The 18.5-kDa Myelin Basic Protein has Loose Tertiary Contacts Regulated by Zinc and Post-Translational Modification

by
Ehsan Fayaz

A Thesis
Presented to
The University of Guelph

In partial fulfillment of requirements
for the degree of
Master of Science
in
Biophysics

Guelph, Ontario, Canada

© Ehsan Fayaz, December, 2011
ABSTRACT

The 18.5-kDa Myelin Basic Protein has Loose Tertiary Contacts Regulated by Zinc and Post-Translational Modification

Ehsan Fayaz
University of Guelph, 2011

Advisor: Professor G. Harauz

Myelin basic protein (MBP) has fascinated researchers and clinicians alike due to its major structural role in myelin and the central nervous system, and its potent auto-immunogenic properties that cause demyelination in animal models. The charge variants of MBP have been of particular interest. The C1 component, the least modified and most cationic of the variants, is the most abundant form of MBP in healthy adult myelin. The C8 component, the most modified and the least cationic variant, has been found in higher proportions in myelin of MS patients and children. Here, an investigation of the structural differences between C1 and C8 components of MBP was conducted. The spectral and hydrodynamic properties of these variants were monitored via a number of biophysical/biochemical techniques. The effect of zinc (Zn$^{2+}$) on the conformational behaviour of MBP was examined. Zn$^{2+}$ is an abundant metal in the brain, and had been previously shown to induce hydrodynamic compaction in MBP. Both variants have a loose tertiary arrangement with subtle differences. This arrangement is deficient in secondary structure and undergoes non-cooperative temperature-induced melting. Zn$^{2+}$ stabilizes a molten globular-like state with enhanced ANS fluorescence, and promotes oligomerization.
ACKNOWLEDGEMENTS

First and foremost I would like to express my deepest gratitude to my supervisor, Dr. George Harauz for his contributions of time, ideas, and funding, which made my research possible. I could not have imagined having a better advisor and mentor for my M.Sc. study.

Of course, I cannot forget the past and present members of the Harauz group for their wonderful friendship, good advice and collaboration. I would specially like to acknowledge Mrs. Janine Voyer-Grant for being a great friend and assisting me in so many ways throughout my research.

My sincere appreciation goes to Drs. Kenrick Vassall and Vladimir Bamm for their great contribution to my research, and for helping me improve my knowledge of protein chemistry.

I would like to thank my advisory committee members: Drs. Rod Merrill and Frances Sharom for their invaluable contribution of time and expertise, as well as for their generous use of instrumentation and facilities.

Many thanks to Yazan Abbas and Dr. Bhushan Nagar at McGill University for helping me collect the Small Angle X-ray Scattering data I needed to write this thesis. Thank you to the Rickey Yada lab group for assisting me in using the Differential Scanning Calorimeter.

I gratefully acknowledge the funding source that made my M.Sc. work possible. I was funded by a Discovery Grant (RG #121541) from the Natural Sciences and Engineering Research Council of Canada to Dr. George Harauz.

Last but not least, I thank my parents and my sister for providing me with a constant source of love and encouragement.

Ehsan Fayaz
University of Guelph
December 2011
# TABLE OF CONTENTS

ABSTRACT ....................................................................................................................... ii
ACKNOWLEDGEMENTS .................................................................................................. iii
TABLE OF CONTENTS .................................................................................................... iv
LIST OF FIGURES ........................................................................................................ vi
LIST OF TABLES ........................................................................................................... vii
ABBREVIATIONS ........................................................................................................... viii

1.0 Introduction .............................................................................................................. 1
  1.1 Intrinsically disordered proteins .............................................................................. 1
    1.1.1 What makes some proteins “disordered”? ....................................................... 2
    1.1.2 Functionality of disordered proteins ............................................................... 2
  1.2 Myelin anatomy ...................................................................................................... 4
    1.2.1 Myelin Basic Protein (MBP) .......................................................................... 4
    1.2.2 MBP in myelin ............................................................................................. 5
    1.2.3 The MBP gene locus ................................................................................... 7
    1.2.4 Post-translational modifications of MBP ..................................................... 8
    1.2.5 Multiple sclerosis and MBP ......................................................................... 11
    1.2.6 Structure of MBP ...................................................................................... 12
  1.3 MBP and zinc ......................................................................................................... 15
  1.4 Compaction ........................................................................................................... 16
  1.5 Oligomerization .................................................................................................... 16
  1.6 Rationale behind research .................................................................................... 17

2.0 Materials and Methods .......................................................................................... 18
  2.1 Materials ............................................................................................................... 18
  2.2 Recombinant protein over-expression and purification ........................................ 19
  2.3 Fluorescence spectroscopy ................................................................................... 19
    2.3.1 Intrinsic fluorescence spectroscopy and quenching .................................... 19
    2.3.2 ANS fluorescence ..................................................................................... 20
  2.4 Gel filtration chromatography .............................................................................. 21
  2.5 Differential scanning calorimetry ....................................................................... 22
LIST OF FIGURES

Figure 1.1 - Representation of MBP in myelin .................................................................6
Figure 1.2 - The murine Golli-MBP gene ........................................................................7
Figure 1.3 - Amino acid sequence of 18.5-kDa MBP .........................................................9
Figure 1.4 - Molecular recognition features .......................................................................13
Figure 3.1 - ANS fluorescence of His-tagged and His-MBP ..............................................27
Figure 3.2 - Far-UV CD spectra of C1, C8 and T95E ..........................................................28
Figure 3.3 - Trp emission spectra of C1 and C8 .................................................................30
Figure 3.4 - Trp emission spectra of C1 and C8 in presence and absence of Zn\(^{2+}\) ....31
Figure 3.5 - Acrylamide quenching of Trp in MBP ............................................................32
Figure 3.6 - ANS fluorescence of C1 ..................................................................................33
Figure 3.7 - ANS fluorescence of MBP variants in presence and absence of Zn\(^{2+}\) ....34
Figure 3.8 - Time-based ANS fluorescence of MBP in presence of Zn\(^{2+}\) .....................35
Figure 3.9 - DSC traces of C8 ............................................................................................36
Figure 3.10 - Thermal unfolding of MBP variants measured by ANS fluorescence .......38
Figure 3.11 - Relative thermal stability of MBP variants ..................................................39
Figure 3.12 - Gel filtration chromatographs in presence and absence of Zn\(^{2+}\) ............41
Figure 3.13 - SAXS scattering data ....................................................................................42
Figure 3.14 - Guinier plot of C8 ........................................................................................43
Figure 3.15 - Glutaraldehyde cross-linking of C8 ..............................................................46
Figure 3.16 - ITC dilution data for C1 ..............................................................................47
LIST OF TABLES

Table 3.1 – Relative thermal stability of MBP variants ..................................................39
Table 3.2 – The SAXS data and MW estimation ..............................................................44
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANS</td>
<td>1-anilino-naphthalene-8-sulfonate</td>
</tr>
<tr>
<td>C1</td>
<td>unmodified C1 charge component of MBP</td>
</tr>
<tr>
<td>C8</td>
<td>pseudo-deiminated C8 charge component of MBP</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DSC</td>
<td>differential scanning calorimetry</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>GdnHCl</td>
<td>guanidine hydrochloride</td>
</tr>
<tr>
<td>I(0)</td>
<td>forward (or zero-angle) scattered intensity</td>
</tr>
<tr>
<td>IDP</td>
<td>intrinsically disordered protein</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>M&lt;sub&gt;app&lt;/sub&gt;</td>
<td>apparent molecular weight</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>NATA</td>
<td>N-acetyl tryptophan amide</td>
</tr>
<tr>
<td>PTM</td>
<td>post-translational modification</td>
</tr>
<tr>
<td>R&lt;sub&gt;g&lt;/sub&gt;</td>
<td>radius of gyration</td>
</tr>
<tr>
<td>R&lt;sub&gt;H&lt;/sub&gt;</td>
<td>hydrodynamic radius</td>
</tr>
<tr>
<td>R&lt;sub&gt;S&lt;/sub&gt;</td>
<td>Stokes radius</td>
</tr>
<tr>
<td>rmMBP</td>
<td>18.5 kDa recombinant murine MBP (A1-R168-Leu-Glu-His6)</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>zinc</td>
</tr>
</tbody>
</table>
1.0 Introduction

1.1 Intrinsically disordered proteins

At the turn of the 20th century, Emil Fischer introduced the “Lock and Key model” to describe the remarkable specificity and rigidity of enzymes (reviewed in 1). This description was extrapolated to all proteins, and was established as a general rule that governs all enzymatic and protein interactions. Subsequent work, involving denaturation experiments, provided compelling evidence in support of what became known as the protein structure-function paradigm (reviewed in 1). This concept regards a rigid folded structure as being a prerequisite for protein function. For almost a century, the structure-function paradigm remained unchanged and guided all subsequent thinking and investigations in protein science. It was not until the late 1970s that missing electron density in protein structures from X-ray crystallography led researchers to suspect some functional proteins to be unstructured (reviewed in 1).

The late 20th century saw the development of advanced computational methods and their application in identifying globular and non-globular sequences in protein databases (2;3). A survey of the Swiss Protein Database found many gene sequences of proteins with major portions that were predicted to form non-globular structures (4;5). Today, the list of biologically active proteins that lack an ordered structure under physiological conditions is rapidly expanding. Since their discovery, various terms have been used in the literature to describe this class of proteins, including flexible, partially folded, mobile, natively denatured, intrinsically unstructured, and intrinsically disordered. Here, ‘intrinsically disordered’ proteins (IDPs) will be used to refer to proteins or regions
(IDRs) that do not form specific three-dimensional (3D) structures under physiological conditions.

1.1.1 What makes some proteins “disordered”?

Both ordered and disordered proteins are polypeptides that arise from the same set of amino acids. Why then do some polypeptides form highly specific globular structures whereas others remain relatively unstructured and flexible? The structural variation in the two classes of proteins lies in their amino acid composition (6). That is, the propensity of a polypeptide to adopt a certain level of order depends on the residues that form it. In general, IDPs are rich in ‘disorder-promoting’ residues like Arg, Gly, Gln, Ser, Pro, and Lys, but lack “order-promoting” residues, which are mostly bulky and hydrophobic. In addition, the combination of low mean hydrophobicity and high net charge has become recognized as a requirement for being disordered. The high net charge introduces electrostatic repulsion, which promotes disorder in polypeptides, whereas weak hydrophobic interactions prevent the formation of a compact structure (6).

1.1.2 Functionality of disordered proteins

The structural plasticity of IDPs enables them to have a diverse functional repertoire. The advantages of being intrinsically disordered are (1) high specificity coupled with low affinity; (2) ability to bind multiple targets; (3) ability to interact with different structures by altering binding interfaces; (4) large interaction surfaces; and (5) faster rates of association and dissociation (7;8). A growing body of experimental
evidence has highlighted the importance of being disordered, and has pointed to the diverse regulatory roles executed by IDPs.

An important feature of IDPs is their ability to adopt a certain degree of structure upon interaction with binding partners (9;10). Induced folding or disorder-to-order transition is central to IDP function. Moreover, function-related transitions almost always involve interactions with folding partners or molecular chaperones (9). These interactions include permanent or transient binding to other proteins such as to kinases, nucleic acids, membranes, or metal ions (7). Folding partners bind to regions within an IDP and increase its propensity to undergo a disorder-to-order transition. IDPs have been demonstrated to bind to various partners, adopting different structures as a result. This process has been termed binding “promiscuity” or “one-to-many signalling” (11).

Regions undergoing induced folding are referred to as Molecular Recognition Features (MoRFs) (9;12;13). Generally, MoRFs are domains within IDPs, which execute distinct, and at times, unrelated functions (11). This concept enables IDPs to have functional diversity, which increases their network complexity. MoRFs typically have high proline content, making poly-proline II (PPII) structures prevalent in these regions (14). The PPII conformations are rigid, left-handed, extended structures that lack internal hydrogen bonds and are hydrated in aqueous solutions. Such characteristics make them a good candidate for regulating protein-protein interactions (14). For example, MBP contains a solvent-exposed proline-rich segment, which adopts a PPII conformation upon phosphorylation. The PPII motif becomes an SH3 (SRC Homology 3)-domain target, enabling the protein to participate in signalling (15-19).
From a thermodynamic viewpoint, the disorder-to-order transitions in MoRFs offer IDPs important functional benefits by allowing for structural and functional variability in an unbound and bound state. In the unbound state, there is coupling of high specificity with low affinity, making IDPs flexible. In the bound state, high affinity is coupled with low specificity. The latter feature enables IDPs to bind to multiple partners in a reversible manner (15;20;21).

1.2 Myelin anatomy

Myelin forms a multilamellar insulating sheath composed of membrane bilayers around nerve axons (22). In the central nervous system (CNS), myelin’s unique segmental assembly enables the rapid transmission of nerve impulses via saltatory conduction. This method of action potential propagation allows for high fidelity signals to be transmitted at high velocity over long distances without the need to increase axonal calibre (23).

1.2.1 Myelin Basic Protein (MBP)

After the proteolipid protein (PLP), MBP is the most predominant protein of myelin, making up about 30% of all myelin proteins. It is characterised by structural polymorphism and multifunctionality (22;24). The major role of MBP includes, but is not limited to, holding together the compact multilamellar architecture of myelin. Because of this function, it has been dubbed the ‘executive molecule of myelin’ (25). In addition to being a ‘molecular Velcro’, MBP has also been shown to perform various other functions through promiscuous interactions with a variety of molecules and domains (22).
keeping with the theme of this review, MBP is a member of the group of proteins that lack a compact tertiary fold; therefore, its adaptable, multifunctional nature seems to be a direct consequence of being an IDP.

1.2.2 MBP in myelin

Until now, most studies attempting to characterize the structure of MBP, including this one, used a water-soluble form of the protein prepared under denaturing conditions (26). Spectroscopic measurements in aqueous conditions revealed a flexible coil-type conformation (27;28). In order to better understand the structure of lipid-bound MBP, two experimental approaches have been reported. One is based on reconstitution of “denatured” lipid-free MBP with lipids in a number of ways (29;30). The alternative approach involves the extraction of lipid-bound MBP using mild detergents (31). In both cases, specific MBP-lipid interactions lead to formation of ordered assemblies resembling the bilayers in myelin (30;32;33). The conformation of MBP within myelin is not known. Figure 1.1 provides a schematic representation of a C-shaped MBP holding together apposing membrane leaflets. This model, also referred to as the hairpin model, has been proposed based on electron microscopy and computational techniques (34;35). This type of conformation, where end-to-end interactions occurs through long-range contacts, has been proposed for other IDPs such as α-synuclein (36;37) and acid-unfolded apo-myoglobin (38).
Figure 1.1 - This figure is a schematic representation of the multilamellar nature of myelin sheath and the role of MBP within it. MBP is localized in the cytosolic side of the oligodendrocyte membrane, where it interacts with negatively-charged lipids as a result of its positive net charge. Myelin is the lipid-rich structure wrapped in a tight spiral around nerve axons, enabling them to transmit nerve impulses efficiently (1).
1.2.3 The MBP gene locus

The Golli (Gene of OLigodendrocyte LIneage) gene complex is alternatively spliced to give rise to the “classic” MBP family (includes a family of proteins ranging from 17.2 kDa to 21.5 kDa in humans) as well as the Golli proteins: J37, BG21, and TP8 (Figure 1.2) (39). The Golli gene complex is a collection of 10 exons in the human gene (11 in mice) with three different transcription start sites (tss). The most downstream tss, tss3, regulates the expression of “classic” MBP mRNAs (Figure 1.2) (39).

![Figure 1.2 - The murine Golli-MBP gene. (A) and (B) show the classic MBP proteins from murine and human, respectively. (C) shows the Golli proteins, BG21, J37, and TP8 (figure adapted from (40)).](image-url)
1.2.4 Post-translational modifications of MBP

The 18.5-kDa MBP is subjected to developmental regulation, namely extensive post-translational modifications (PTMs), including deimination, phosphorylation, deamidation, methylation, and N-terminal acylation (Figure 1.3) (15). These modifications, deimination in particular, result in the successive reduction of net positive charge, and generate a continuum of charge variants, C1-C8. Component C1 is the least modified, most positively-charged component (+19 at pH 7.4). The remaining components, C2 through C8, have successive additional losses of a single unit of positive charge, so that C8 is the most modified, least positively-charged component (+13 or lower at pH 7.4) (24).

Deimination, the irreversible conversion of arginine to citrulline by the enzyme peptidylarginine deiminase II (PADII), has attracted more interest compared to other modifications. Increased proportions of deiminated MBP to non-deiminated MBP have been observed in brains of Multiple Sclerosis (MS) patients (41). The reaction involves the release of ammonia, and the loss of one unit of positive charge from MBP (41). As a consequence, deimination compromises the ability of MBP to bind and aggregate lipids, leading to MBP’s failure to assemble bilayers into compact multilayers (15;42). Aside from altering protein-lipid interactions, deimination has been shown to be responsible for a number of other structural changes and functional deficits in MBP.
Figure 1.3 - Amino acid sequence of the C1 charge component from 18.5-kDa murine MBP with a suggested zinc binding site. Residues highlighted red are changed to glutamine (Q) as a mimic of citrulline to give the C8 charge isoform. The twisted ribbons represent regions with the potential to form amphipathic α-helices that would associate with the lipid bilayer in myelin (43). A highly-conserved central region containing threonyl residues is phosphorylated by MAP-kinases. This region is proposed to function as the primary immunodominant epitope in MS (44).
Phosphorylation increases charge heterogeneity, resulting in the addition of two negative charges. Consequently, phosphorylated MBP isoforms have a reduced ability to aggregate lipid vesicles (45). Unlike deimination, phosphorylation is a reversible and ubiquitous event, and has been observed at lower levels in MS compared to healthy adults (40). *In vitro* studies suggest that MBP is phosphorylated by various kinases found in myelin, including protein kinase C (PKC), protein kinase A, glycogen synthase kinase, calmodulin-dependent kinase, and mitogen-activated protein kinase (MAPK) (46).

From a structural viewpoint, phosphorylation has been suggested to be an important regulator of MBP conformation. Systematic study of MBP and other IDPs indicates that phosphorylation occurs predominantly in disordered regions of proteins (7;40). This analysis was performed with a web-based predictor of protein phosphorylation sites called DISPHOS (DISorder-enhanced PHOSphorylation predictor). Following phosphorylation, MBP undergoes a disorder-to-order transition, gaining ordered secondary structure in disordered regions (44). Experimental evidence, as well as predicted models, suggests that phosphorylation induces and maintains β-structure in MBP. Phosphorylation of the different MBP charge components by PKC leads to conformational differences among components. None of the components gain α-helical structure, whereas a 35-40% increase in amount of β-structure is observed in all charge isomers. (45). Two threonines in the center of the polypeptide have been indicated to be critical phosphorylation sites for stabilizing secondary structure (47). This site is within the phosphorylation hotspot, the conserved TPRTPPP segment (residues 92-98 in murine MBP), which is suspected of forming a PPII structure and functioning as a target for SH3 domains. The two threonines (Thr92 and Thr95) are phosphorylated by MAPK in *in vitro
Phosphorylation of this site has been shown to affect local structure, protein-protein interactions, and ultimately cause the destabilization of the preceding amphipathic $\alpha$-helix (Val83-Thr92) that anchors MBP to the lipid bilayer. For this reason, this site is referred to as the “molecular switch” of MBP (15;48).

1.2.5 Multiple sclerosis and MBP

MS is an inflammatory disease of the CNS, typically affecting young and middle-aged people. It is characterized by chronic inflammation and damage to axons and the myelin sheath. This leads to substantial disability through deficits in sensation, vision, hearing, memory, balance and mobility. Canada has one of the highest rates of MS in the world (49). Based on clinical characteristics, MS has been classified under two major forms, the more frequent relapsing-remitting (RR)-MS, and the less common primary progressive (PP)-MS. In RRMS, patients experience periods of relapse and remission until they enter the secondary progressive stage when the damage from recurring tissue injury can no longer be reversed by the nervous system. In PPMS, MS is manifested as a gradual worsening of symptoms (50). The aetiology of MS has yet to be elucidated, but two generally accepted hypotheses have emerged (51;52). The immunologic hypothesis involves an autoimmune attack mounted by T-cells against antigens in myelin. The neurodegenerative hypothesis involves changes occurring in components of myelin, which ultimately lead to the destabilization of its compacted multilamellar structure (41). MBP is a major constituent of myelin and has been implicated in the immunologic and neurodegenerative processes, both as an antigen and in myelin destabilization. For this
reason, the focus of the remainder of this overview is on the myriad of structural and functional features of MBP.

1.2.6 Structure of MBP

The major hurdle in the structural characterization of MBP is the lack of a well-defined 3D structure, and our inability to crystallize this protein for the purpose of X-ray crystallography studies. The primary amino acid sequence of MBP, as shown in Figure 1.3, lacks cysteines, meaning the protein is deficient in disulfide bonds, a major stabilizing factor in folded extracellular proteins. Also, compared to well-folded proteins, it has a lower aromatic residue content, a higher positively-charged residue content (numerous lysyl and arginyl residues), and a higher overall hydropathy. The combination of low mean hydrophobicity and relatively high net charge shifts the conformational equilibrium of MBP towards an unfolded state (53). Accordingly, in an aqueous environment, and in a non-bound state, MBP is expected to be found in a dynamic and relatively extended conformation. With that said, MBP conformational behaviour is not consistent with that of a random coil model, in that it contains some non-random structure.

1.2.6.1 Secondary structure

In an aqueous environment, MBP is more or less deficient in fully formed secondary structural elements that can be detected via CD or infrared spectroscopy (54). Physiologically relevant solvent conditions, however, do stabilize some secondary structural elements (16). Bioinformatic tools like Predictor of Naturally Disordered
Regions (PONDR) predict a primarily extended structure with a significant highly mobile portion (about 35%) (Figure 1.4).

**Figure 1.4** – The putative interacting domains in 18.5-kDa MBP. (A) Regions with strong α-helical propensity identified by solution NMR spectroscopic studies of MBP in 30\% trifluoroethanol-d2 (43). (B) C-terminal segment binding site for calmodulin in solution (43). (C) Regions undergoing disorder-to-order transition upon interacting with actin shown by solid-state NMR studies (55). (D) Regions of MBP immobilized by interactions with artificial membranes and (E) regions which remained mobile in MBP-lipid interactions. (F, G) Regions predicted by PONDR to be disordered (F) or ordered (G) (56). (H) Recombinant peptides: (A22–K56) and (S133–S159) bind to Ca^{2+}-calmodulin, and (A22-K56) has actin polymerization and bundling activity (57).
Solution NMR spectroscopy (chemical shift index analysis, secondary structure propensity, and relaxation measurements) have been used to detect regions with propensity to form α-helical segments (T33-D46, V83-T92, T142-L154) upon interaction with the membrane or other cellular components. These α-helices correspond to segments within the MBP sequence with an inherent propensity to be ordered. Thus, secondary structure in MBP is likely transient and limited to small helical regions consistent with the MoRF hypothesis (Figure 1.4).

1.2.6.2 Long range contacts - global structure

It is clear that MBP lacks a rigid tertiary structure (58). However, given that a true random coil practically never exists, not even in strong denaturing conditions, MBP must possess some form of residual tertiary arrangement (59). IDPs have been shown to be significantly more compact compared to random coil models of the same length (60). Regions within IDPs are expected to approach or contact each other. Weak conformational forces (e.g., electrostatic forces within a highly polar protein like MBP) may bias specific interactions leading to transient and relatively compact tertiary arrangement (61). The extent and quality of the tertiary structure in MBP is under investigation. The biggest challenge of this endeavour is the dynamic and flexible nature of MBP. Global structure of MBP is best represented as an ensemble with a broad distribution of rapidly interconverting conformations, which are more compact than a random coil model. A static model representing a time and ensemble-averaged conformation would be able to show bias towards a specific conformation. A number of groups have undertaken this approach for other IDPs. The microtubule-assembling
protein tau, despite being largely unfolded and lacking secondary structure, has been shown to adopt a paperclip-like conformation (end-to-center long-range interaction) using fluorescence resonance energy transfer (FRET) (62). Paramagnetic relaxation enhancement (PRE) NMR spectroscopy and ensemble molecular dynamics (MD) simulations provide evidence of long range contacts between the highly charged C-terminus and a central hydrophobic domain within α-synuclein (36;37). Similarly, acid-unfolded apo-myoglobin is proposed to form long range end-to-end contacts (38). The tertiary arrangement of the 18.5-kDa MBP is still unknown, but a C-shaped model has been proposed based on single particle electron microscopy and molecular modeling (35).

1.3 MBP and zinc

Zn$^{2+}$ is one of the most abundant metals in the brain (63;64). It plays an important, yet ill-defined role in neurochemistry. Both an excess and a deficiency would lead to neurological disorders (64). In myelin, Zn$^{2+}$ seems to be important in stabilizing MBP-membrane association. The interplay between myelin, MBP, and the membrane maintains the integrity of the myelin sheath (65). Up to now, the functional and structural implication of Zn$^{2+}$ binding to MBP is still not understood. Calorimetric data suggest a single site per MBP molecule that binds zinc in the micromolar range (54;66). Given the pH dependence of zinc binding to MBP, histidyl residues are likely involved in the interaction (67;68).
1.4 Compaction

By now, a number of studies have provided evidence of induced ordered secondary structure in MBP, upon association with a binding partner (22;69). However, it is still unclear how the tertiary conformation of MBP changes with ligand association. A nanopore analysis study reported a global compaction, induced by divalent cations Zn\(^{2+}\) and Cu\(^{2+}\) (70). Compaction in IDPs is assessed by hydrodynamic radius (\(R_h\)), where \(R_h\) is the radius of a hypothetical sphere that would diffuse at the same rate as the non-globular protein of interest. Net charge, number of proline residues, and poly-histidine tags appear to be the main contributors to the extent of compaction in IDPs (60). At low pH, the heterogeneous conformational ensemble of \(\alpha\)-synuclein shifts to a more homogeneous ensemble represented by a compact conformation with strong long-range interactions in the C-terminal region (71;72). The strength of repulsive electrostatic forces is to a large extent determined by phosphorylation and deimination, making post-translational modifications a regulator of compaction in IDPs. Conformational changes that lead to compaction or expansion could have important functional consequences.

1.5 Oligomerization

An intriguing hallmark of IDPs that has recently emerged is physiologically relevant oligomerization. Recently it was suggested that a 58 kDa \(\alpha\)-synuclein tetramer may be the more abundant and functional species in the cell compared to the 14 kDa monomer (73). Disorder is a common characteristic of hub proteins. On the other hand, oligomerization is known to be a feature of receptor-mediated signal transduction. Taken together, these observations provide a link between disorder and oligomerization. The
C/EBP homologous protein, Smll, and UmuD(2), are examples of hub proteins, which have been found to self-associate in solution (74;75).

1.6 Rationale behind research

Like many other IDPs, MBP differs from denatured proteins or a statistical coil model, in that it has some non-random structure and is more compact than denatured proteins of the same length. A number of factors, such as intramolecular charge distribution, create conditions for the formation of a tertiary arrangement, which can be stabilized by interactions with physiologically relevant binding partners (60;62). PTMs reduce the net charge, and significantly alter the intramolecular interactions that hold together the higher-order topology of MBP. This may explain why deimination is correlated with the severity of MS. The relative proportion of deiminated MBP to unmodified MBP is much higher in individuals with chronic MS (76).

The primary goal of this thesis is to investigate the conformational behaviour of C1 and C8 variants in solution. To mimic deiminated MBP, as found in myelin of MS patients, a variant form of the recombinant murine MBP with 5 Arg and 1 Lys residues substituted with Gln was used (77). The pseudo-deiminated variant, henceforth referred to as C8, has been deemed a viable model for evaluating the effects of deimination. Phosphorylation is another PTM that is capable of changing MBP’s conformation. Phosphorylation sites are found in regions of the protein predicted to be disordered (58;78). In general, phosphorylation appears to have structure-stabilizing effects in MBP (47). In order to investigate the consequence of phosphorylation on the conformation of MBP, a pseudo-phosphorylated variant of C1, referred to as T95E, was used. This variant
has a single Thr to Glu substitution at position 95. Thr95 is one of two MAPK phosphorylation sites, the other being Thr92. Molecular dynamics simulations have shown that phosphorylation at one or the other of these sites could lead to changes in MBP conformation (48). The pseudo-phosphorylation strategy has been used successfully in studies of α-synuclein (79) and tau (80-82).

The objective of this thesis was to characterize the proposed tertiary arrangement in charge variants of the 18.5 kDa MBP, and its putative stabilization by site-specific phosphorylation and Zn$^{2+}$. A number of biophysical and biochemical techniques were used. Far-UV CD spectra revealed a lack of secondary structure. The Trp fluorescence emission indicated a largely solvent exposed Trp close to the center of molecule. High affinity for the hydrophobic probe, ANS, however, suggested residual tertiary conformation as opposed to a fully expanded polypeptide. It was also found that Zn$^{2+}$ has significant effects on fluorescence and hydrodynamic parameters, suggesting a possible role of the divalent cation on MBP.

2.0 Materials and Methods

2.1 Materials

Electrophoresis grade acrylamide, ultrapure Tris base, and ultrapure Na$_2$EDTA were purchased from ICN Biomedicals (Costa Mesa, CA). Other chemicals were reagent grade and acquired from either Fisher Scientific (Unionville, ON) or Sigma-Aldrich (Oakville, ON). The Ni$^{2+}$-NTA (nitrilotriacetic acid) agarose beads were purchased from Qiagen (Mississauga, ON).
2.2 Recombinant protein over-expression and purification

The untagged versions of the C1, C8, and the pseudo-phosphorylated C1 isoform, T95E were expressed and purified as previously described (54). Hexa-histidine tagged variants (His+ C1 and His+ C8) were purified by Ni\(^{2+}\)-affinity column chromatography with an additional ion-exchange chromatography step for higher purity (77). Higher purity was achieved for the purposes of glutaraldehyde cross-linking via reversed-phase high-performance liquid chromatography (HPLC). A Waters (Mississauga, ON) apparatus was used as described previously (83).

2.3 Fluorescence spectroscopy

Fluorescence measurements were done with a PTI Alphascan-2 spectrofluorimeter (Photon Technology International, London, ON). All measurements were carried out with the cell holder temperature and circulating bath set to 22 °C. Data were acquired using Felix (version 1.4) software. The spectra represent the average of three replicates, whereas time-based ANS fluorescence measurements were done in duplicate. The spectra were blank-subtracted using the software.

2.3.1 Intrinsic fluorescence spectroscopy and quenching

Changes in fluorescence emission properties of the single tryptophan were measured for various MBP isoforms. With emission and excitation slits set to 4 nm, the sample was excited at 295 nm, and emission was scanned from 305 to 450 nm in 1 nm steps. Samples had an initial volume of 40 µL with a constant MBP concentration of 5 µM in buffer (20 mM Tris-HCl, 100 mM NaCl, pH 7.4). Acrylamide was added in 1 µL
aliquots from a 2 M stock solution. To test the effect of Zn$^{2+}$ on the degree of fluorescence quenching, a final concentration of 20 mM ZnCl$_2$ was added to the buffer. Data were corrected for dilution, scattering, and inner filter effect using the following equation (84):

$$F_{icorr} = (F_{exp} - B) \left( \frac{V_i}{V_0} \right) 10^{0.5b(A_{\lambda_{ex}}+A_{\lambda_{em}})}$$

where $F_{icorr}$ and $F_{exp}$ are the corrected and experimental fluorescence intensities, respectively, B is the background fluorescence intensity, $V_0$ is the initial volume, $V_i$ is the volume after each addition, b is the path length in cm, $A_{\lambda_{ex}}$ and $A_{\lambda_{em}}$ are the absorbance of the sample at the excitation and emission wavelengths, respectively. The data were fitted to the following non-linear form of Stern-Volmer equation (85).

$$\frac{F_0}{F} = (1 + K_{SV}[Q])e^{V[Q]}$$

where $F_0$ and $F$ are fluorescence intensities at 350 nm in the absence and presence of acrylamide, respectively, [Q] is the concentration of acrylamide in M, $K_{SV}$ (M$^{-1}$) and V (M$^{-1}$) are the dynamic and static quenching constants, respectively.

2.3.2 ANS fluorescence

The ANS concentration was determined by assuming an ε350 value of 5000 M$^{-1}$ cm$^{-1}$ (86). The concentration of ANS in the sample was kept at 75 µM or at a 7.5:1 molar
ratio with protein. The spectra were collected by setting emission and excitation slits to 4 nm, exciting the sample at 350 nm, and scanning the emission from 400 nm to 600 nm in 2 nm steps. The concentration of MBP variants was kept at 10 µM. All samples were prepared in 20 mM Tris-HCl (pH 7.4), 100 mM NaCl. As in previous experiments, 20 mM ZnCl₂ was used to test the effect of Zn²⁺. The ANS fluorescence was also used in monitoring the thermal stability and unfolding of MBP variants. Here, the same concentrations of protein and ANS were used; however, due to the temperature sensitivity of Tris-buffer, 20 mM HEPES-NaOH (pH 7.4) with 100 mM NaCl was used instead. A sample in a 0.5 cm path-length cuvette was excited at 350 nm and its emission was recorded at 485 nm over a temperature range of 5 °C to 90 °C and a heating rate of 2 °C/min. A drop of mineral oil was placed on top of the sample in order to prevent changes in concentration due to evaporation at high temperatures. The reversibility of traces was checked by doing a reverse cooling.

2.4 Gel filtration chromatography

The C1 and C8 (500 µg) variants of MBP were injected onto a Superdex G-75 column (GE Healthcare Life Sciences, Baie d’Urfe, Quebec). Samples were eluted at room temperature with a flow rate of 0.8 mL/min using two buffer conditions, “zinc” and “no zinc”. The buffers were identical (20 mM Tris-HCl, 100 mM NaCl, pH 7.4) with the exception of 20 mM ZnCl₂ added to the zinc buffer. The column was previously calibrated with molecular standards under the same conditions. Chromatographic data were expressed in terms of the distribution coefficient, \( K_{av} \), derived from the equation:
\[ K_{av} = \frac{V_e - V_0}{V_t - V_0} \]

where \( V_0 \) is void volume of the column, \( V_e \) is the elution volume of sample, and \( V_t \) is the total volume of the gel bed. In order to relate the Stokes radius (\( R_s \)) to \( K_{av} \) values, the following equation was used:

\[ R_s = \frac{\sqrt{-\log(K_{av})} - 0.395}{0.015} \]

For an estimation of the apparent molecular mass (\( M_{app} \)) of the samples, the following equation was used:

\[ M_{app} = 10^{\frac{(1.958 - K_{av})}{0.378}} \]

Since the hydrodynamic dimension reflects the degree of compactness, the values of the hydrodynamic volumes, \( V_h \), were calculated from the corresponding value of \( R_s \), using the following equation:

\[ V_h = \frac{4}{3} \pi (R_s)^3 \]

2.5 Differential scanning calorimetry

Differential Scanning Calorimetry (DSC) was performed using a MicroCal VP-DSC (MicroCal Inc., Northampton, Massachusetts) controlled by the VP-viewer program and equipped with 0.51 mL cells. The DSC traces were obtained from 54 µM MBP in 20 mM HEPES-NaOH and 100 mM NaCl, pH 7.4. Traces were also obtained from MBP in buffer with 20 mM ZnCl₂. The heating rate was 1 °C min⁻¹ the heating range from 10°C
to 110°C. Blank measurements were performed for baselines. All experimental solutions were degassed for 10 min under vacuum.

2.6 Circular dichroism (CD) spectroscopy

Far-UV CD spectra were acquired on a Jasco J-815 spectropolarimeter (Japan Scientific, Tokyo). Samples of MBP in aqueous solution (20 mM Tris-HCl, 100 mM NaCl, pH 7.4) were 70 µL in volume, containing 1.3 mg/mL of protein. Spectra of samples in the presence of ZnCl$_2$ were collected at a metal to protein molar ratio of 2000:1. Five successive scans were taken from each sample over a range of 250-190 nm and a scanning speed of 50 nm/min. The data averaging and smoothing (using the Savitzky-Golay algorithm) operations were accomplished with OriginPro (Version 8, OriginLab Corporation, Northampton, Massachusetts).

2.7 Small Angle X-ray Scattering (SAXS)

The SAXS data were collected on a SAXSess instrument (Anton Paar, Austria) equipped with a PANalytical PW3830 X-ray generator and a Roper/Princeton CCD detector. The beam length was set to 18 mm, and the beam profile was recorded using an image plate for subsequent desmearing. All measurements were carried out at 4°C. Buffer conditions were 20 mM Tris-HCl, 100 mM NaCl, pH 7.4, with addition of 5 mM ZnCl$_2$ for zinc samples. Protein samples were concentrated to a range of 12 to 15 mg/mL using Amicon Ultra 3K centrifugal filter (Millipore, Billerica, Massachusetts). To ensure that samples were mono-disperse and free of aggregation, they were further purified via size exclusion chromatography (Superdex G-200 column, GE Healthcare Life Sciences,
Baie d'Urfe, Quebec). In order to match the solvent and blank as closely as possible, protein samples were dialyzed against the appropriate buffer, and the last step dialysate was used as a blank. Initial data processing, such as dark current correction, scaling, buffer subtraction, binning, and desmearing, were performed on SAXSquant 3.0 (Anton Paar, Austria). Data were collected from different protein concentrations and exposure times, as listed in Table 3.2.

2.7.1 Molecular mass (MW) determination

The values of I(0) and radii of gyration (R<sub>g</sub>) were obtained from the Guinier plots plotted with PRIMUS. The value of I(0) is related to the molecular mass of the scattering particle. The MW of a sample with known protein concentration can be estimated using I(0) and standard proteins with known MW. Lysozyme (14.3 kDa) and bovine serum albumin (BSA, 66.2 kDa) were used as reference samples. The reported MW of samples is the averaged MW calculated from lysozyme and BSA. Only the MW values estimated within the acceptable error of 10% are reported.

2.8 Isothermal Titration Calorimetry (ITC)

A VP-ITC instrument (Microcal Inc., Northampton, MA) was used for isothermal titration calorimetry. C1 and C8 variants were concentrated, dialyzed against buffer (20 mM HEPES, pH 7.4, 100 mM NaCl), and filtered through a 0.22 µm pore size filter. Protein in Zn<sup>2+</sup> was prepared in the same manner except for the addition of 5 mM ZnCl<sub>2</sub> in the dialysis buffer. Samples were degassed in a Thermovac (Northhampton, MA) at 25 °C for 10 min. Concentrated protein was injected into buffer in the sample cell.
Preliminary injections of 2 μL were followed by a total of 29 injections of 10 μL spaced 300 s apart. Experiments were carried out in triplicate, data were plotted as a function of the molar ratio, and the binding isotherms obtained were fitted to the Origin “one set of sites” model for both proteins (Origin 5.0, Microcal) (87-89).

2.9 Glutaraldehyde cross-linking

The C1 and C8 variants in buffer consisting of 20 mM HEPES, pH 7.4, 100 mM NaCl were incubated in 0.0035% glutaraldehyde for 1 h at room temperature and at 4 °C (to be consistent with SAXS conditions). The cross-linking reaction was stopped by 100 mM Tris-HCl, pH 7.4, to quench the unreacted glutaraldehyde. The samples were analyzed by Tricine polyacrylamide gel electrophoresis (90).

3.0 Results

3.1 Histidine tags

Originally, MBP variants under study contained hexa-histidine (His) tags for the purposes of purification via Ni²⁺ affinity chromatography. In light of recent evidence indicating a large effect on the hydrodynamic behaviour of IDPs, purification protocols were altered to exclude these tags (60). An additional reason for removing the His tags was the probable effect of histidines in Zn²⁺ binding studies. Indeed, ANS fluorescence of tagged proteins was shown to be significantly different from that of untagged proteins (Figure 3.1). This observation may be attributed to a difference in tertiary arrangement between histidine-tagged and untagged proteins. For these reasons, untagged proteins
were used in all other experiments. For simplicity, henceforth, untagged variants will be referred to as C1, C8, and T95E.

3.2 Structural rearrangement of MBP upon zinc binding

As we previously reported, Zn$^{2+}$ binds to MBP with a micromolar dissociation constant followed by concomitant compaction of protein structure as determined by nanopore analysis (54;70). This compaction does not result in significant changes in the far-UV CD spectra of either C1 or C8 MBP, suggesting no formation of specific well-defined secondary structural elements (Figure 3.2). Molar residue ellipticity spectra were consistent with unfolded protein with little secondary structure. The minimum intensity occurred at 202 nm for C1, 203 nm for C8, and 201 nm for T95E. Although the curves appear to be superimposable, they are different in intensity. The negative intensity of C8 is 1.8 times higher than that of C1 in the region of the minimum, and at 222 nm, the negative intensity of C8 is 2.5 times higher compared to that of C1. This observation reflects the existence of more secondary structure in the C8 variant. The negative ellipticity near 200 nm and small ellipticity at 222 nm in these spectra is typical of disordered proteins. The negative shoulder in the 220-230 nm region instead of a positive band reflects a higher extent of disorder than the spectrum for PPII structure (9). According to the double wavelength plot ($\theta$222 versus $\theta$200), which distinguishes coil-like proteins from pre-molten globule-like proteins based on proteins with known hydrodynamic properties (10), both MBP variants are more pre-molten globule-like (data not shown).
Figure 3.1 – Spectra of ANS fluorescence and the effects of hexa-histidine tags. Removing His tags significantly changes the ANS fluorescence of each variant. This change may represent perturbations in the tertiary conformation of MBP.
Figure 3.2 - Far-UV CD spectra of C1, C8, and T95E. Protein concentration was 1.3 mg/mL in buffer containing 20 mM HEPES-NaOH, pH 7.4, 100 mM NaCl. The minimum intensity is at 202 nm for C1, 203 nm for C8, and 201 nm for T95E. Each spectrum represents the average of three measurements.
Here, we have investigated the change in intrinsic tryptophan fluorescence upon the addition of Zn$^{2+}$ to both C1 and C8 MBP variants (Figures 3.3 and 3.4). The murine MBP sequence has a lone tryptophan residue at position 113 (Figure 1.3). Protein tryptophan fluorescence is sensitive to both local structural changes and global structural changes resulting in the formation of new tertiary contacts (91). The intrinsic tryptophan fluorescence spectra of C1 and C8 MBP in the absence of Zn$^{2+}$ are remarkably similar (Figure 3.3). The maximum emission wavelength ($\lambda_{\text{max}}$) of Trp in C1 and C8 is 353 nm. Considering N-acetyl-tryptophanamide (NATA) has a $\lambda_{\text{max}}$ of 356 nm, Trp in MBP is highly solvent-exposed. After addition of 4 M guanidine hydrochloride (GdnHCl), $\lambda_{\text{max}}$ of C1 and C8 were 355 nm and 354 nm, respectively. Taken together, these observations are consistent with the IDP status of MBP. In the presence of Zn$^{2+}$, $\lambda_{\text{max}}$ of C1 and C8 were 355 nm and 352 nm, respectively. The lack of Stokes shift upon addition of Zn$^{2+}$ suggests that the divalent cation does not change the polarity of the Trp microenvironment in MBP. With that said, Zn$^{2+}$ does change the fluorescence intensity (FI) of the two variants. The change in C1 is small, whereas FI is increased about 25% in C8 when Zn$^{2+}$ is added (Figure 3.4).
Figure 3.3 - Trp emission spectra of C1 and C8. Fluorescence intensity is normalized. Polarity of the Trp environment is probed in presence of Zn$^{2+}$ and in denaturing conditions (4 M GdnHCl). Samples with concentration of 10 µM protein were excited at 295 nm to ensure selective excitation of the single Trp residue.
Figure 3.4 - Intrinsic Trp fluorescence of C1 and C8. The addition of 20 mM ZnCl$_2$ does not result in a shift in $\lambda_{max}$ in either variant. However, the addition of Zn$^{2+}$ does induce a measurable increase in fluorescence emission intensity, particularly in C8.

The collisional quencher acrylamide was used to further probe the degree of solvent accessibility of the Trp residue in MBP. Acrylamide decreases the FI of Trp through physical contact; hence, the extent of quenching demonstrates the “exposure” of the residue and its microenvironment to solvent (92). As mentioned above, the Trp emission spectra of both C1 and C8 variants suggest a fairly solvent-exposed microenvironment. However, Trp is quenched to a lesser extent than NATA, indicating that the conformation of MBP provides some steric shielding in the region of its Trp (Figure 3.5). Addition of Zn$^{2+}$ further reduces Trp exposure to solvent, suggesting a change in conformation. The upward curvature of the Stern-Volmer plots (Figure 3.5) indicates that both static and dynamic quenching are occurring in the samples.
Figure 3.5 – Acrylamide quenching of Trp fluorescence in MBP. The Trp analog NATA was used as a reference. The extent of quenching, assessed by $F_0/F$, is used as a measure of solvent exposure of Trp in MBP. Zn$^{2+}$ reduces solvent exposure in both variants, probably through changes in tertiary conformation. This effect is more pronounced in C1.

The conformational change in C1 and C8 MBP upon Zn$^{2+}$ binding was additionally probed using the fluorescent dye ANS. This dye is a sensitive probe for the formation of hydrophobic pockets in proteins upon conformational change. It has been widely used in the characterization of protein folding intermediates, in particular, those with a molten globule type structure. Upon binding to hydrophobic pockets, the fluorescence spectrum of ANS typically undergoes large increases in intensity and a blue shift in the fluorescence maximum (93). The ANS fluorescence emission spectrum has enhanced fluorescence intensity, when bound to MBP, with a fluorescence maximum
centered at approximately 480 nm (Figure 3.6). This observation represents a hypochromic shift compared to the fluorescence maximum for ANS in the absence of protein, at 529 nm.

![Fluorescence emission spectra of Tris buffer (black) along with ANS in buffer alone (blue), and with C1 (red). ANS fluorescence is greatly enhanced and blue-shifted to lower wavelengths with the addition of C1. Thus, MBP must provide a hydrophobic environment for the ANS.](image)

Figure 3.6 - Fluorescence emission spectra of Tris buffer (black) along with ANS in buffer alone (blue), and with C1 (red). ANS fluorescence is greatly enhanced and blue-shifted to lower wavelengths with the addition of C1. Thus, MBP must provide a hydrophobic environment for the ANS.

The enhancement in fluorescence is reversed upon the addition of 6 M GdnHCl (Figure 3.8) suggesting that MBP, although intrinsically disordered, is not fully unfolded. The maximum ANS fluorescence intensity, however, is higher for the C8 variant, indicating an increased number of ANS binding sites and/or presence of more non-polar binding sites compared to the C1 variant. The differences in ANS fluorescence between the variants represent a difference in global conformation between the C1 and C8 variants. The ANS fluorescence spectra of C1 and C8 MBP bound to Zn$^{2+}$ have the same basic shape and fluorescence maxima. However, for both MBP variants, the presence of
Zn$^{2+}$ leads to a significant ANS fluorescence enhancement of ~1.5-fold for C1 and ~1.3 fold for C8 MBP. These effects are reversed with the addition of the metal chelating agent EDTA (Figure 3.8), which indicates that Zn$^{2+}$ changes the global conformation of MBP in a way that enhances ANS fluorescence.

Figure 3.7 – The ANS fluorescence spectra of C1 and C8 in the presence and absence of Zn$^{2+}$. Protein concentration was kept at 10 μM with 75 μM ANS. Emission maximum does not shift with addition of Zn$^{2+}$, remaining at 487 nm. Enhancement of ANS fluorescence in both variants is indicative of a conformational change induced by Zn$^{2+}$. The increased ability of C8 compared to C1 in enhancing ANS fluorescence suggests a difference in their tertiary arrangement.
3.3 Thermal stability of MBP

The conventional paradigm for well-folded proteins holds that their denaturation is cooperative. Expanded proteins unfold in a manner that is non-cooperative or only marginally cooperative. To determine the presence or absence of a stable global tertiary conformation, DSC was used to obtain information on the melting temperature of MBP. The absence of a heat absorption peak in the corresponding calorimetric curve reflects the lack of a rigid tertiary structure. In the presence and absence of Zn$^{2+}$, MBP unfolding is broad and non-cooperative (Figure 3.9).

![Graph showing changes in ANS binding/fluorescence over time. A 10 µM sample of C1, incubated in buffer containing 20 mM ZnCl$_2$ and 75 µM ANS is excited at 350 nm and its emission intensity is read at 485 nm. At the 15 min time point, 45.5 mM EDTA is added. Samples containing 4 M GdnHCl remain relatively non-fluorescent, while samples lacking GdnHCl exhibit high fluorescence intensity initially, but with addition of EDTA, ANS fluorescence is quenched. Thus, Zn$^{2+}$ induces a reversible conformational change in MBP.]

Figure 3.8 - Changes in ANS binding/fluorescence over time. A 10 µM sample of C1, incubated in buffer containing 20 mM ZnCl$_2$ and 75 µM ANS is excited at 350 nm and its emission intensity is read at 485 nm. At the 15 min time point, 45.5 mM EDTA is added. Samples containing 4 M GdnHCl remain relatively non-fluorescent, while samples lacking GdnHCl exhibit high fluorescence intensity initially, but with addition of EDTA, ANS fluorescence is quenched. Thus, Zn$^{2+}$ induces a reversible conformational change in MBP.
Figure 3.9 - DSC traces of C8 - The absence of a cooperative transition in the thermal unfolding of MBP suggests a highly disordered state, even in the presence of Zn$^{2+}$. The concentration of C8 was 54 µM in 20 mM HEPES-NaOH and 100 mM NaCl, pH 7.4, buffer. 20 mM of ZnCl$_2$ was used in samples containing Zn$^{2+}$. Similar traces were obtained for C1 (data not shown).
The thermal stability of the MBP variants both with and without Zn$^{2+}$ was probed by measuring the change in the fluorescence intensity of bound ANS across a broad temperature range (Figure 3.10) as previously carried out for other proteins (94;95). The differences in intensity among the variants are greatest at the lowest temperature, and gradually decrease such that at the highest temperature surveyed, the differences in intensity are minimal. It is important to note that the changes in ANS fluorescence with temperature are reversible, indicating that the decrease in intensity is related to structural changes associated with denaturation as opposed to irreversible protein aggregation. The convergence of ANS fluorescence intensity values for the variants at high temperature suggests that they have similar ANS binding properties when fully unfolded. The very broad and gradual loss of ANS fluorescence intensity with temperature demonstrates the lack of cooperativity in MBP unfolding. Raw data from traces in Figure 3.10 were analyzed in order to attain the relative thermal stability of MBP variants in the presence and absence of Zn$^{2+}$ (Figure 3.11). Here, ANS fluorescence at the lowest temperature surveyed represents 100% folded protein, whereas protein is assumed to be completely unfolded at the highest temperature measured. The C1 and T95E traces are similar in shape. At 37 $^0$C, C1 is 64% unfolded and T95E is 68% unfolded. The C8 trace undergoes a small transition, represented as a subtle bump in the region of 40-65 $^0$C. At 37 $^0$C, C8 is 58% unfolded (Table 3.1), suggesting that the mutations mimicking deimination may actually increase stability of MBP. In C1 and T95E, the addition of Zn$^{2+}$ leads to changes in ANS fluorescence, which may represent formation of a transition-state. Relative thermal stability is increased with Zn$^{2+}$ in C8 and T95E by 3% to 7%, respectively.
whereas C1 stability seems to remain unchanged until above 45 °C (Figure 3.11).

![Figure 3.10 - Thermal unfolding of MBP variants in the presence and absence of Zn²⁺ were monitored by ANS fluorescence at 485 nm. The ANS fluorescence is greatest at lower temperatures when the protein is in a partially unfolded conformation with accessible hydrophobic clusters. The ANS fluorescence diminishes with increasing temperatures as the protein completely unfolds.](image-url)
Figure 3.11 - Comparison of the relative stability of MBP variants with temperature. The ANS fluorescence at 5 °C was assumed to reflect 100% folded protein.

Table 3.1 - Relative stability of MBP variants at 37 °C. In C8 and T95E, Zn\(^{2+}\) increases thermal stability.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percent unfolded protein at 37 °C</th>
<th>Percent change in stability with addition of Zn(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>64.0</td>
<td>0</td>
</tr>
<tr>
<td>C1 + Zn(^{2+})</td>
<td>64.0</td>
<td></td>
</tr>
<tr>
<td>C8</td>
<td>58.0</td>
<td>3</td>
</tr>
<tr>
<td>C8 + Zn(^{2+})</td>
<td>55.0</td>
<td></td>
</tr>
<tr>
<td>T95E</td>
<td>68.0</td>
<td>7</td>
</tr>
<tr>
<td>T95E + Zn(^{2+})</td>
<td>61.0</td>
<td></td>
</tr>
</tbody>
</table>
3.4 Dimerization and compaction

In order to ascertain whether the Zn\(^{2+}\)-induced conformational changes in MBP result in oligomerization (as occurs for Cu\(^{2+}\) (96)), size-exclusion chromatography as well as SAXS and glutaraldehyde cross-linking experiments were performed. The size-exclusion chromatograms (Figure 3.12) for C1 and C8 MBP have a main broad peak centered at an elution volume corresponding to 13.6 mL and 13.7 mL, respectively. In the presence of Zn\(^{2+}\), C1 had an elution volume of 14.1 mL, whereas C8 eluted at 13.9 mL. These values are larger than those expected for a compact MBP monomer and consistent with a largely unfolded monomer. The broadness of the peak suggests the presence of multiple interconverting MBP conformations, which could include dimeric or trimeric oligomers. It is noteworthy that the addition of Zn\(^{2+}\) causes a slight shift in the elution profile to higher elution volumes, suggesting a compaction of the monomer in agreement with previous nanopore analysis (70). Additionally, Zn\(^{2+}\) binding results in the presence of an additional small peak in the void volume of the column, which corresponds to a very large oligomer or aggregate.
Figure 3.12 - Gel filtration chromatograms of 500 µg of C1 and C8 eluted from a Superdex G-75 column with ‘no Zn\(^{2+}\)’ buffer (dashed lines) and buffer with Zn\(^{2+}\) (solid line). The main peak is broad and is representative of the various populations of MBP conformations. Addition of Zn\(^{2+}\) leads to a subtle shift and the appearance of an additional peak in the void volume.

The SAXS experiments on MBP could only be carried out at protein concentrations lower than 2 mg/mL, as higher protein concentrations led to random aggregation. The use of these lower protein concentrations led to a weaker scattering intensity, and consequently lower signal to noise at higher angles (higher resolution)
(Figure 3.13). Analysis of data collected at low angle through a Guinier plot (Figure 3.14) generated estimates of the radius of gyration ($R_g$) and the apparent molecular weight ($M_{app}$) of MBP (Table 3.2).

![Graph](image)

*Figure 3.13 - Experimental SAXS data. Measurements were carried out at 4°C. Buffer conditions were 20 mM Tris-HCl, 100 mM NaCl, pH 7.4, with the addition of 5 mM ZnCl$_2$ for Zn$^{2+}$ conditions. The concentration of C1 was 1.13 mg/mL with an exposure time of 8 h. The C1+Zn$^{2+}$ concentration was 1.14 mg/mL with an exposure of 1 h. The C8 and C8+Zn$^{2+}$ samples were both exposed for 10 h, with concentrations of 1.14 mg/mL and 0.87 mg/mL, respectively. At higher angles, scattering data become noisy, particularly for C1+Zn$^{2+}$, which had a short exposure time.*
Figure 3.14 – Guinier plot for C8 scattering data under Zn²⁺ and non-Zn²⁺ conditions. A linear fit of scattering data within $sR_g<1.3$ (data points 10-85 were used here) allows for calculation of $R_g$ and $I(0)$, from the slope and y-intercept, respectively. Since the slopes (determined via linear regression) of the two samples are nearly parallel, the $R_g$ values are similar.

The scattering data were used to estimate the MW of C1 and C8 variants (Table 3.2). The addition of 5 mM ZnCl₂ approximately doubled the MW, suggesting that Zn²⁺ may induce dimerization of MBP. The $R_g$ values calculated for dimeric species is similar to $R_g$ found for monomeric C1 and C8. This result indicates that dimeric Zn²⁺-containing species are similar in size to the Zn²⁺-free MBP monomer. It is probable that Zn²⁺-induced dimerization of MBP is accompanied by significant compaction of the protein subunits. This suggestion is not surprising, as two IDP molecules are known to undergo a mutual or “synergistic” folding at the interaction interface upon homo-dimerization (97).
Table 3.2 - The SAXS data were collected from a range of protein concentrations and at different exposure times. Some samples that contained Zn\(^{2+}\) showed considerable aggregation (*), which was removed via gel filtration. I(0)/concentration method was used in estimating MW. I(0) was obtained from the linear region of a Guinier plot (s.Rg <1.3). At high protein concentrations, samples were aggregated. Samples that passed the data quality control had MW estimates within 10\% of the actual M_r of C1 and C8.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (mg/mL)</th>
<th>Exposure time (h)</th>
<th>I (0)</th>
<th>R_g (Å)</th>
<th>MW (lysozyme)</th>
<th>MW (BSA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>1.13</td>
<td>8</td>
<td>0.03</td>
<td>31.50</td>
<td>19.61</td>
<td>18.20</td>
</tr>
<tr>
<td></td>
<td>1.91</td>
<td>5</td>
<td>0.06</td>
<td>34.56</td>
<td>22.06</td>
<td>20.47</td>
</tr>
<tr>
<td></td>
<td>2.72</td>
<td>1/2</td>
<td>0.10</td>
<td>34.70</td>
<td>25.22</td>
<td>23.41</td>
</tr>
<tr>
<td></td>
<td>1.72</td>
<td>1/2</td>
<td>0.56</td>
<td>42.50</td>
<td>35.81</td>
<td>33.23</td>
</tr>
<tr>
<td>C1 + Zn(^{2+})</td>
<td>1.14</td>
<td>1</td>
<td>0.07</td>
<td>40.60</td>
<td>43.68</td>
<td>40.53</td>
</tr>
<tr>
<td></td>
<td>2.14</td>
<td>1/2</td>
<td>0.17</td>
<td>41.40</td>
<td>54.46</td>
<td>50.54</td>
</tr>
<tr>
<td></td>
<td>5.01</td>
<td>1/2</td>
<td>0.55</td>
<td>46.90</td>
<td>75.12</td>
<td>69.71</td>
</tr>
<tr>
<td></td>
<td>1.13 *</td>
<td>2</td>
<td>0.05</td>
<td>31.56</td>
<td>35.38</td>
<td>32.83</td>
</tr>
<tr>
<td>C1 + Zn(^{2+}) (EDTA)</td>
<td>1.16</td>
<td>2</td>
<td>0.03</td>
<td>33.90</td>
<td>19.76</td>
<td>18.34</td>
</tr>
<tr>
<td></td>
<td>1.14</td>
<td>10</td>
<td>0.03</td>
<td>33.60</td>
<td>20.70</td>
<td>19.21</td>
</tr>
<tr>
<td></td>
<td>1.94</td>
<td>6</td>
<td>0.05</td>
<td>32.92</td>
<td>18.09</td>
<td>16.79</td>
</tr>
<tr>
<td></td>
<td>3.16</td>
<td>1/2</td>
<td>0.11</td>
<td>35.18</td>
<td>24.46</td>
<td>22.71</td>
</tr>
<tr>
<td></td>
<td>5.85</td>
<td>1/2</td>
<td>0.26</td>
<td>36.78</td>
<td>30.57</td>
<td>28.36</td>
</tr>
<tr>
<td></td>
<td>10.06</td>
<td>1/2</td>
<td>0.49</td>
<td>38.44</td>
<td>33.62</td>
<td>31.20</td>
</tr>
<tr>
<td>C8 + Zn(^{2+})</td>
<td>1.59</td>
<td>1/2</td>
<td>0.12</td>
<td>39.75</td>
<td>52.31</td>
<td>48.54</td>
</tr>
<tr>
<td></td>
<td>0.87 *</td>
<td>10</td>
<td>0.05</td>
<td>31.56</td>
<td>35.38</td>
<td>32.83</td>
</tr>
</tbody>
</table>
It is important to note that although SAXS data on the Zn\(^{2+}\)-bound species indicated a dimer, this result does not rule out a monomer-dimer equilibrium. The scattering profile is dominated by the higher molecular mass dimer, which could mask the presence of the monomeric species (98). This point is well-illustrated by our glutaraldehyde cross-linking data (Figure 3.15). The cross-linking experiments conducted for C1 (not shown) and C8 (Figure 3.15) under the same conditions as SAXS shows the presence of predominantly monomeric, but also significant amounts of dimer, as well as smaller amounts of higher order oligomers, in the presence of Zn\(^{2+}\). Cross-linking experiments hence support the conclusion that binding of Zn\(^{2+}\) to both MBP C1 and C8 promotes MBP self-association. Temperature also seems to be a factor in self-association, as incubation at 4\(^{0}\)C seems to be more effective in increasing cross-linked product in comparison to room temperature. The sample incubated with Zn\(^{2+}\) at 4\(^{0}\)C had high MW oligomeric product or aggregates in the stacking gel. This observation is consistent with the gel filtration chromatograms (Figure 3.12), which show Zn\(^{2+}\)-induced aggregation.
Figure 3.15 - Tricine-polyacrylamide gel (16%) shows self-association of C8 MBP detected via glutaraldehyde cross-linking. Samples were incubated for 1 h with 0.0035% glutaraldehyde (+) or buffer (-) before the cross-linking reaction was terminated with 100 mM Tris-HCl, pH 8 buffer. Glutaraldehyde was left to react with samples containing 20 mM Zn\(^{2+}\) at either 4 °C or room temperature (RT).

The reliability of the scattering data was assessed via several methods. First, the low q region in the plot of log [I(q)] versus log(q) was checked for a clear flat region, which is indicative of a mono-disperse sample. Then, the linearity of the Guinier plot as well as consistency in the slope (R_g) and I(0) values were verified. The presence of an upward or a downward turn in the lowest q\(^2\) region of this plot is symptomatic of samples with aggregation and inter-particle interference, respectively. This effect was observed in samples with concentrations exceeding 2 mg/mL. To rule out concentration-dependent inter-particle effects, the I(0)/concentration was calculated at various sample concentrations.
In order to quantify the homo-dimeric interaction, isothermal titration calorimetry (ITC) was used (89). Previously, ITC has been used to report the strength of homo-dimeric interaction via dilution experiments (89). Concentrated protein solution was injected into the calorimeter cell containing the buffer making the solution more dilute with every injection. We were unable to detect heat arising from dissociation of MBP oligomer (Figure 3.16).

![Figure 3.16 - ITC dilution data for the dissociation of C1 dimers in buffer solution. A) The raw data for 10 µL injections of 8.4 mg/mL C1 into buffer solution at 4° C. B) Control data showing calorimetric response for blank buffer injections.](image)
4.0 Discussion

The extremely high net positive charge (pI > 10) of MBP is essential in how the protein functions as an adhesive molecule to maintain the compact structure of myelin. Loss of positive charge would disrupt the electrostatic association with negatively-charged lipids (42;99). To this end, a change to MBP’s positive charge has emerged as a plausible hypothesis in representing the neurodegenerative process in MS. PTMs, which lead to the successive loss of positive charge, have been shown to reduce MBP’s ability to aggregate lipids (99). In the most extreme case, C8 has multiple arginines converted to neutral citrullines (78). The C8 component is proposed to have altered functional and structural properties compared to C1, the most abundant isoform in healthy human myelin (42). Here, both the secondary and tertiary structure of these MBP variants was probed.

The term tertiary structure or fold traditionally refers to a three-dimensional arrangement of different secondary structural elements within a polypeptide. IDPs are often too flexible to have a well-defined arrangement, or lack secondary structure altogether. They exist as a heterogeneous population of conformations, which are rapidly interconverting due to marginal thermodynamic stability. For this reason, when describing the conformational behaviour of IDPs, the term “tertiary structure” is often avoided. Instead, IDPs exhibit long-range tertiary contacts as a result of weak conformational forces.

The highly dynamic structure of MBP makes structural analysis by conventional techniques difficult. The interconverting conformational species of MBP have collapsed structures of varying degrees of compactness. Compactness in IDPs is primarily governed
by net charge (60). The variety of PTMs, namely deimination, methylation, and phosphorylation, could represent a mechanism for regulating compaction. SAXS yields the radius of gyration ($R_g$), which in conjunction with the protein MW approximation provides a measure of compactness. The $R_g$ values measured for C1 and C8 are similar in monomeric state (Table 3.2). However, as the estimated MW nearly doubles with the addition of Zn$^{2+}$, the $R_g$ of both variants changes only slightly. Thus, dimerization results in considerable compactness in MBP. Association-induced folding is not uncommon among IDPs (100;101). Sml1, a highly disordered protein, when in dimeric form, becomes significantly more resistant to proteolytic degradation (75). Self-association may also be a prerequisite for signalling (102). Zn$^{2+}$-dependent dimerization has been reported for a number of proteins such as human growth hormone and amyloid-β-derived peptides (103;104). In the presence of Zn$^{2+}$, protein disulfide isomerase (PDI), an expanded monomer, forms a stable dimer, which aggregates into high order oligomeric forms exceeding 600 kDa over time (105).

IDPs are often conceptualized as the transition-state structures that appear during the folding process of globular proteins. They come in various degrees of disorder ranging from more compact molten globules to pre-molten globules and nearly fully unfolded forms. MBP possesses a tertiary conformation that lacks secondary structure and has a more denatured-like tertiary arrangement. The hydrophobic probe ANS interacts much more strongly with proteins exhibiting molten globular behaviour compared to well-folded or completely unfolded proteins (86;93). Changes in buffer conditions shift the conformational equilibrium of MBP towards either a more compact or a more expanded state, as shown by ANS fluorescence. I have shown here that Zn$^{2+}$
enhances ANS fluorescence in MBP, indicating the creation of hydrophobic binding sites (Figure 3.7). Conversely, in denaturing conditions such as in the presence of GdnHCl, MBP unfolds, losing ANS to the solution, and leading to a drop in ANS fluorescence intensity (Fig 3.8). Thus, even in solution, MBP seems to have a certain amount of residual tertiary structure that is enhanced by Zn\(^{2+}\). It is possible that Zn\(^{2+}\) enables the formation of a molten globule-like tertiary conformation with hydrophobic regions through folding or formation of tertiary contacts (106). Indeed, other techniques suggest that Zn\(^{2+}\) may stabilize a more compact conformation through formation of tertiary contacts (70). Previously, Zn\(^{2+}\) has been shown to induce both secondary and tertiary structure in a number of proteins (107). In other cases, Zn\(^{2+}\) resulted in hydrophobic exposure and/or aggregation in proteins (108).

Here, ANS was further used in the analysis of MBP conformational stability. DSC ruled out the presence of a cooperatively unfolding tertiary structure (Figure 3.9). Thus, the stability of the tertiary arrangement in MBP was judged by the steepness of temperature-induced unfolding curves, represented by ANS fluorescence intensity. The MBP variants tested unfold in a non-cooperative manner. Addition of Zn\(^{2+}\) induces more pronounced changes in the unfolding trajectories of C1 and T95E, particularly at higher temperatures. Intrinsic fluorescence of MBP is not consistent with the Zn\(^{2+}\)-induced formation of partially folded MBP. The polarity of the single Trp in MBP, which is already highly solvent-exposed, does not change in the presence of Zn\(^{2+}\) (Figure 3.3). Acrylamide quenching of Trp fluorescence measuring the extent of solvent-exposure in the vicinity of Trp113 was decreased in both C1 and C8 upon
addition of Zn\(^{2+}\) (Figure 3.5). These observations are in line with formation of a tertiary conformation which offers Trp a certain degree of molecular shielding.

### 5.0 Conclusions and Future Directions

The aim of this study was to detect and describe the residual tertiary structure or transient long-range contacts in MBP, in the hope that such information may prove informative regarding the protein’s associated biological function. The C1 variant, the prominent component in healthy myelin, was found to have subtle differences in its tertiary arrangement in comparison to C8, the disease-associated isoform. The divalent cation Zn\(^{2+}\) caused changes in this arrangement. It has been reported that upon interaction with binding partners such as actin, Ca\(^{2+}\)-activated calmodulin, and lipids, MBP undergoes disorder-to-order transitions accompanied by formation of \(\alpha\)-helical structure in distinct regions (55). The conformational transition resulting from Zn\(^{2+}\) binding does not involve the formation of distinct secondary structure, but rather involves alterations in long-range interactions resulting in global conformational change, compaction of the protein, and increased self-association tendency. These changes were accompanied by enhanced thermal stability and oligomerization. The Zn\(^{2+}\)-mediated self-association of MBP could be important physiologically, allowing the protein to carry out its proposed central role in holding together the apposing cytoplasmic leaflets in the oligodendrocyte membrane.

Future work may involve further characterization of the oligomeric state of MBP via analytical ultracentrifugation (AUC). Sedimentation equilibrium AUC, specifically, will report on the monomer/oligomer equilibrium as well as the stoichiometry and
equilibrium constants for the species present. Fluorescence (Förster) Resonance Energy Transfer (FRET) analysis would be able to show long-range tertiary contacts in MBP’s tertiary arrangement. Acquisition of higher signal-to-noise SAXS scattering data should also permit the determination of additional structural information on the compact denatured state.
6.0 References


membrane-mimetic solvent conditions for studying the conformational adaptability of the 18.5 kDa isoform of myelin basic protein (MBP). Eur. Biophys. J. 37, 1015-1029.


