoforms and isomers), the patterns of charge microheterogeneity in the developing mouse brain were very different from that of human. Isomers of murine 18.5 kDa MBP species, including the most cationic 18.5 kDa isoform, were most abundant during stages before post-natal day 14-15, just before the peak of myelination. With myelin maturation in mouse brain, an increase in the production of the more cationic isomers (14 kDa) was observed. Thus, cationicity of MBP seems important in both murine and human brain for myelin maturation and membrane stabilization.

The production of the 14 kDa species during active myelination suggests that this isoform may be involved in the compaction of myelin. A similar conclusion was proposed by Kimura et al (1989) from their studies on their transgenic mouse which was generated by introducing a transgene encoding the 14 kDa MBP onto a *shiverer* background. It was
demonstrated that the introduction of the 14 kDa transgene allowed for compact myelin formation suggesting that myelination can occur in the presence of the 14 kDa MBP. Although identified by immunohistochemistry on brain tissue slices, the 14 kDa MBP isoform was not isolated and, therefore, not characterized. It would be interesting to see whether charge microheterogeneity with the 14 kDa MBP isoform exists in order to determine whether the compact myelin is forming in the presence of a post-translationally modified or unmodified 14 kDa isoform.

In the following studies, we attempted to determine how MBP is affected in natural mutant and transgenic mice. The first mouse model of study was the shiverer mutant, a mouse model that carries a deletion of exons 3 to 7 in the MBP gene (Roach et al. 1983) and is characterized by the absence of the four major MBP isoforms (Barbarese et al., 1983). Although small amounts of MBP have been reported in shiverer brain (Cammer and Zimmerman, 1983; Roach et al., 1983), very little is known about the identity of the MBP. From Western blot analysis of shiverer brain homogenates, HMW proteins were immunoreactive with an anti-MBP antibody (Barbarese et al., 1983) suggesting that these HMW proteins may account for the MBP observed in shiverer brain.

In our studies with the shiverer mutant, observations similar to those of Barbarese et al. (1983) were made. Following the isolation of acid-soluble proteins from homozygous shiverer brain, Western blot analysis of the isolated material demonstrated the presence of HMW proteins immunoreactive with MBP-specific antibodies. The most interesting finding was that of a doublet that migrated with apparent molecular weights of 30 and 35 kDa and was reactive with polyclonal antibody reactive with bovine MBP. Using monoclonal antibodies that recognized exon 3 peptide sequences in MBP, immunoreactivity with one of the proteins (~35 kDa) representing the doublet was completely unexpected. N-terminal sequencing and amino acid analysis of the 35 kDa protein suggested that the protein may (1) be blocked at the N-terminus and (2) correspond to the J37 golli-mbp isoform. The blocked N-terminus may be represented by (1) a
formylated methionyl residue or (2) a glycyl residue carrying a blocked amino group should the N-terminal methionine be removed following translation. These findings suggested that in the shiverer brain, a protein carrying MBP exon 3 peptide sequences is being expressed and that the gene encoding this particular protein is not affected by the shiverer mutation i.e. deletion of exons 3 to 7 of the MBP gene.

HMW proteins carrying MBP-peptide sequences have been reported (Campagnoni et al. 1993: see Introduction). Alternative splicing of the golli-mbp primary transcript gives rise to three isoforms. BG21 (31 kDa), J37 (35 kDa) and TP8 (7.5 kDa) (Campagnoni et al. 1993). The two major isoforms, BG21 and J37, possess sequence identity with MBP: BG21 carries exon 1 of the classical MBP gene whereas exons 1, 3, 4 and 7 are present in J37. Previous studies have shown that recombinant BG21 and J37 proteins were immunoreactive with anti-golli-mbp and anti-MBP antibodies (Landry et al. 1996). Western blot analysis of 14 and 28 day old normal mouse brain homogenates have shown that BG21 was present at both ages (Landry et al. 1996). Although J37 was not detected in the brain homogenates, it was suggested by Landry et al (1996) that the J37 isoform represented a minor species of golli-mbp. Landry et al (1996) also detected BG21 (31 kDa) in shiverer mouse brain homogenates that were analyzed by Western blot although the presence of the J37 (35 kDa) isoform cannot be ruled out. In our study, we were able to identify the presence of both isoforms by Western blot analysis following the isolation of protein from shiverer brain using an acid-extraction technique.

In order to confirm the identity of these HMW proteins, Western blot analysis of acid-soluble proteins isolated from shiverer brain using an anti-golli-mbp antibody as probe would confirm whether these HMW proteins represent translational products of golli-mbp. The use of antibodies reactive with MBP exons 1, 4 and 7 would confirm the presence of the BG21 and J37 golli-mbp isoforms and whether the reported MBP peptide sequences in these proteins correspond with the immunoreactivity. A final method to confirm the presence of golli-mbp is to perform peptide sequencing of CNBr-cleaved fragments of
these HMW proteins following SDS-PAGE and transfer onto Immobilon membrane. The confirmation for the presence of golli-mbp (BG21 and J37 isoforms) in the shiverer brain would suggest that the golli-mbp and MBP genes are two separate entities and that the former is not affected by the shiverer mutation.

Another study involving the chromosomal mapping of both golli-mbp and MBP genes in mouse brain would confirm whether the genes exist as two separate entities. Both the MBP gene (Roach et al., 1985) and the shiverer mutation (Sidman et al., 1985) have been localized to chromosome 18 in mouse although the loci were not provided. If the golli-mbp gene was separate from the MBP gene, we should expect to find an intact golli-mbp gene at (1) a site upstream or downstream from the shiverer locus on chromosome 18 or (2) on a separate chromosome.

Although golli-mbp isoforms have been detected in post-natal mouse brain (14 and 28 days) (Landry et al., 1996), the expression levels of golli-mbp isoforms during embryonic and early post-natal development (prior to post-natal day 14) has not been studied. However, transcript levels of the BG21 and J37 isoforms have been reported to be highly expressed in the developing embryo and during early stages of development (Campagnoni et al., 1993) suggesting that the expression of golli-mbp represents an early developmental event. In our studies, the expression of these isoforms in the adult shiverer mouse brain suggests that the events in the shiverer brain may reflect an immature stage of development.

The transgenic shiverer mouse was another interesting mouse model. Briefly, these mice were generated by introducing the wild-type MBP gene onto a shiverer background (Readhead et al., 1987). In theory, the transgenic mouse should be able to synthesize all MBP isoforms but our data proves otherwise. The major MBP species in the 30 day old transgenic mouse was the 18.5 kDa isoform which was modified in manner that lead to charge microheterogeneity. Interestingly, the MBP charge microheterogeneity, as well as the absence of phosphorylation on threonine-95, in the adult transgenic shiverer
brain was similar to that of a 4 day old normal mouse suggesting that the developmental stage at which the MBP is presently in is similar to that in an immature animal.

The presence of the 18.5 kDa isoform and corresponding isomers in the transgenic shiverer brain suggested that the mouse carried the machinery to (1) alternatively splice the 21.5 kDa transcript and (2) post-translationally modify the 18.5 kDa isoform. Even if the 18.5 kDa species may appear sufficient for the myelin compaction observed by Readhead et al (1987). the study by Shine et al (1992) demonstrated that there were significant morphological differences between the transgenic and normal mouse. In the transgenic brain, approximately 75% of the axons were not myelinated. Of the myelinated axons, the myelin thickness was almost half of that in normal (Shine et al., 1992). The study of Shine et al (1992), along with our data, suggest that the 18.5 kDa isoform is not enough for normal myelin formation and that other isoforms, particularly the 14 kDa, are needed.

Another interesting observation is the absence of MBP phosphorylation on Thr-95 in the transgenic shiverer brain. Because immunoreactivity of P12 (the antibody that recognizes the MBP phosphorylated at Thr-95) has been localized to the major dense line (MDL) of myelin (Yon et al., 1996). the absence of P12 immunoreactivity with the MBP in the transgenic shiverer brain suggests that there may be a defect in the assembly of the MDL although an intact MDL was demonstrated by Readhead et al (1987). A comparative study involving the dimensional analysis of the MDL following electron microscopy of cryosections of brain tissue from normal and transgenic shiverer brain should determine whether alterations in the MDL exist.

Because the MBP transgene was expressed on a shiverer background, it is possible that certain events arising from the shiverer background may prevent the production of the 14 kDa MBP (and other isoforms) and thus, prevent the transgenic mouse from developing further. In order to determine whether the expression of the 14 kDa isoform is required for myelin formation and maturation in the transgenic shiverer mouse, an interesting experiment would involve morphological and MBP characterization studies on
mice produced from crossing the Readhead et al (1987) transgenic mouse (used in our study) with the 14 kDa transgenic mouse of Kimura et al (1989), both of which have shiverer backgrounds.

In the ND4 mouse, normal expression of MBP was observed (this study: Mastronardi et al. 1996). However, defects in the myelin sheath in the maturing ND4 mouse brain were attributed to changes in the MBP charge microheterogeneity suggesting that MBP cationicity in necessary for myelin stabilization. Charge isomer production appeared normal from post-natal day 11 to 3 months. At 3 months, morphological analysis has shown that the myelin in the ND4 mouse brain appears normal (Mastronardi et al. 1993). After 3 months, alterations in the charge microheterogeneity, particularly C-8 levels, paralleled the morphological changes observed by Mastronardi et al (1993) i.e. loosening of the myelin membrane. The increasing C-8 levels lead to a high cationic index (high C-8/C-1 ratio) which has been reported in early myelinating normal mice and in humans, both normal infants and victims of multiple sclerosis (Moscarello et al. 1994). These studies suggest that alterations in MBP charge microheterogeneity in the adult ND4 mouse brain may be the events that lead to destabilization of the myelin membrane.

Although murine MBP expression and charge microheterogeneity appeared necessary for myelinogenesis, the roles of individual isoforms or isomers are still not well-understood. In addition, the question concerning the biological significance of HMW proteins carrying sequence identity with MBP (i.e. golli-mbp) still remains unanswered. Therefore, the continued use of mouse models in myelin research will remain powerful tools for understanding further the relationship of MBP with myelinogenesis and disease.
APPENDIX

MURINE MBP CHARGE MICROHETEROGENEITY IN MYELIN-CONTAINING FRACTIONS COLLECTED FROM DISCONTINUOUS SUCROSE DENSITY GRADIENT CENTRIFUGATION

Introduction

Because of its buoyant properties, myelin can be isolated from whole brain or white matter using discontinuous sucrose density centrifugation. This technique was used to fractionate human myelin isolated from white matter into fractions that were morphologically and biochemically distinct (Cruz and Moscarello, 1985). The fractionation depended upon the protein-to-lipid ratios: density of a fraction increased proportionally with protein-to-lipid ratios. The light myelin fractions contained compact myelin (mature form) whereas loose myelin (immature form) was demonstrated in heavier fractions. In light, compact myelin fractions, the MBP population was enriched in the more cationic isomers (i.e., C-1, C-2 and C-3), relative to heavier fractions. Less compact myelin was enriched in the less cationic isomers (C-6, C-7 and C-8).

Characterization of myelin fractions isolated from mouse brain has been performed at both the morphological and biochemical levels (Barrese, 1995). This study has shown that each of these fractions was morphologically different: fraction A (the lightest fraction) consisted of compact myelin; fraction B, less compact myelin; fraction C, unilamellar fragments of myelin plus cellular components; and fraction D (the pellet), myelin fragments and cellular debris (Barrese, 1995). The fractionation procedure from whole mouse brain is shown in Figure A.1. The protein and lipid levels in these myelin-containing fractions were well-characterized. However, patterns of MBP charge isomers in myelin fractions were not demonstrated. This study will focus on characterization of MBP charge microheterogeneity in the myelin fractions isolated from whole mouse brain.
Methods

Isolation of MBP from Myelin-Containing Fractions

The following method was adapted from the procedure described by Cruz and Moscarello (1985). Approximately 5 mouse brains (2 to 2.5 g wet weight) were homogenized in 12 mL 0.25M sucrose. The homogenate was diluted to 36 mL with 0.25M sucrose. The sucrose gradient was prepared by underlaying 12 mL of the following solutions into tubes: 0.8M, 1.0 M and 1.2M sucrose. The homogenate was gently layered on top of the 0.8M sucrose layer. Following application of the homogenate layer, the tubes were centrifuged at 20,000 rpm for 90 minutes at 4°C using an SW 25.2 rotor (Beckman). Interfacial layers were collected and suspended in 50 mL cold ddH2O. The suspensions were osmotically shocked (shaken vigourously) and centrifuged (using a JA-20 rotor. Beckman) at 8000 rpm for 20 minutes at 4 °C. The
supernatants were decanted while the pellets were resuspended in ddH₂O and centrifuged as before. The washing of the pellets was repeated a third time. After the final centrifugation, the pellets, which corresponded to the individual myelin fractions, were resuspended in ddH₂O and lyophilized.

The extraction of MBP from the lyophilized myelin fractions was performed as follows. The lyophilized material was homogenized with 0.2N H₂SO₄ and mixed overnight at 4°C. The homogenate was centrifuged at 9000 rpm for 20 minutes at 4°C using a JA-20 rotor (Beckman). The supernatants (containing acid-soluble material) were collected and the pellets were discarded. To the supernatant, an equal volume of absolute ethanol was added to precipitate the MBP from the acid-soluble material. The supernatant-ethanol mixture was incubated overnight at -20°C. The precipitate was pelleted by centrifuging at 9000 rpm, 20 minutes and -10°C. The supernatants were discarded. Washing of the pellet with absolute ethanol was repeated; 90% ethanol was used in a third wash. After the final wash, the pellet was dried under nitrogen gas to remove any traces of ethanol, suspended in ddH₂O and lyophilized. The lyophilized material was stored at -20°C for future use.

Results

Using discontinuous sucrose density gradient centrifugation, whole mouse brain homogenate (3 to 4 months) was fractionated into four distinct bands at sucrose interfaces. These fractions were collected and lyophilized as described in Methods.

In order to isolate MBP from the lyophilized fractions, the fractions were homogenized in 0.2N H₂SO₄ and mixed overnight. Following centrifugation of the homogenate, the supernatants were removed and treated with ethanol to precipitate the MBP from the acid-soluble fractions. The resulting ethanol precipitate was pelleted, washed, dried under nitrogen gas, suspended in double-distilled H₂O and lyophilized. The lyophilized material was subjected to characterization by SDS-PAGE, Western blot and alkaline-urea gel electrophoresis.
Figure A.2  Characterization of Murine MBP isolated from myelin-containing fractions.

Acid-soluble material isolated from myelin-containing fractions A, B, C and D was characterized by (A) SDS-PAGE, (B) Western blot and (C) alkaline-urea gel electrophoresis. In (A) and (B), lane assignments were: lane 1, molecular weight standards; lanes 2 to 5, fractions A to D, respectively; lane 6, (B) only, mouse MBP C-1 (14 kDa); lane 7, (B) only, human MBP C-1 (18.5 kDa). In (C), lanes 1 to 4, fractions A to D, respectively; lane 5, murine MBP; lane 6, human MBP. Rabbit anti-bovine MBP antibody was used in (B).
The MBP from each of the myelin fractions (A to D) was electrophoresed on SDS-polyacrylamide gels (Figure A.2.A). The 14 and 18.5 kDa MBP isoforms were present in all myelin fractions. Several acid-soluble proteins were also present in all myelin fractions although high-molecular weight proteins were more abundant in heavier fractions.

Following electrophoresis by SDS-PAGE, the MBP was transferred onto nitrocellulose and probed with a polyclonal antibody against bovine MBP (Figure A.2.B). Immunoreactivity demonstrated decreasing levels of the 14 and 18.5 kDa isoforms from myelin fractions A to D (Lanes A to D, Figure A.2.B). Faint bands corresponding to the 17 and 21.5 kDa isoforms were also detected in myelin fractions A and B (Lanes A and B, respectively) but not in heavier fractions.

MBP charge microheterogeneity in these myelin fractions was characterized by alkaline-urea gel electrophoresis (Figure A.2.C). The population of MBP components in compact myelin (lane A) consisted of more cationic isomers than in loose myelin (lane B) suggesting that cationicity of MBP is important for myelin compaction. In myelin fractions C and D (lanes C and D, respectively), MBP charge microheterogeneity is detected and is less cationic than that in the myelin-containing fractions A and B. This finding suggested that less cationic forms of MBP can be found in cellular compartments other than myelin although cross-contamination from other fractions cannot be ruled out.

Summary

Compact and loose myelin fractions were isolated from whole normal mouse brains using discontinuous sucrose density gradient centrifugation. MBP charge microheterogeneity in these fractions was demonstrated. MBP populations were generally more cationic in compact, mature myelin than in loose, immature myelin. These findings suggested that the more cationic isomers associate with mature forms of myelin whereas less cationic isomers, immature forms of myelin. In order to follow-up
this study. A developmental study would provide information concerning whether the initial changes in MBP charge microheterogeneity occur in mature and immature myelin. A similar study on the ND4 mice will establish whether the MBP expression during demyelination is being altered in mature or immature myelin.
REFERENCES


