The interaction of zinc with membrane-associated 18.5 kDa myelin basic protein: an attenuated total reflectance-Fourier transform infrared spectroscopic study

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Abstract Myelin basic protein (MBP) is an essential structural protein required for tight compaction of the myelin sheath of the central nervous system, and belongs to the family of intrinsically disordered proteins. It contains a high proportion of polar and charged amino acids, and has an adaptive conformation depending on its environment and binding surfaces (membranes) or partners (other proteins or small ligands including divalent cations). Zinc is an important stabilizing component of myelin and its concentration is substantially higher than that of any other trace element in the brain. In this study, we investigate the effect of zinc on different variants of 18.5 kDa MBP, including new recombinant forms lacking hexahistidine tags which would interfere with the binding of the cation. Isothermal titration calorimetry showed the dissociation constant to be in the micromolar range for all variants. Circular dichroism spectroscopy showed that there was minimal effect of zinc on the secondary structure on MBP in aqueous solution. When MBP was reconstituted with myelin-mimetic membranes, attenuated total reflectance-Fourier transform infrared spectroscopy revealed that there was a rearrangement of secondary structure components upon addition of zinc that was subtly different for each variant, indicative of a synergistic protein–membrane–cation interaction.

Keywords Myelin basic protein (MBP) · Intrinsically disordered protein · Induced folding · Isothermal titration calorimetry (ITC) · Circular dichroism (CD) · Fourier transform infrared (FTIR) spectroscopy · Attenuated total reflectance (ATR) spectroscopy

Abbreviations

ATR Attenuated total reflectance
CD Circular dichroism
CNS Central nervous system
Cyt-LUV Large unilamellar vesicle with lipid composition, of inner oligodendrocyte membrane leaflet
ddH₂O Double-distilled water
EDTA Ethylenediamine tetraacetic acid
FTIR Fourier transform infrared
HEPES N-(2-Hydroxyethyl) piperazine-N’-2-ethanesulfonic acid
HPLC High-performance liquid chromatography
IDP Intrinsically disordered protein
IEX Ion-exchange (chromatography)
IPTG Isopropyl-β-D-thiogalactopyranoside
ITC Isothermal titration calorimetry
MBP Myelin basic protein
OD Optical density
PAGE Polyacrylamide gel electrophoresis
PCR Polymerase chain reaction
rmC1 Recombinant murine 18.5 kDa MBP, unmodified (LEH₆-tagged)
rmC8  Recombinant murine 18.5 kDa MBP, pseudo-deiminated (LEH₆-tagged)
SDS   Sodium dodecyl sulphate
UT-rmC1 Untagged recombinant murine 18.5 kDa MBP, unmodified
UT-rmC8 Untagged recombinant murine 18.5 kDa MBP, pseudo-deiminated

Introduction

Myelin basic protein (MBP), particularly the 18.5 kDa isoform, is essential for maintaining the stability of the myelin sheath of the central nervous system (CNS) (Boggs 2006, 2008; Harauz et al. 2009). Ablation of or mutations in the MBP gene result in the loss of compact myelin in the CNS, e.g., shiverer (shi) mice (Readhead and Hood 1990) or the Long Evans shaker (les) rat (Carré et al. 2002). Myelin basic protein can be classified as an intrinsically disordered protein (IDP) (Uversky 2009) because it has a considerable proportion of predicted disorder (Libich and Harauz 2008a; Harauz and Libich 2009; cf., Bailey et al. 2001). This intrinsic flexibility facilitates interaction with a diverse variety of surfaces, other proteins, or small ligands, indicative of a complex structure–function relationship as recently reviewed (Harauz et al. 2004, 2009; Harauz and Libich 2009). In all associations that have so far been investigated structurally (phospholipid membrane, Ca²⁺-calmodulin, actin), the 18.5 kDa MBP isoform has demonstrated a partial induced disorder-to-order transition, including several α-helical molecular recognition fragments (Libich and Harauz 2008a; Harauz and Libich 2009; Libich et al. 2010). All complexes can be described as “fuzzy” since much of the protein remains highly mobile within them, meaning that there is no rigid tertiary structure in the usual sense in which it is conceived (Libich et al. 2010; Tompa and Fuxreiter 2008; Mittag et al. 2009). The conformational polymorphism of MBP is consistent with its in vivo roles as a scaffold, linker, and signaling hub for, e.g., membranes, cytoskeleton, Ca²⁺-calmodulin, SH3-proteins (Harauz et al. 2009).

Another stabilizing component of CNS myelin is zinc, with a concentration (about 50 μM) that is substantially higher than that of any other trace element (Iyengar et al. 1978; Koh 2001). Zinc deficiency leads to abnormal CNS development and neuropsychological impairment (Keen et al. 1993; Sandstead et al. 2000; Koh 2001; Takeda 2001; Unal et al. 2005). Abnormally high levels of zinc have been reported in neurofibrillary tangles associated with various neurodegenerative diseases (e.g., reviewed in Mo et al. 2009). Using X-ray diffraction, Inouye and Kirschner (1984) showed that zinc caused peripheral nervous system myelin to retain sharp reflections (i.e., a compact structure), and it is conceivable that the same result would be obtained with CNS myelin in which MBP predominates. Almost all of the zinc in brain white matter is protein-bound (Itoh et al. 1983), and MBP has been identified specifically as a zinc-binding protein via its histidyl residues (Berlet et al. 1994; Tsang et al. 1997) with a reported dissociation constant of 0.44 μM (Saedien et al. 2001). When the MBP is solubilized in phosphate buffer, the addition of zinc results in large aggregates (Cavatorta et al. 1994; Riccio et al. 1995), and the cation is thus believed to stabilize the protein’s association to myelin membranes (Earl et al. 1988; Berlet et al. 1991). It has been shown by X-ray absorption spectroscopy of reconstituted Langmuir–Blodgett multilayers that Zn²⁺ ions bind to both MBP and phospholipid headgroups (Morante 2001; Nuzzo et al. 2002; Benfatto et al. 2004). In multiple sclerosis patients, zinc levels in erythrocytes have been observed to increase dramatically (~3×), perhaps due to disruption of normal compartmentalization of zinc during demyelination (Ho et al. 1986).

In this study, we test specifically the hypothesis that zinc induces ordered secondary structure in 18.5 kDa MBP, as it does for several other IDPs (Gatewood et al. 1990; Uversky et al. 2000; Uversky 2009). The previous studies cited above have used MBP preparations obtained from brain myelin, which are heterogeneous mixtures of charged components comprising myriad post-translational modifications (Kim et al. 2003; Harauz et al. 2004). Our group has introduced the use of recombinant murine MBP isoforms that yield very pure (compositionally homogeneous) protein samples for biochemical and structural studies. Our two main prototypes are unmodified 18.5 kDa murine MBP (generically rmMBP, specifically rmC1) (Bates et al. 2000), which emulates the predominant, minimally modified C1 component of healthy myelin, and rmC8 with six R/K-to-Q substitutions to mimic citrullination in the highly modified C8 component, which is found in increased proportion in multiple sclerosis (Bates et al. 2002). The net charges at neutral pH are +19 and +13 for rmC1 and rmC8, respectively. In previous solution NMR spectroscopic experiments on rmC1, we have shown that there was negligible effect of Ca²⁺ on the ¹H–¹⁵N heteronuclear single quantum coherence spectra of the protein, but that Zn²⁺ perturbed the spectra subtly (Libich and Harauz 2008b). We concluded that future studies of the interactions of Zn²⁺ with rmMBP required the construction of variants lacking the hexahistidine tag (which would interact with any cation).

In this current work, we describe new protein constructs lacking this His₆-tag (UT-rmC1 and UT-rmC8, respectively) and the optimization of their purification. Using circular dichroic (CD) spectroscopy, we show that there is...
minimal effect of zinc on the secondary structure of either UT-rmC1 or UT-rmC8 in aqueous solution. However, when these proteins were reconstituted with myelin-mimetic membranes, attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy demonstrated a subtle conformational shift, primarily a slight increase in α-helicity, in both of these new protein variants.

Materials and methods

Construction of UT-rmMBP expression plasmids

The previously described pET22b-rmC1 and pET22b-rmC8 plasmids containing the complete open reading frame for murine 18.5 kDa MBP were used as templates to construct the un-tagged pET22b-UT-rmC1 and pET22b-UT-rmC8 protein expression plasmids, respectively. These constructs were created by introducing a stop codon between the hexahistidine tag and the open reading frame for mMBP using Quikchange site-directed mutagenesis (Stratagene, La Jolla, CA, USA). For all polymerase chain reactions (PCR), synthetic oligonucleotides were purchased from Sigma-Aldrich (Oakville, ON, USA). The forward and reverse primers (FP and RP, respectively) used to produce pET22b-rmC1 and pET22b-rmC8 were:

rmC1-FP: 5' CCATGGCGAGACGC
rmC1-RP: 5' GGTGGTGGTGCTCGAGTCAGCGTCTCGCCATGG
rmC8-FP: 5' CCATGGCGAGACGG
rmC8-RP: 5' GGTGGTGGTGCTCGAGTCATTGTCTCGCCATGG

Each 50 μL mutagenesis reaction consisted of 1 μL of 250 ng stock of plasmid (template) DNA, 1 μL of a 150 mM stock of mutagenic forward and reverse primer, 5 μL of 10× Pfu Ultra Buffer, 41.5 μL ddH2O, and 0.5 μL of Pfu Ultra DNA polymerase (2.5 units μL⁻¹). The PCR site-directed mutagenesis was performed using a BioRad thermocycler PCR system with the following cycling parameters: initial denaturing temperature of 95°C for 30 s, followed by 18 cycles of 95°C (denaturing temperature) for 30 s, 55°C (annealing temperature) for 1 min, 68°C (extension temperature) for 12 min followed by a final extension at 68°C for 10 min, with the completed reaction being held at 4°C. Any remaining non-mutated parental DNA was eliminated by adding 0.5 μL of DpnI (10 units μL⁻¹) restriction enzyme directly to the PCR reaction. The restriction digest was incubated at 37°C for 4 h, and 5 μL was subsequently transformed into E. coli DH5α and plated on Luria–Bertani plates containing 50 μg mL⁻¹ of ampicillin. A single colony obtained from each transformation reaction was grown in Luria–Bertani media. The plasmid DNA was extracted using Roche High Pure Plasmid isolation kit (Roche Diagnostics, IN, USA) and was confirmed by sequencing (Laboratory Services Division, University of Guelph).

Protein over-expression and purification

Plasmids that were confirmed by sequencing to encode the UT-rmC1 and UT-rmC8 proteins were transformed into E. coli BL-21-CodonPlus (DE3)pLysS cells (Stratagene) and were expressed and purified as described below. A 150 mL Luria–Bertani culture containing 50 μg mL⁻¹ ampicillin and 34 μg mL⁻¹ chloramphenicol was inoculated using an overnight culture of E. coli transformed with either the pET22b-UT-rmC1 or pET22b-UT-rmC8 plasmid, and grown at 37°C to an optical density (600 nm) of 0.6–0.8. This culture was centrifuged at 4,000×g for 15 min at room temperature and was transferred to a 1 L culture of pre-warmed M9 minimal media containing the same concentrations of antibiotic mentioned above. Again, cultures were grown to an optical density (OD at 600 nm) of 0.6 and were induced for 6 h with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside).

The cell pellet was resuspended in 100 mL of lysis buffer containing 8 M urea, 100 mM NaH2PO4, 10 mM Tris-base, 500 mM NaCl and 1% Tween-20 (v/v), pH 9.5. The mixture was stirred at room temperature for about 40 min, and then freeze-thawed twice. Afterwards, the lysate was centrifuged at 25,000×g for 45 min; the supernatant was collected and dialyzed against ion-exchange chromatography buffer (IEX buffer) consisted of 6 M urea, 80 mM glycine, pH 9.5 (three changes). Next, the dialysate was filtered through a 0.8/0.2 μm membrane filter and was loaded onto a 5 mL CM-HighTrap FF column (GE Heathcare Bio-sciences Inc.) that was previously charged using 50 mL of 1 M NaCl in IEX buffer, followed by an equilibration using 50 mL of IEX buffer. The column was washed with IEX buffer until the absorbance reading at 280 nm was under 0.01. The protein was eluted using 50 mL of 0.0–0.2 M NaCl linear gradient in IEX buffer at a flow rate of 1 mL min⁻¹. All fractions were analyzed using SDS-PAGE and absorbance was measured at 280 nm to determine purity and concentration, respectively (vide infra). Pure protein fractions were pooled and dialyzed slowly in a sequential fashion to remove salts and denaturing agents, using tubing with a Mr cutoff of 6,000–8,000 Da. Dialysis buffers were as follows: (a) 50 mM Tris–HCl, pH 7.4, 250 mM NaCl (two changes); (b) 100 mM NaCl (two changes); (c) double-distilled water.
(four changes). After the final dialysis step, the protein was frozen and finally lyophilized.

Two other proteins containing the hexahistidine tag, rmC1 and rmC8, were purified using our standard protocols (Bates et al. 2000, 2002; Hill et al. 2003). Throughout rmC1 and rmC8, were purified using our standard protocols frozen and finally lyophilized.

After the final dialysis step, the protein was filtered (0.22 µm pore size) and the concentration was measured from the absorbance at 280 nm. The extinction coefficients used were 0.667 L g⁻¹ cm⁻¹ for rmC1, 0.672 L g⁻¹ cm⁻¹ for rmC8, 0.705 L g⁻¹ cm⁻¹ for UT-rmC1, and 0.711 L g⁻¹ cm⁻¹ for UT-rmC8 (as calculated by SwissProt for protein in 6.0 M guanidine hydrochloride, 0.02 M phosphate, pH 6.5). The accuracy of this approach had previously been verified by amino acid analysis (Bates et al. 2000, 2002). Protein preparations were routinely analyzed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie Brilliant Blue R250 (Fisher Scientific, Unionville, ON, USA).

### Reversed-phase HPLC

Reversed-phase high performance liquid chromatography (HPLC) was employed to assess the final purity of UT-rmC1 and UT-rmC8 proteins following the purification procedure, using a previously published protocol with small modifications (Bamm and Harauz 2008). The HPLC system comprised a Waters 626 Gradient Pump, a Waters 2487 Variable Wavelength Dual Channel UV Detector, 50 µL sample loop, and a Waters Symmetry 300™ C18 column (5 µm, 4.6 × 150 mm²). Detection was at 214 nm, the flow rate was 1 mL min⁻¹ and 0.5 mL min⁻¹ for UT-rmC1 and UT-rmC8, respectively, and the column was maintained at 25°C. Acetonitrile (ACN) and trifluoroacetic acid (TFA) were used as the mobile phase and ion-pairing agent, respectively. After 2 min of loading time at 10% acetonitrile and 0.1% TFA, the elution gradients started from 10 to 50% acetonitrile, and 0.1% trifluoroacetic acid, at a rate of 1% acetonitrile per min, followed by 5% per min for an additional 10 min.

### Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) experiments were carried out using a VP-ITC instrument from Microcal Inc. (Northampton, MA, USA). Lyophilized variants of rmMBP were dissolved in buffer (20 mM HEPES–NaOH, pH 7.4, 100 mM NaCl), and extensively dialyzed against the same solution (at least 4 changes). Following the dialysis, the protein was filtered (0.22 µm pore size) and the concentration was measured from the absorbance at 280 nm. The stock of 2.5 mM ZnCl₂ was prepared in the same solution prior to each experiment. Samples were degassed in a Thermovac (Northhampton, MA, USA) at 25°C for 10 min. The Zn²⁺ solution (2.5 mM) was injected into the sample cell, containing 50 µM of rmMBP variant in the above solution. Typically, the titrations were carried out with a preliminary injection of 2 µL followed by 24 injections of 5 µL of ligand solution, and then 17 injections of 7 µL of ligand solution, with a 300 s spacing between each injection. All experiments were carried out in triplicate at 25°C. Before analysis, data from the preliminary 2 µL injection were discarded, and heats of dilution of the ligand (Zn²⁺) into solution of 20 mM HEPES–NaOH, pH 7.4, 100 mM NaCl (in the absence of rmMBP variants) were subtracted from the ligand (Zn²⁺) into rmMBP experiments. The corrected data were integrated and plotted as a function of the molar ratio, and the binding isotherms obtained were fitted to the Origin “one set of sites” and/or “two sets of sites” models (Origin 5.0, Microcal) (cf., Majava et al. 2008).

### Circular dichroism spectroscopy

Circular dichroism (CD) was performed using a Jasco J-815 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) using an initial protein concentration of 1.3 mg mL⁻¹ in a sample volume of 70 µL in a quartz cuvette with a path length of 0.1 mm. The far-UV CD spectra (190–250 nm) were scanned at a rate of 50 nm min⁻¹, with data collection at 1 nm intervals, at 25°C. The blank was subtracted after each measurement, and the spectra from six successive scans were averaged for each sample. The molar ellipticities were calculated using the protein concentrations and molar mass. The CD measurements were carried out for each protein variant in two different aqueous solutions: (1) 20 mM HEPES–NaOH, pH 7.4, 100 mM NaCl; and (2) 20 mM HEPES–NaOH, pH 7.4, 100 mM NaCl, 1.3 mM ZnCl₂.

### Sample preparation for ATR-FTIR spectroscopy

Protein samples were dissolved in a HEPES buffer (20 mM HEPES–NaOH, pH 7.4, 100 mM NaCl) at a concentration of approximately 4 mg mL⁻¹, determined by absorbance at 280 nm. Protein solutions were stored at −20°C to prevent degradation. The ZnCl₂ was dissolved in the same HEPES buffer at a concentration of 10 mM and stored at room temperature.

Large unilamellar vesicles (LUVs) with lipid composition similar to that of the cytoplasmic monolayer of myelin (Cyt-LUVs; cholesterol:phosphatidylethanolamine:phosphatidylerine:phosphatidylcholine:sphingomyelin:phosphatidyl-inositol in 44:27:13:11:3:2 M ratios) (Inouye and Kirschner 1988) were prepared by extrusion as previously described (Boggs et al. 1997; Musse et al. 2006; Zhong et al. 2007). Lipids were purchased from Avanti Polar Lipids (Alabaster,
Various lipid stocks in chloroform mixtures (chloroform:H2O:methanol 1:2:1, vol), were mixed to the desired molar ratio. Chloroform was then evaporated under a mild nitrogen flow and subsequently kept under vacuum overnight for complete removal of the chloroform remnants. Lipid mixtures were rehydrated in the same HEPES buffer at 45°C overnight with vigorous shaking and three freeze–thaw cycles. Large unilamellar vesicles (LUVs) were formed by extruding the lipid mixture 61 times through a polycarbonate membrane with a 100 nm pore size, at 45°C. The sizes of vesicles were confirmed to be around 100 nm using a Zetasizer Nano-S model ZEN1600 (633 nm “red” laser; Malvern Instruments) Dynamic Light Scattering (DLS) instrument. The final concentration was determined by a modified Micro-Bartlett Phosphorus Assay (Bartlett 1959).

Protein solutions were diluted with the above HEPES buffer to a concentration that matched the lipid vesicles before mixing (around 4 mg mL\(^{-1}\)). In the experiments studying the effect of Zn\(^{2+}\), concentrated Zn\(^{2+}\)-containing HEPES buffer was added to the protein solution first (to reach the final Zn\(^{2+}\) concentration of 20 times molarity as that of protein), followed by a 5 min incubation at room temperature and subsequent addition of the Cyt-LUV solution to reach a 1:1 (mass:mass) protein–lipid ratio. This relatively high protein–lipid ratio was necessary to obtain an adequate signal-to-noise ratio in the ATR-FTIR experiments. (We do not believe that there is significant protein–protein interaction under these conditions, because \(^{13}\)C spectra recorded for different protein:lipid mass ratios up to 0.7 were essentially identical as ascertained by solid-state NMR spectroscopy (Zhong et al. 2007). A subsequent incubation of 10-15 min allowed a further equilibration of the protein–lipid interaction.

### ATR-FTIR spectroscopy and data analysis

The spectra were recorded using a BrukerOptics Vertex70 FTIR spectrometer equipped with a liquid nitrogen-cooled mercury cadmium telluride (MCT) detector. A vertical PIKE MIRacle Micro ATR accessory (PIKE technologies, USA), combined with a three-reflection diamond crystal unit (diameter 6 mm), was used. The diamond three-reflection ATR crystal was cleaned by double-distilled H\(_2\)O, followed by isopropanol to eliminate trace water. For each spectrum, 200 interferograms were collected and Fourier-transformed to give a resolution of 2 cm\(^{-1}\). During measurement, the optics compartment and the sample compartment of the spectrometer were purged continuously with dry nitrogen gas to eliminate spectral contributions from atmospheric water vapor. All experiments were conducted at room temperature (\(\sim 22^\circ\)C).

After incubation, the protein-LUV complex (with or without Zn\(^{2+}\)) was spun down in a table-top centrifuge at 14,000 rpm (18,000 \(\times g\)) for 1 h. The supernatant was then removed. Aggregates were carefully spread onto the surface of the three-reflection diamond ATR crystal. A stream of nitrogen gas, containing rich D\(_2\)O vapor, flowed over the crystal until the sample was completely deuterated. Upon \(^{1}\)H/\(^{2}\)H exchange, the absorption band associated with the random secondary structure shifts from about 1,655 cm\(^{-1}\) to about 1,642 cm\(^{-1}\). This change permits differentiation of the \(\alpha\)-helical and the random secondary structures (Susi et al. 1967; Byler and Susi 1986). Spectra in the range from 950 to 1,750 cm\(^{-1}\) were collected. A typical measurement required approximately 200 \(\mu\)g of each variant of rmMBP.

The overlapping bands were resolved by Fourier self-deconvolution using OMNIC software (Thermo Fisher Scientific, Waltham, MA, USA). The bandwidth value was set to 15 and the enhancement value was set to 1.8. The frequencies of these component bands were subsequently used in PeakFit software (Seasolve Software Inc., San Jose, CA, USA; version 4.12) as input parameters for curve-fitting analysis of the original spectrum. The amide I region (\(\sim 1,620\) to \(\sim 1,700\) cm\(^{-1}\)) involves the peptide backbone C=O stretch and N–H groups, and bond angles were analyzed for protein secondary structure composition. The spectra of the rmMBP variants in Cyt-LUVs were deconvolved using a mixed Gaussian and Lorentzian band shape of standard deviation 8.5. Auto-fits of the second derivative of the original spectra were performed until the coefficient of determination (\(r^2\)) was larger than 0.99. The integrated areas derived from the curve-fitting analyses were used in calculating the various conformational states assigned to individual bands.

### Results and discussion

#### Purification and yield of UT-rmC1 and UT-rmC8

Previously we have described procedures to purify and extract His\(_6\)-tagged versions of rmC1 and rmC8 using nickel affinity followed by ion-exchange chromatography to achieve an overall purity of \(\sim 95\%\) (Bates et al. 2000, 2002). Subsequent optimization resulted in yields of at least 10 mg isotopically labeled rmMBP per liter of M9 minimal media for NMR spectroscopy (Libich et al. 2004). In this study, we have described a new purification protocol that isolates un-tagged (UT) versions of these recombinant proteins using a denaturing lysis procedure followed by ion-exchange chromatography. The denaturing lysis procedure was introduced to extract rmMBP from the inclusion bodies to produce a sample suitable for ion-exchange chromatography (Fig. 1). A one-step purification protocol employing ion-exchange chromatography allowed for isolation of UT-rmC1 and UT-rmC8 with purity greater than
96%. Using HPLC, the UT-rmC1 and UT-rmC8 preparations were determined to have a purity of 97 and 96.4%, respectively, by assessing the integrated peak areas of each individual profile (Fig. 2). A typical yield from 1 L of M9 medium for UT-rmC1 and UT-rmC8 was 8 and 6 mg, whereas the maximal amount of protein isolated from the purification procedure was 17 and 12 mg, respectively. We were thus able to optimize the purification procedure for UT-rmC1 and UT-rmC8 to have equal purity and nearly equal protein yield as for our hexahistidine-tagged versions.

Binding of Zn$^{2+}$ assessed by isothermal titration calorimetry

Previous studies have indicated that MBP binds divalent cations, particularly Zn$^{2+}$ and Cu$^{2+}$ (Berlet et al. 1994). These interactions were shown to inhibit dissociation of MBP from the membrane (Earl et al. 1988). Thus, we decided to probe the interaction of different variants of MBP with Zn$^{2+}$ quantitatively using isothermal titration calorimetry (ITC). Figure 3 presents results from a typical ITC experiment, which was performed in triplicate for each recombinant murine MBP variant (Wiseman et al. 1989;
Velazquez-Campoy et al. 2004). The parameters derived are summarized in Table 1. The dissociation constants for all MBP variants appeared to be in a low micromolar range, specifically 15.4 \( \mu M \pm 12% \) for rmC1 (Fig. 3a), 22.3 \( \mu M \pm 14% \) for rmC8 (Fig. 3c), 41.8 \( \mu M \pm 6.7% \) for UT-rmC1 (Fig. 3b), and 41.6 \( \mu M \pm 10% \) for UT-rmC8 (Fig. 3d). The major differences between the tagged and untagged versions of MBP were observed at higher \( Zn^{2+} \)-MBP ratios. At that region of the curve, the data points deviated slightly from the mathematical fit in the case of the rmC1 and rmC8 variants, but this effect disappeared with the untagged proteins. In fact, for two MBP variants containing the hexahistidine tag, rmC1 and rmC8, the better mathematical fit was obtained using “two sets of sites model” (data not shown). After employing that model, the parameters of one set of binding sites appeared to be very similar to those generated by the “one set of sites” model, but the other set had higher dissociation constants (220 and 51 \( \mu M \)) with the values of \( n \) (number of ligands bound) equal to 5.1 and 4.7 for rmC1 and rmC8, respectively. This observation can be explained by less specific and physiologically irrelevant binding of \( Zn^{2+} \) to the His6-tag of the proteins.

Effects of \( Zn^{2+} \) on rmMBP and UT-rmMBP in aqueous solution—CD spectroscopy

We next examined the characteristics of the UT-rmMBPs compared to His6-tagged rmMBPs by using circular dichroism (CD), in aqueous solution in the absence of lipids. Previous CD spectroscopic experiments have shown that rmC1 and rmC8 in aqueous solution are primarily unstructured (Bates et al. 2000, 2002), and both UT-rmC1 and UT-rmC8 demonstrated similar characteristics, as anticipated (Fig. 4). After recording the CD spectra for UT-rmC1 and UT-rmC8 in the absence of divalent cation, we then added \( Zn^{2+} \) to a final concentration of 20 times molarity of the protein (1.3 mM). The \( Zn^{2+} \) induced only a slight increase in the large minimum and a slight decrease in the small minimum in both UT-rmC1 and UT-rmC8. It would be expected that solution NMR spectroscopy would reveal any local perturbations (Libich and Harauz 2008b), but here there were no detectable (by CD spectroscopy) global conformational changes of the protein interacting with the cation in aqueous solution.

Effects of \( Zn^{2+} \) on rmMBP and UT-rmMBP reconstituted with Cyt-LUVs—ATR-FTIR spectroscopy

It has long been known that detergents and lipids induce ordered secondary structure in MBP, particularly \( \alpha \)-helix (e.g., Stuart 1996; Polverini et al. 1999); reviewed in (Harauz et al. 2004). However, reconstituting the recombinant MBP variants into a more myelin-mimetic environment, i.e., with Cyt-LUVs, yields semi-solid aggregates unsuitable for CD or, indeed, any type of solution spectroscopy (Libich and Harauz 2008b). Thus, ATR-FTIR spectroscopy (Haris and Chapman 1995; Barth 2007) was used next to examine the secondary structure composition of the four protein preparations in a lipid environment in the presence and absence of \( Zn^{2+} \).

In our analyses, the ATR-FTIR spectra of all four rmMBP and UT-rmMBP variants in D$_2$O solution were normalized based on the amide I’ (deuterated amide I) absorbance. Since it has been reported that \( Zn^{2+} \) ions interact with the phospholipid headgroups (Nuzzo et al. 2002), we first present the spectra of a control experiment,

![Fig. 3](https://example.com/fig3.png)
the Cyt-LUV lipid system (Fig. 5)—it is apparent that the membrane alone makes no significant contribution to the amide I region in the ATR-FTIR spectra.

In Fig. 6, we present the ATR-FTIR spectra of rmC1, rmC8, UT-rmC1, and UT-rmC8 protein variants associated with Cyt-LUVs, in the absence and presence of Zn$^{2+}$ at a 20-fold molar excess (4.1 mM). In Fig. 7, we present summaries of the ordered secondary structure predictions for all samples, derived from analyses of multiple spectra within the range 1,680 and 1,620 cm$^{-1}$ corresponding to the amide I band (Byler and Susi 1986; Barth 2007). It should be cautioned that the calculations of overall secondary structure composition from such spectra might vary depending on the type of peak identification and fitting performed (Barth 2007; Goormaghtigh et al. 2009; Laird et al. 2009). Thus, comparisons with previous FTIR spectroscopic studies on natural MBP preparations in different reconstituted lipid systems (Surewicz et al. 1987; Nabet et al. 1994; Stuart 1996) would be confusing, so we restrict ourselves here simply to reporting on trends.

First, all spectra are characterized by a maximum around 1,639 cm$^{-1}$, and all show reproducible perturbations upon the addition of cation. Second, the addition of Zn$^{2+}$ does not alter the degree of ordered secondary structure ($\alpha$-helix, parallel and anti-parallel $\beta$-sheet, or $\beta$-turn) of any protein sample dramatically, but there are subtle variations. For

### Table 1  Summary of ITC parameters

<table>
<thead>
<tr>
<th>Recombinant MBP variant</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$\Delta H$ (kcal mol$^{-1}$)</th>
<th>$\Delta S$ (cal mol$^{-1}$ K$^{-1}$)</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>rmC1</td>
<td>$(6.5 \pm 0.8) \times 10^4$</td>
<td>$-6.50 \pm 0.19$</td>
<td>0.2</td>
<td>$2.5 \pm 0.1$</td>
</tr>
<tr>
<td>rmC8</td>
<td>$(4.5 \pm 0.6) \times 10^4$</td>
<td>$-6.92 \pm 0.31$</td>
<td>$-1.9$</td>
<td>$2.1 \pm 0.1$</td>
</tr>
<tr>
<td>UT-rmC1</td>
<td>$(2.4 \pm 0.2) \times 10^4$</td>
<td>$-5.69 \pm 0.20$</td>
<td>0.9</td>
<td>$1.9 \pm 0.1$</td>
</tr>
<tr>
<td>UT-rmC8</td>
<td>$(2.4 \pm 0.2) \times 10^4$</td>
<td>$-2.31 \pm 0.31$</td>
<td>12.28</td>
<td>$2.3 \pm 0.1$</td>
</tr>
</tbody>
</table>

All measurements were carried out in triplicate at 25°C. The shown data represent the fitting parameters obtained using the “one set of sites” model. For each variant, “$K_a$” is the association constant, “$\Delta H$” is change in enthalpy, “$\Delta S$” is the change in entropy, and “$n$” is the number of ligands bound.

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**Fig. 4** CD spectroscopy of **a** UT-rmC1 and **b** UT-rmC8 in the absence *(solid line)* and presence *(dashed line)* of Zn$^{2+}$. The protein concentration was 1.3 mg mL$^{-1}$ in a buffer containing 20 mM HEPES-NaOH, pH 7.4, 100 mM NaCl. Upon the addition of Zn$^{2+}$, the divalent cation was present at 1.3 mM concentration, or 20 times the molarity of protein. All spectra were collected at 25°C. Each CD spectrum shown in this figure is the average of three separate spectra. *Insets* in the bottom right corner of each plot depict the change in the averaged CD spectrum upon addition of Zn$^{2+}$.

**Fig. 5** ATR-FTIR spectroscopy of Cyt-LUVs alone in the absence *(solid line)* and presence *(dashed line)* of 4.1 mM Zn$^{2+}$. Note that there were no major changes observed in the amide I region (1,620–1,700 cm$^{-1}$).

In Fig. 6, we present the ATR-FTIR spectra of rmC1, rmC8, UT-rmC1, and UT-rmC8 protein variants associated with Cyt-LUVs, in the absence and presence of Zn$^{2+}$ at a 20-fold molar excess (4.1 mM). In Fig. 7, we present summaries of the ordered secondary structure predictions for all samples, derived from analyses of multiple spectra within the range 1,680 and 1,620 cm$^{-1}$ corresponding to the amide I band (Byler and Susi 1986; Barth 2007). It should be cautioned that the calculations of overall secondary structure composition from such spectra might vary depending on the type of peak identification and fitting performed (Barth 2007; Goormaghtigh et al. 2009; Laird et al. 2009). Thus, comparisons with previous FTIR spectroscopic studies on natural MBP preparations in different reconstituted lipid systems (Surewicz et al. 1987; Nabet et al. 1994; Stuart 1996) would be confusing, so we restrict ourselves here simply to reporting on trends.

First, all spectra are characterized by a maximum around 1,639 cm$^{-1}$, and all show reproducible perturbations upon the addition of cation. Second, the addition of Zn$^{2+}$ does not alter the degree of ordered secondary structure (*$\alpha$*-helix, parallel and anti-parallel *$\beta$*-sheet, or *$\beta$*-turn) of any protein sample dramatically, but there are subtle variations. For
either rmC1 or rmC8, the changes are negligible except perhaps for rmC8, which appears to have a slight increase (a few percent) in amount of $\alpha$-helix with a concomitant decrease in amount of $\beta$-turn. These same trends were observed more clearly for the untagged variants, both UT-rmC1 and UT-rmC8. These findings suggest that, in a lipid environment, UT-rmC1 and UT-rmC8 are more susceptible than rmC1 or rmC8, respectively, to secondary structural changes induced by Zn$^{2+}$-binding. Removal of the hexahistidine tag from the rmC1 and rmC8 variants is thus important to examine the proteins’ ability to interact with metal ions in a lipid environment, and justifies the construction of the UT-rmC1 and UT-rmC8 variants. Although the conformational transitions induced by Zn$^{2+}$-binding are subtle, they are of a nature consistent with the idea of zinc being a stabilizing component of myelin.

Zinc-induced secondary structure transitions in membrane-associated MBP

Structural studies of IDPs such as MBP must take into account the sensitivity of the protein’s conformation to exposure to different environments (Harauz et al. 2004, 2009; Zhong et al. 2007; Libich and Harauz 2008b; Harauz and Libich 2009; Libich et al. 2010; Uversky 2009). Here, partly in this context, we have tested specifically the hypothesis that Zn$^{2+}$ induces ordered secondary structure in 18.5 kDa MBP. Divalent cations such as Zn$^{2+}$ have been shown to lead to a disorder-to-order transition in several other IDPs (Gatewood et al. 1990; Uversky et al. 2000; Uversky 2009), but this is not necessarily the case for all IDPs (Uversky et al. 2002; Permyakov et al. 2002). The 18.5 kDa MBP has long been known to have a strong association with copper (Moscarello et al. 1968), and subsequent studies have reported that the protein binds divalent cations, Hg$^{2+}$ > Cu$^{2+}$ > Zn$^{2+}$ > Mg$^{2+}$ > Cd$^{2+}$ > Co$^{2+}$ in decreasing order of affinity (Berlet et al. 1994). We focused here on zinc for physiological relevance, as discussed in the “Introduction”.

It is seen in Fig. 7 that removal of the hexahistidine tag resulted in a subtly different pattern of conformational changes of the recombinant proteins when complexed with an excess of Zn$^{2+}$ in a myelin-mimetic lipid environment, particularly a small increase in $\alpha$-helix and concomitant decrease in $\beta$-turn content. One explanation may be as follows. Deuterated arginine side chains absorb at wave-numbers of 1,607 and 1,583 cm$^{-1}$. At both of these frequencies, there are significant absorbance increases upon the addition of Zn$^{2+}$ in all four rmMBP constructs studied here (rmC1, rmC8, UT-rmC1, and UT-rmC8). This observation suggests that zinc may compete with the arginine residues for interaction with the phospholipid headgroups, resulting in the “release” of these side chains from the membrane (cf., Cabiaux et al. 1994), and a concomitant conformational change. Moreover, the observation that Zn$^{2+}$ added to any protein variant alone (i.e., in

**Fig. 6** ATR-FTIR spectroscopy of membrane-reconstituted a His-tagged rmC1, b His-tagged rmC8, c UT-rmC1, d UT-rmC8 in the absence (solid line) and presence (dashed line) of 4.1 mM Zn$^{2+}$. Protein samples and large unilamellar vesicles (LUV) were prepared in 20 mM HEPES-NaOH, pH 7.4, 100 mM NaCl at concentration of 4 mg mL$^{-1}$. We mixed 1 mg protein with lipid at 1:1 mass ratio (for details see “Materials and Methods”). Arrows at 1,657 cm$^{-1}$ denote the presence of $\alpha$-helix, and those at 1,629 cm$^{-1}$, and 1,675 cm$^{-1}$ denote the presence of $\beta$-sheet structures. Insets in the upper right show the difference between spectra collected before and after the addition of Zn$^{2+}$.
aqueous solution, Fig. 4) did not significantly alter the secondary structure composition confirmed that there is a synergy of MBP-membrane-cation interactions (Cavatorta et al. 1994; Riccio et al. 1995; Morante 2001; Nuzzo et al. 2002; Benfatto et al. 2004).

Two other recent biophysical studies support our conclusion of zinc-induced conformational changes in MBP. First, Majava et al. (2009) have independently shown changes in synchrotron CD spectra of natural porcine 18.5 kDa MBP dissolved in phosphate buffer, upon titration with Zn$^{2+}$. Their use of phosphate buffer (in contrast to our aqueous solution) may have yielded aggregates of protein, zinc, and phosphate (Cavatorta et al. 1994), thus representing a synergistic association that we mimicked in the solid-state. Secondly, Baran et al. (2010) have recently used nanopore analysis to demonstrate a cation-induced compaction of the same rmMBP variants studied here, suggestive of a higher-order topology such as the “paperclip” fold of tau (von Bergen et al. 2006; Jeganathan et al. 2006; Mukrasch et al. 2009). The conditions in this separate nanopore study required very high salt concentrations (1 M KCl), however, which made correlative secondary structure analysis by CD spectroscopy impossible. Conversely, nanopore analysis could not be performed on the semi-solid membrane-reconstituted protein samples that we have studied here. Nevertheless, the formation of a more compact tertiary fold in the presence of the cation may also account for some of the changes in secondary structure composition that we have observed here.

Conclusions

Two new recombinant forms of the classic 18.5 kDa MBP family, namely UT-rmC1 and UT-rmC8, were overexpressed and purified to greater than 96% purity via a native lysis procedure and ion-exchange chromatography, without the use of an appended purification tag. From 1 L of *E. coli* bacterial culture, 8 and 6 mg of UT-rmC1 and UT-rmC8 were isolated, respectively, and their secondary structures were probed in the absence and presence of Zn$^{2+}$ using CD spectroscopy and ATR-FTIR spectroscopy. Circular dichroism revealed that there was little to no change in secondary structure in either protein variant alone upon the addition of Zn$^{2+}$. In contrast, using ATR-FTIR spectroscopy in a reconstituted membrane environment, Zn$^{2+}$ caused a small but reproducible shift in overall ordered secondary structure of both UT-rmC1 and UT-rmC8, compared to their tagged counterparts. These studies suggest that divalent cations such as Zn$^{2+}$ would have a stabilizing effect on MBP reconstituted within increasingly complex myelin-mimetic environments for solid-state NMR spectroscopic studies (Zhong et al. 2007; Ahmed et al. 2009; Libich et al. 2010).

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