Biophysical characterization of 21.5-kDa myelin basic protein (MBP) and the effects of zinc on its structure

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Abstract

Myelin Basic Protein (MBP) is a highly abundant protein in central nervous system (CNS) myelin that has a critical role in its proper formation and functioning. The 21.5-kDa isoform of MBP has been shown to be selectively imported into the nucleus of myelin-producing cells, oligodendrocytes, and may be involved in signaling pathways that affect the formation and recovery of CNS myelin. The first step in understanding potential nuclear binding partners of 21.5-kDa MBP is to characterize the structure of the protein. In this study, circular dichroism and fluorescence spectroscopy were used to analyze the structure of 21.5-kDa rmMBP (recombinant murine MBP) *in vitro* in the presence and absence of Zn^{2+} , an abundant trace metal in CNS myelin that has been suggested to affect MBP structure. Fluorescence spectroscopy with a probe for hydrophobic protein regions showed that Zn^{2+} may affect the conformation of 21.5-kDa MBP in aqueous solution.

Keywords: myelin basic protein (MBP); intrinsically-disordered protein; circular dichroism; fluorescence spectroscopy

Introduction

The mammalian central nervous system (CNS) is composed of neurons that conduct electrochemical signals between cell groups, and glial cells that support and protect the neurons. Oligodendrocytes, a subset of glial cells, produce projections of lipid-rich membrane that wrap around the neuronal axons at intervals to facilitate rapid saltatory signal conduction between nodes, areas of the axon not insulated by myelin (Yamazaki et al. 2007). Demyelination, the loss of myelin due to damage and degradation, results in the loss of efficient axonal signal conduction and is associated with a number of debilitating diseases, including Multiple Sclerosis (MS), which often result in the loss of motor control and other symptoms (Hanafy et al. 2011).

Myelin basic protein (MBP) is the second most abundant protein associated with CNS myelin, and is the only known structural protein required for myelin formation (Moscarello et al. 1997; Harauz and Boggs 2013). This importance is exemplified in the *shiverer* mutant mouse, which has a deletion in the *MBP* gene, and which almost completely lacks compact CNS myelin (Tyszkla et al. 2006). MBP is a family of intrinsically-disordered proteins that are encoded by the Gene in the Oligodendrocyte Lineage (*Golli*) and can be subdivided into classical MBPs and *Golli*-specific MBPs (Bamm and Harauz 2008). The classical MBPs arise from transcription start site 3, and differential splicing of a

single mRNA transcript produces splice isoforms ranging from 14.0 to 21.5 kDa in mass (Campagnoni and Campagnoni 2008). The most abundant of these isoforms in mature CNS myelin is the highly basic 18.5-kDa isoform, which can be post-translationally modified by processes that include phosphorylation and deimination (or citrullination) (Vassall et al. 2013). This isoform is a peripheral membrane protein that localizes to the cytosolic leaflet of the myelin membrane and attaches it to the opposing cytosolic leaflet, effectively condensing myelin layers around the nerve axon and forming the major dense line (MDL) (Readhead et al. 1990). It has been shown that this protein is also involved in a number of other processes, including cytoskeletal assembly (Boggs et al. 2001; Boggs et al. 2005; Harauz and Boggs 2013; Smith et al. 2012a) and voltage-operated calcium channel modulation (Smith et al. 2011).

Unlike 18.5-kDa MBP, the 21.5-kDa MBP isoform contains exon-II of the *Golli* gene complex, which encodes 26 amino acid residues (Smith et al. 2012b). Whereas 18.5-kDa MBP has primarily been observed only in the cytoplasm *in vivo*, 21.5-kDa MBP localizes to the cell nucleus as well as the cytoplasm (Pedraza et al. 1997; Smith et al. 2012a, 2012b, 2013; Harauz and Boggs 2013; Ozgen et al. 2014). Exon-II has been shown to contain a non-traditional nuclear localization signal (Smith et al. 2012b) and contains a higher

proportion of basic residues than the remainder of the protein (Hill and Harauz 2005). At high cell density in N19-oligodendrocyte cell culture, 21.5-kDa MBP was shown to translocate out of the nucleus, indicating that 21.5-kDa MBP may be involved in cell proliferation signaling and regulatory pathways (Smith *et al.* 2013).

The over-expression of 21.5-kDa MBP has been shown to activate multiple kinases that are suggested to have involvement in oligodendroglial differentiation, and thus may be involved in the secretion of a cell proliferation factor (Younes-Rapozo *et al.* 2009; Harauz and Boggs 2013; Smith *et al.* 2013; Ozgen *et al.* 2014). Involvement in the regulation of secreted growth factors is implicated in a study in which neurite growth and process branching complexity was increased in N2a neuronal cells that were co-cultured with N19-oligodendrocytes transfected with 21.5-kDa MBP, and as well in N2a cells grown in media obtained from these N19-oligodendrocyte cultures (Smith *et al.* 2013). These effects were no longer observed when 21.5-kDa MBP had a double-nuclear export signal at its N-terminus, or when a double-nuclear localization signal was added to the N-terminus of 18.5-kDa MBP, indicating that they were specific to 21.5-kDa MBP and that exon-II is a critical component for MBP's involvement in the stimulation of cell growth (Smith *et al.* 2013; Ozgen *et al.* 2014).

The expression of 21.5-kDa MBP is highest in oligodendrocytes during active myelination, and has been shown to be up-regulated during attempts at remyelination in multiple sclerosis, suggesting that 21.5-kDa MBP has a role in the regulation of myelination (Barbarese *et al.* 1978; Capello *et al.* 1997; Pedraza *et al.* 1997; DeBruin *et al.* 2006; Uruse *et al.* 2014). The implications of the potential role of 21.5-kDa MBP in regulating myelination and remyelination are significant to the understanding of demyelinating diseases like MS. It is, therefore, crucial to investigate potential nuclear targets of 21.5-kDa MBP to characterize signaling pathways in which it may be involved.

To understand fully the nature of the protein-protein or protein-DNA interactions of 21.5-kDa MBP, a biophysical analysis must be conducted on the protein to investigate its secondary and tertiary structure. One technique used to analyze protein structure is Circular Dichroism (CD) spectroscopy, which measures a protein's differential absorbance of circularly polarized light. In the far-ultraviolet (UV) range (190-250 nm) it can yield information about secondary structures in the protein based on comparison to previously developed well-defined spectra. In the near-UV range (250-350 nm), we can get information on the tertiary structure of the protein (Whitmore and Wallace 2007).

In addition to CD, fluorescence spectroscopy can be used in conjunction with a probe such as 8-anilidonaphthalene-1-sulfonic acid (ANS) to study the changes in the tertiary structure of the protein in different conditions. The ANS probe has a high affinity for hydrophobic environments, and produces a strong emission spectrum between ~450-500 nm only when sequestered in such an environment. When a protein is partially folded, the

proportion of localized, solvent-exposed hydrophobic pockets to which ANS may bind is theoretically increased, and a stronger emission signal can be detected (Brand and Gohlke 1972).

Lastly, it is crucial to investigate the effects of the divalent cation Zn^{2+} on MBP structure due to this metal's high prevalence in CNS myelin. It has been previously suggested that Zn^{2+} may have an effect on MBP folding (Cuajungco and Lees 1997; Tsang *et al.* 1997; Majava *et al.* 2010; Smith *et al.* 2010).

It is essential to understand myelin architecture to decipher the onset and pathogenesis of MS. The objectives of our research group are to determine how this key basic protein of the myelin sheath interacts with membranes and other proteins to form and stabilize healthy myelin, and how modifications to this protein participate in the onset and pathogenesis of MS. The study presented here is part of this overall program.

Materials and Methods

Reagents

1-Anilidonaphthalene-8-Sulfonic Acid (ANS) was purchased from Invitrogen (Carlsbad, CA) and electrophoresis grade acrylamide was purchased from ICN Biomedicals (Costa Mesa, CA). Other chemicals were reagent grade and purchased from either Fisher Scientific (Unionville, ON) or Sigma-Aldrich (Oakville, ON).

Buffers

Lysis Buffer (8 M urea, 100 mM NaH_2PO_4 , 500 mM NaCl, 5 mM Tris-HCl, 10 mM imidazole, 1% v/v Tween-20, pH 8.0); FPLC Ni^{2+} -affinity Wash Buffer (8 M urea, 100 mM NaH_2PO_4 , 500 mM NaCl, 5 mM Tris-HCl, 10 mM imidazole, pH 8.0); FPLC Ni^{2+} -affinity Elution Buffer (8 M urea, 100 mM NaH_2PO_4 , 500 mM NaCl, 5 mM Tris-HCl, 10 mM imidazole, pH 4.5); cation exchange IEX Buffer (6 M urea, 80 mM glycine, pH 10.0); cation exchange Charge Buffer (1 M NaCl); final dialysis Buffer A (50 mM Tris-HCl, 250 mM NaCl, pH 7.4); final dialysis Buffer B (100 mM NaCl).

Recombinant protein over-expression

The *Escherichia coli* BL-21-Codon Plus (DE3)-RP cells (Stratagene) were transformed with pET22b(+) plasmid containing the gene for 21.5-kDa rmMBP (recombinant murine MBP) with a C-terminal hexa-histidine tag, under the control of the *lac* operon. Transformed *E. coli* was grown in 300 μ L SOC media at 37°C with shaking for one hour, and was then added to 10 mL LB media with 50 μ g/mL ampicillin and 34 μ g/mL chloramphenicol, for incubation overnight at 37°C with shaking. The overnight culture was added to 250 mL LB media containing 50 μ g/mL ampicillin and 34 μ g/mL chloramphenicol, and incubated at 37°C with shaking until the absorbance at 600 nm (A_{600}) reached between 0.6-0.8 (arbitrary units), as measured by an

Ultraspec-2000® (Pharmacia-Biotech, QC, Canada). The culture was centrifuged at 2000 rpm (Beckman-Coulter Rotor JA-10) for 15 minutes at 20°C. The cell pellet was re-suspended in 1.5 L of M9 media, pre-warmed at 37°C, containing 30 mL of 20% glucose solution, 3 mL of 1 M MgSO₄, 150 µL of 1 M CaCl₂, 1.5 mL of 50 µg/mL ampicillin, and 1.5 mL of 34 µg/mL chloramphenicol. The M9 culture was incubated at 37°C with shaking until the A₆₀₀ reached 0.8. A 1-mL pre-induction sample was taken, and then 1.5 mL of 0.238 g/mL isopropyl β-D-1-thiogalactopyranoside (IPTG), a molecular mimic of allolactose, was added to the M9 media to induce expression of His₆-tagged 21.5-MBP.

The M9 culture was then incubated at 37°C with shaking for approximately three-four hours. A 1-mL post-induction sample was taken, and the culture was centrifuged at 6000 rpm (Beckman-Coulter Rotor JA-10) for 25 minutes at 4°C. The pellet was stored at -20°C, and pre- and post-IPTG induction samples were analyzed using a 12% bis-acrylamide sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Supplementary Figure S1).

The cell pellet was thawed on ice and homogenized in Lysis Buffer (8 M urea, 100 mM NaH₂PO₄, 500 mM NaCl, 5 mM Tris-HCl, 10 mM imidazole, 1% v/v Tween-20, pH 8.0) at room temperature. Cell lysate was sonicated for 6 pulses of 10 seconds, with 10-second increments between pulses, and stirred at room temperature for 20 minutes. The lysate was then centrifuged at 15,000 rpm (Beckman-Coulter Rotor JA-25.5) for 30 minutes at 4°C. The resulting supernatant was filtered through a 0.45-µm membrane.

Recombinant protein purification by Ni²⁺-chelation chromatography

Fast Protein Liquid Chromatography (FPLC) was first used to purify 21.5-kDa MBP from the aforementioned supernatant using a BioRad-DuoFlow® apparatus (BioRad Laboratories Ltd., Mississauga, ON). An amount of 8 mL of a 50% slurry of Qiagen Ni-NTA agarose resin (QIAGEN Sciences, Germantown, MD, USA) was used to produce a 4 mL column bed volume. The mobile phase was composed of Wash Buffer (8 M urea, 100 mM NaH₂PO₄, 500 mM NaCl, 5 mM Tris-HCl, 10 mM imidazole, pH 8.0) and Elution Buffer (8 M urea, 100 mM NaH₂PO₄, 500 mM NaCl, 5 mM Tris-HCl, 10 mM imidazole, pH 4.5). Each buffer was filtered through a 0.45-µm membrane prior to chromatography. The run was performed at 1 mL min⁻¹ and protein absorbance was measured at 280 nm. The MBP was eluted with 20 mL Elution Buffer, and 1-mL fractions were collected automatically. The composition of the fractions, flow-through, and wash steps was then analyzed using SDS-PAGE (Supplementary Figure S2). All of the fractions containing a band at ~22-kDa on the SDS-PAGE gel were pooled in 6000-8000 MWCO dialysis tubing, and dialyzed overnight against 2 L of buffer containing 6 M urea and 80 mM glycine at a pH of 10.0, to remove the NaCl prior to the next step.

Cation exchange chromatography

The post-dialyzed sample was further purified by cation exchange chromatography using FPLC with a 1-mL Pall AcroSep® cation exchange column (Pall Life Sciences, Ann Arbor, MI, USA). The mobile phase was composed of IEX Buffer (6 M urea, 80 mM glycine, pH 10.0) and Charge Buffer (IEX Buffer with additional 1 M NaCl), both of which had been filtered through a 0.45-µm membrane prior to chromatography. Chromatography was run at 1 mL min⁻¹ and the protein absorbance was measured at 280 nm. A linear salt gradient was established over 10 mL to a final salt concentration of 500 mM, and 1-mL fractions were collected automatically. The composition of the fractions, flow-through, and wash steps was then analyzed using SDS-PAGE (Supplementary Figure S2). All fractions containing a band at the ~22-kDa position on the SDS-polyacrylamide gel were pooled in 6000-8000 MWCO dialysis tubing, and dialyzed against 2 x 2L of Buffer A (50 mM Tris-HCl, 250 mM NaCl, pH 7.4), 2 x 2L Buffer B (100 mM NaCl), and 4 x 2 L double-distilled water. Buffer changes were made approximately every four hours. The purity of the post-dialysis sample was analyzed using high-performance liquid chromatography HPLC (Supplementary Figure S3), and the protein was flash-frozen in liquid nitrogen and lyophilized.

Figure 1. An HPLC chromatogram for analysis of purity of 21.5-kDa MBP purified by HPLC. The mobile phase consisted of acetonitrile, and the ion-pairing agent was 0.1% TFA. Detection was performed at 214 nm and 280 nm (data not shown). The elution gradient began at 75% sterile water + 0.1% TFA and 25% acetonitrile and 0.1% TFA. Acetonitrile + 0.1% TFA was increased to 40% over 15 minutes at 1% per minute. It was then increased further to 100% over 2 minutes. The peak occurring at ~15.00 minutes corresponds to 21.5-kDa MBP monomers, and the peak at ~15.50 minutes corresponds to 21.5-kDa MBP dimers. The dialyzed cation-exchange fractions were further purified using HPLC chromatography.

High-performance liquid chromatography

The His₆-tagged 21.5-kDa MBP was purified in a final step using reversed-phase high-performance liquid chromatography (HPLC) on a Waters (Mississauga, ON) apparatus with a Symmetry 300, C18, 5 mm, 4.6 x 250 mm column (Waters Corp., Milford, MA, USA) (Figure 1). The mobile phase consisted of acetonitrile, and the ion-pairing

Figure 2. Near-UV CD spectroscopy of 25 μM 18.5-kDa and 21.5-kDa MBP, in the presence and absence of 10X excess Zn^{2+} , with 20 mM HEPES buffer. Here, 1 mM TCEP was added to the 21.5-kDa MBP samples to reduce dimers linked by cysteine residues.

Figure 3. Far-UV CD spectroscopy of 5 μM 18.5-kDa and 21.5-kDa MBP in 10 mM phosphate buffer. The 21.5-kDa MBP sample also contained 1 mM TCEP to reduce dimers linked by cysteine residues.

Figure 4. Far-UV CD spectroscopy of 5 μM 21.5-kDa MBP, in the presence and absence of 10X excess Zn^{2+} , in 10 mM HEPES buffer with 1 mM TCEP to reduce dimers linked by cysteine residues.

agent was 0.1% trifluoroacetic acid (TFA). The column temperature was maintained at 40°C, and the flow-rate at 1 mL min^{-1} . Detection was performed at 214 nm and 280 nm. Protein was eluted from the column beginning at an elution gradient of 75% Solvent A (sterile water and 0.1% TFA) and 25% Solvent B (acetonitrile and 0.1% TFA), followed by increasing Solvent B at 1% per minute to 40%. Solvent B was then increased to 100%. Fractions containing MBP were collected and flash-frozen in liquid nitrogen and lyophilized.

Assessing protein purity

In addition to protein purification, HPLC was used as an analytical tool to assess the purity of the resultant MBP stock (Bamm *et al.* 2011). The MBP was solubilized in water to a concentration of 2 mg mL^{-1} and a 50 μL sample was applied to the apparatus following the protocol outlined for purification. Protein concentration was determined by A_{280} and the extinction coefficient ($\epsilon = 19,940 \text{ M}^{-1} \text{ cm}^{-1}$).

SDS-PAGE was also used to confirm the purity of the protein stock and gain insight into the potential identity of constituents other than full-length 21.5-kDa MBP. This technique also allowed for the identification of homodimers of MBP that would be expected to be located at approximately 44 kDa on the SDS-polyacrylamide gel.

MBP dimer reduction

To eliminate disulfide-linked covalent homodimers of 21.5-kDa MBP, the reducing agent tris(2-carboxyethyl)phosphine (TCEP) was added to 2 mg mL^{-1} 21.5-kDa MBP to give a final concentration of 1 mM. The reaction mixture was incubated at room temperature for one hour prior to analysis by HPLC at 214 nm, as described above.

Incubation of Zn^{2+} with 21.5-kDa MBP

The MBP at concentrations of 0.5 mg mL^{-1} and 0.05 mg mL^{-1} was incubated in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer for one hour at room temperature and at 37°C, in the presence and absence of 10X excess Zn^{2+} . Each sample condition was incubated in duplicate, and one of each sample was mixed with either reducing (0.05% β -mercaptoethanol) or non-reducing sample buffer, boiled for ~45 seconds, and analyzed by SDS-PAGE.

Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy was performed using a Jasco J-815 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) with both 18.5-kDa and 21.5-kDa MBP. Near-UV (250–350 nm) and far-UV CD scans (190–250 nm) were carried out at protein concentrations of 25 μM and 5 μM , respectively, and a total sample volume of 200 μL in a quartz cuvette of path length of 1 mm (Figures 2–4). Data were collected at room temperature, and each scan was repeated three times on a single protein preparation. The 21.5-kDa and 18.5-kDa MBP were each solubilized in 10

Figure 5. A 14% SDS-polyacrylamide gel of 21.5-kDa MBP incubated in the presence and absence of TCEP and 10X excess Zn^{2+} . **A:** 20- μL samples contained 20 mM HEPES and were prepared by the addition of non-reducing loading dye to 16 μL of each sample. Odd lanes contain 0.05 mg mL^{-1} protein (0.8 μg per well) and even lanes contain 0.5 mg mL^{-1} protein (8.0 μg per well). Lanes 5-8 contain 10X excess Zn^{2+} . Samples were incubated for one hour prior to loading at either room temperature (lanes 1, 2, 5, and 6), or at 37°C (lanes 3, 4, 7, and 8). Samples were then boiled for two minutes prior to loading. Lane 1 contains the Pink Plus Protein Ladder from Biorad. **B:** Samples were prepared as the samples in gel A except a reducing dye was used for loading. The gels were stained with Coomassie Brilliant Blue for 10 minutes, and destained with 2% methanol and 1% acetic acid.

mM phosphate buffer, pH 7.4, and 21.5-kDa MBP was also solubilized in 10 mM HEPES buffer, pH 7.4, with and without 50 μM ZnCl_2 . All samples of 21.5-kDa MBP contained 1 mM TCEP-HCl to reduce dimers. Buffer blanks were subtracted from each measurement, which was taken as an average of the 3 scans for each sample. Data were normalized to mean residue ellipticity.

Fluorescence spectroscopy

The 21.5-kDa and 18.5-kDa MBP were solubilized to a final concentration of 5 μM in 20 mM HEPES, pH 7.4, with and without 50 μM ZnCl_2 , in a total volume of 80 μL . Samples also contained 50 μM of the dye 8-anilino-1-naphthalene-sulfonic acid (ANS), which was used as a probe for the presence of solvent exposed hydrophobic pockets within MBP. A Photon Technology International (PTI) fluorimeter (Photon Technology International Inc., London, ON), and a 45- μL quartz cuvette with 3-mm path-length, were used to measure each sample in triplicate at room temperature. The slit widths were set to 2 nm for excitation and 6 nm for emission, and gain was set to 1.0 V at 350 nm for all measurements. Samples were excited at 350 nm, and emission was measured at 400-600 nm. Scans of buffer samples were subtracted from protein-containing sample scans before presentation and analysis.

Results and Discussion

Purification and yield of 21.5-kDa rmMBP

This study utilized a purification protocol for hexahistidine-tagged 21.5-kDa rmMBP that employed Ni^{2+} -chelation chromatography, cation exchange chromatography, and high-performance liquid chromatography in tandem. One notable modification in our procedure is the use of a different pH rather than a different imidazole concentration for the

binding and elution buffers from the Ni^{2+} -affinity column – the pI of histidine is 7.6, so this residue would be fully protonated at pH 4.5 and would elute from the column. The yield from 1.5 L M9 media was roughly 12 mg of protein, and the purity (as determined by the integrated peak areas on the HPLC profile) was consistently above 90%. This purification procedure produced protein that was pure enough to be used for CD and fluorescence spectroscopy analysis.

Analysis of purity of 21.5-kDa MBP

In preparation for the biophysical characterization of 21.5-kDa MBP using circular dichroism and fluorescence spectroscopy, the MBP stock was analyzed using HPLC to confirm purity and ensure that the protein was in monomeric form. The HPLC chromatogram of the absorbance of 21.5-kDa MBP at 214 nm showed a slightly smaller peak tightly associated with the main peak in a 50 μL analytical sample (**Figure 1**). This product was hypothesized to represent MBP homodimers due to disulfide bonding of the single cysteine residue encoded within exon-II. A 2 mg mL^{-1} sample of the MBP stock was then analyzed using SDS-PAGE (32 μg protein per well) and non-reducing loading dye to observe whether a band representative of MBP dimers would appear at approximately 44 kDa. Four bands appeared on this gel: one at ~23 kDa, representing full-length 21.5-kDa MBP; one at ~12 kDa (faint and visible only on original gel), which was likely a degraded form of 21.5-kDa MBP; one at ~46 kDa, representing homodimers of 21.5-kDa MBP; and one at ~35 kDa, which was likely a heterodimer of full-length and degraded MBP (**Figure 5A**). Identical samples loaded onto an SDS-polyacrylamide gel with reducing buffer produced only a single band at approximately 24 kDa (**Figure 5B**). The number and location of bands were consistent across samples and within treatments (reducing or non-reducing

sample buffer). From these results it was concluded that the smaller peak that was tightly associated with the larger, main peak on the HPLC chromatogram represented MBP homodimers.

Figure 6. An HPLC chromatogram for analysis of the effects of 1 mM TCEP on peak profile. The 1 mM TCEP-HCl was added to 2 mg mL⁻¹ of 21.5-kDa MBP and incubated at room temperature for one hour before HPLC. The mobile phase consisted of acetonitrile, and the ion-pairing agent was 0.1% TFA. Detection was performed at 214 nm and 280 nm (data not shown). The elution gradient began at 75% sterile water + 0.1% TFA and 25% acetonitrile and 0.1% TFA. Acetonitrile + 0.1% TFA was increased to 40% over 15 minutes at 1% per minute. It was then increased further to 100% over 2 minutes. The peak occurring at ~13.50 minutes corresponds to 21.5-kDa MBP monomers, and the peak at ~15.75 minutes corresponds to 21.5-kDa MBP dimers.

The presence of MBP homodimers would greatly skew the data obtained from CD and fluorescence spectroscopy, which were used in this study to characterize the monomeric structure of the protein. To eliminate dimers, 1 mM of the reducing agent TCEP was added to a sample of 2 mg mL⁻¹ of 21.5-kDa MBP, and incubated for one hour at room temperature. The purpose of this step was to observe whether the disulfide bond would be successfully reduced, producing a sample containing only the monomeric form of the protein. This reaction was then analyzed using HPLC. A large reduction in this second peak, to less than 10% of the total protein, was observed in a 50-μL sample (**Figure 6**).

Circular dichroism spectroscopy of MBP in the presence and absence of 10X Excess Zn²⁺

In this study, the CD spectra of 18.5-kDa and 21.5-kDa MBP in aqueous solution were compared to investigate any structural differences caused by the inclusion of the residues encoded by exon-II in the sequence of 21.5-kDa MBP, as well as any differences caused by Zn²⁺-binding.

Near-UV CD spectroscopy of 5 μM 18.5-kDa and 21.5-kDa MBP in aqueous solution, in the presence and absence of 10X excess Zn²⁺, produced very similar mean residue ellipticity profiles between 250 nm and 350 nm (**Figure 6**). Between 290 nm to 350 nm, the mean residue ellipticities for

all samples oscillated around zero. The 21.5-kDa MBP sample yielded an overall maximum between 250 nm and 290 nm, whereas 18.5-kDa MBP experienced an overall minimum in this range. The low signal-to-noise ratio of the curve in this region made it difficult to discern whether there were any true structural differences between the two isoforms of MBP.

Far-UV CD spectroscopy of 5 μM 18.5-kDa and 21.5-kDa in 10 mM phosphate buffer produced similar mean residue ellipticity profiles between 200 nm and 250 nm (**Figure 6**). The spectrum for 18.5-kDa MBP did show a slight increase between 218 nm and 222 nm, and a decrease between 200 nm and 212 nm, in comparison to 21.5-kDa MBP. Far-UV CD spectroscopy of 21.5-kDa MBP with and without 10X excess zinc in 10 mM HEPES buffer produced similar spectra between 200 nm and 250 nm.

In the near-UV range (250-350 nm), there did not appear to be any detectable differences between 18.5-kDa and 21.5-kDa MBP preparations (**Figure 6**). In the far-UV range (200-250 nm), there appeared to be slight differences at 200 nm and 220 nm. Since exon-II encodes a high proportion of positively-charged residues that would have a repulsive effect on other basic areas of the protein, it is unlikely that 21.5-kDa MBP would have significantly more ordered structure in aqueous solution than 18.5-kDa MBP, which has been shown to be extended and flexible in the absence of a binding partner (Libich and Harauz 2008a, 2008b; Harauz *et al.* 2009; Libich *et al.* 2010; Nalugapalli *et al.* 2012). However, the CD data suggest that some slight structural differences between the two isoforms may be present.

Zinc is present at high concentrations in myelin and has been found to have a role in the compaction of 18.5-kDa MBP *in vitro* (Baran *et al.* 2010). Incubation of 50X excess Zn²⁺ with 0.05 and 0.5 mg mL⁻¹ 21.5-kDa MBP in 20 mM HEPES buffer did not produce any detectable differences in mobility on an SDS-polyacrylamide gel from samples incubated without zinc. The effects of Zn²⁺-binding on the structure of 21.5-kDa MBP were also analyzed using CD spectroscopy (**Figures 2-4**). The far- and near-UV spectra for 21.5-kDa MBP in the presence and absence of Zn²⁺ were very similar, and there was no noticeable effect of Zn²⁺ on the secondary structure of 21.5-kDa MBP.

Fluorescence spectroscopy of MBP in the presence and absence of 10X excess Zn²⁺

Previously in our laboratory, ANS fluorescence had been used to probe the compaction of 18.5-kDa MBP by zinc (Fayaz 2011). Then it was found that the spectral shift of ANS fluorescence occurs only when this fluorophore is sequestered in a hydrophobic pocket proffered by the protein, not in solution alone. Here, the ANS-binding measured by fluorescence spectroscopy was further used to analyze the effects of Zn²⁺ on 18.5- and 21.5-kDa MBP conformation in solution (**Figure 7**). The fluorescence of ANS showed a steep increase in a 5 μM sample of 21.5-kDa MBP with 10X excess Zn²⁺, compared to samples without Zn²⁺, of about

Figure 7. Fluorescence spectroscopy of 5 μ M 18.5-kDa and 21.5-kDa MBP in 20 mM HEPES buffer with 10X ANS fluorescent dye. The 21.5-kDa MBP samples also contained 1 mM TCEP to reduce dimers linked by cysteine residues. Buffer scans containing all non-protein components were subtracted from protein scans.

450,000 fluorescence units (FU). Comparatively, ANS fluorescence increased approximately 150,000 FU between a 5 μ M sample of 18.5-kDa MBP to a 5- μ M sample of 18.5-kDa MBP containing 10X excess Zn^{2+} . The maxima of all four fluorescence curves occurred at approximately 480 nm.

In this experiment, samples of 18.5-kDa and 21.5-kDa MBP containing 10X excess Zn^{2+} showed an enhancement in ANS fluorescence, centered at approximately 480 nm, compared to samples lacking Zn^{2+} . The Zn^{2+} appeared to induce approximately 66% more of a conformational change in 21.5-kDa MBP than in 18.5-kDa MBP, as measured by fluorescence units.

Conclusions

The enhancement of ANS fluorescence upon protein binding can be sensitive to both local and global conformational changes. In circular dichroism spectroscopy, however, far-UV CD is specifically sensitive to secondary structural changes, whereas near-UV CD is sensitive to tertiary structural changes. Hence, given that CD data for 18.5- and 21.5-kDa MBP were very similar both with and without Zn^{2+} , it is likely that the observed changes in ANS fluorescence are due to local conformational changes rather than global changes.

Future Directions

The product derived from a recombinant murine gene for 21.5-kDa MBP that is linked to a hexa-histidine tag is thus far the most commonly used form of the protein for over-expression and purification, and was the system used for this study. Although the relatively small size of the His₆-tag renders data collected from experiments on the tagged protein fairly reliable, it is desirable to produce an expression system for a completely native form of the protein. This would eliminate the concern that the tag may be affecting data in any way. It would thus be beneficial to perform polymerase chain reaction (PCR) mutagenesis to remove the

His₆-tag from the rmMBP 21.5-kDa gene, as has already been achieved for several 18.5-kDa MBP variants (Smith *et al.* 2010). The CD and ANS fluorescence spectroscopy experiments could then be repeated on the native protein and compared to those performed in this study to observe any effects of the hexa-histidine tag.

Following these spectroscopic analyses, Isothermal Titration Calorimetry (ITC) would be worthwhile to perform to investigate the nature of any interactions between 21.5-kDa MBP and zinc, as has already been performed by us for the 18.5-kDa MBP isoform (Smith *et al.* 2010), and for plant dehydrins (Rahman *et al.* 2011). Parameters that can be determined in this way include stoichiometry and association constants.

Last but not least, this basic (positively-charged) protein may very well function as a transcription co-factor via its interactions with nuclear DNA sequences, yet to be elucidated (Harauz and Boggs 2013). In this light, chromatin immunoprecipitation (ChIP) would be a logical next step towards the characterization of the potential nuclear binding partners of 21.5-kDa MBP (Buck and Lieb 2004).

In MS, hyperdeimination and hypophosphorylation of 18.5-kDa recapitulate an early developmental pattern, and contribute to physical demyelination and induction of autoimmunity. Disruption of its involvement in essential interaction networks in early childhood when myelin is still developing has been hypothesized to cause inherent initial myelin instability, and to confound remyelination attempts during MS (Bessonov *et al.* 2010, 2013; Bamm and Harauz 2014). The 21.5-kDa MBP isoform is operative in myelin formation (as reviewed above). Understanding its functions in the oligodendrocyte nucleus during proliferation, for example, may clear the way to greater insights into the mechanisms that lead to demyelination and remyelination in MS, and may lead to potential new targets for therapy.

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Supplementary Information

Abbreviations

ANS	8-Anilino-naphthalene-1-Sulfonic Acid
CD	Circular Dichroism
CNS	Central Nervous System
FPLC	Fast Protein Liquid Chromatography
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HPLC	High-Performance Liquid Chromatography
IDP	Intrinsically-Disordered Protein
IPTG	Isopropyl β -D-1-thiogalactopyranoside
MBP	Myelin Basic Protein
MS	Multiple Sclerosis
PCR	Polymerase Chain Reaction
rmMBP	recombinant murine MBP
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SOC	Super Optimal Broth with Catabolite repression
TCEP	Tris(2-Carboxyethyl)Phosphine
TFA	Trifluoroacetic Acid
Tris	tris(hydroxymethyl)aminomethane

Supplementary Figures

Figure S1. A 14% SDS-polyacrylamide gel of 21.5-kDa MBP purified using Ni^{2+} -affinity column chromatography. The 20- μL samples were prepared by the addition of 5X reducing loading dye to 16 μL each of post-IPTG induction (lane 1) and pre-IPTG induction (lane 2) samples, pre- Ni^{2+} column crude protein sample (lane 3), Ni^{2+} column flow-through and wash samples (lanes 4 and 5), and Ni^{2+} column fractions (lanes 6-14). Samples were boiled for two minutes prior to loading. Lane 1 contained a Pink Plus Protein Ladder from BioRad. The gel was stained with Coomassie Brilliant Blue for 10 minutes, and destained with 2% methanol and 1% acetic acid. The fractions that clearly contained over-expressed 21.5-kDa MBP, within the range represented by lanes 7 through 13, were collected for dialysis and further purification.

Figure S2. A 14% SDS-polyacrylamide gel of 21.5-kDa MBP purified using cation-exchange chromatography. The 20 μ L samples were prepared by the addition of 5X reducing loading dye to 16 μ L of each fraction (lanes 1-7). Samples were boiled for two minutes prior to loading. Lane 1 contained a Pink Plus Protein Ladder from BioRad. The gel was stained with Coomassie Brilliant Blue for 10 minutes, and destained with 2% methanol and 1% acetic acid. The fractions that clearly contained over-expressed 21.5-kDa MBP, within the range represented by lanes 3 through 6, were collected for dialysis and further purification.

Figure S3. An HPLC chromatogram for analysis of purity of 21.5-kDa MBP purified by cation-exchange chromatography. The mobile phase consisted of acetonitrile, and the ion-pairing agent was 0.1% TFA. Detection was performed at 214 nm and 280 nm (data not shown). The elution gradient began at 75% sterile water + 0.1% TFA and 25% acetonitrile and 0.1% TFA. Acetonitrile + 0.1% TFA was increased to 40% over 15 minutes at 1% per minute. It was then increased further to 100% over 2 minutes. The peak occurring at ~15.00 minutes corresponds to 21.5-kDa MBP monomers, and the peak at ~15.50 minutes corresponds to 21.5-kDa MBP dimers. The dialyzed cation-exchange fractions were further purified using HPLC chromatography.