Eur Biophys J (2008) 37:1015–1029
DOI 10.1007/s00249-008-0334-8

ORIGINAL PAPER

Solution NMR and CD spectroscopy of an intrinsically disordered, peripheral membrane protein: evaluation of aqueous and membrane-mimetic solvent conditions for studying the conformational adaptability of the 18.5 kDa isoform of myelin basic protein (MBP)

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Received: 9 February 2008 / Revised: 9 April 2008 / Accepted: 11 April 2008 / Published online: 1 May 2008

Abstract The stability and secondary structure propensity of recombinant murine 18.5 kDa myelin basic protein (rmMBP, 176 residues) was assessed using circular dichroic and nuclear magnetic resonance spectroscopy (^{1}H–^{15}N HSQC experiments) to determine the optimal sample conditions for further NMR studies (i.e., resonance assignments and protein-protein interactions). Six solvent conditions were selected based on their ability to stabilise the protein, and their tractability to currently standard solution NMR methodology. Selected solvent conditions were further characterised as functions of concentration, temperature, and pH. The results of these trials indicated that 30% TFE-d_2 in H_2O (v/v), pH 6.5 at 300 K, and 100 mM KCl, pH 6.5 at 277 K were the best conditions to use for future solution NMR studies of MBP. Micelles of DPC were found to be inappropriate for backbone resonance assignments of rmMBP in this instance.

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Keywords Myelin basic protein · Intrinsically disordered protein · Circular dichroism · Solution NMR spectroscopy · Chemical shift index · HSQC spectrum

Abbreviations

BMRB	Biological Magnetic Resonance Data Bank
C1	Charge component 1 of 18.5 kDa MBP (least-modified, most highly-charged)
CaM	Calmodulin
CD	Circular dichroism
ddH_2O	Distilled, deionised water
DPC($-d_{38}$)	Dodecylphosphocholine (perdeuterated)
DSS	2,2-Dimethyl-2-silapentane-5-sulfonic acid
EPR	Electron paramagnetic resonance
G_{M1}	Monosialoganglioside G_{M1}
HPLC	High-performance liquid chromatography
HSQC	Heteronuclear single quantum coherence
IDP	Intrinsically disordered protein
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Luria-Bertani
LUV	Large unilamellar vesicle
MeOH	Methanol
NTA	Nitrilotriacetic acid
OD_{600}	Optical density at 600 nm
PAGE	Polyacrylamide gel electrophoresis
PI_4P	Phosphatidylinositol-4-phosphate
ppm	Parts per million
rmMBP	Recombinant murine 18.5 kDa isoform
SDSL	Site-directed spin-labelling
SDS	Sodium dodecyl sulphate
TFE($-d_2$)	2,2,2-Trifluoroethanol (perdeuterated), (CF_3–CH_2–OH, or CF_3–CD_2–OH)
TMAO	Trimethylamine-*N*-oxide
TRIS	Tris(hydroxymethyl)aminomethane

Introduction

Myelin basic protein (MBP) is a family of developmentally regulated and translocatable isoforms involved in the formation of the myelin sheath of the central nervous system (Campagnoni et al. 2001; Givogri et al. 2001). The 18.5 kDa isoform of MBP is most common in adult humans and exists as a series of highly post-translationally modified charge isomers, or components (Kim et al. 2003). The in vivo environment of this isoform is the major dense line of myelin, where it maintains the cytoplasmic leaflets of the oligodendrocyte membrane in close apposition. In its deiminated form (arginyl residues are converted to citrulline), MBP is also a candidate autoantigen in the autoimmune disease multiple sclerosis (Warren et al. 2006; Musse et al. 2007). The various isoforms of this protein potentially have roles in signalling pathways during myelin development (Boggs 2006), yet our understanding of these phenomena is severely limited by the lack of detailed structural knowledge of any MBP variant (Harauz et al. 2004). Since all forms of MBP belong to the intrinsically disordered proteome (Hill et al. 2002), such knowledge has been impossible to obtain using X-ray or electron crystallography.

The conformation of MBP is highly dependent on its environment (Harauz et al. 2004)—it is highly extended and flexible in aqueous solution, but attains ordered secondary structure in the presence of detergents and lipids, as well as in organic solvents such as trifluorethanol (TFE) (Liebes et al. 1975; Smith 1992; Polverini et al. 1999; Bates et al. 2000; Hill et al. 2002; Farès et al. 2006). Site-directed spin-labelling (SDSL) and electron paramagnetic resonance (EPR) experiments on a recombinant form of the 18.5 kDa isoform of murine MBP (rmMBP) in protein-vesicle pellets have defined how different regions of the protein associate with myelin-like membranes, especially the topology of a central amphipathic α-helical segment (Bates et al. 2003a, 2004; Musse et al. 2006). As an extension of these interaction studies, the secondary and tertiary structures of full-length rmMBP, and other early developmental MBP isoforms, are being investigated in our group using high-resolution NMR spectroscopy (Libich et al. 2004, 2007, 2008; Ahmed et al. 2007).

Sample preparation for solution NMR spectroscopy is critical to ensure that high quality data are obtained. In general, samples for biomolecular NMR experiments should be free of precipitates which may cause magnetic field inhomogeneities, have a relatively low ionic strength to prevent sample heating, and should have as high a concentration of analyte as possible (Cavanagh et al. 1996; Rule et al. 2006). The latter two of these requirements are not always readily achievable for biological samples. Extended sample stability should also be considered, since many of the biomolecular NMR experiments used for resonance assignments require hours to days to complete (Kay 1995; Kanelis et al. 2001). Thus, it was imperative first to investigate various solvent and physical conditions (e.g., concentration, temperature, and pH) before undertaking lengthy NMR experiments on rmMBP. There have been several reviews of the process of evaluation of sample conditions for integral membrane proteins and peptides (Krueger-Koplin et al. 2004; Sanders et al. 2006; Page et al. 2006; Da Costa et al. 2007) which served as a guide to this work, but which could not be definitive. The reason is that MBP is a peripheral (not integral) membrane protein, in addition to being intrinsically disordered, creating a more complex situation (Eliezer 2007).

In the present study, solution conditions that satisfied the following criteria were sought: (1) can maintain high solubility and stability of rmMBP, (2) are readily available in deuterated form, and (3) are usable in NMR samples at reasonable concentrations. Using the literature, the aforementioned criteria, and a priori observations, six solvent conditions were selected for further screening: potassium chloride (KCl), trimethylamine-*N*-oxide (TMAO), trifluoroethanol (TFE), methanol (MeOH), sodium dodecyl sulphate (SDS), and dodecylphosphocholine (DPC). As a potential stabilising factor, the effect of Zn^{2+} was also examined at different temperatures (Riccio et al. 1995; Tsang et al. 1997). A further consideration for the selection of appropriate conditions for NMR studies of MBP was to build a basis for studies of the interaction with calmodulin (CaM) and other proteins (Ikura et al. 1990; Libich et al. 2003, 2008). Initially in this work, CD spectroscopy was used to screen potential solution environments and physical parameters (i.e., temperature and pH) but the final selection of sample conditions was made through the qualitative analysis of (^{1}H–^{15}N) heteronuclear single quantum coherence (HSQC) spectra.

Materials and methods

Materials

Materials of electrophoresis grade or higher, including ultrapure urea, potassium phosphate, glycine, acetate, and TMAO were purchased from ICN Biochemicals (Costa Mesa, CA). Electrophoresis grade SDS was purchased from Bio-Rad Laboratories (Mississauga, ON). Synthetic DPC was purchased in powder form from Avanti Polar Lipids (Alabaster, AL). The Ni^{2+}-NTA agarose beads were obtained from Qiagen (Mississauga, ON). Perdeuterated triflouroethanol (TFE-d_2), perdeuterated dodecylphosphocholine (DPC-d_{38}), D_2O, and $^{15}NH_4Cl$ were acquired from Cambridge Isotope Laboratories (Andover, MA). All other chemicals were reagent grade and purchased from either

Fisher Scientific (Unionville, ON) or Sigma-Aldrich (Oakville, ON).

Protein purification

The purification of C-terminal Leu–Glu–His_6-tagged rmMBP (quasi-C1 variant of the 18.5 kDa classic isoform) was by nickel-chelation chromatography, and assessment of purity by SDS-PAGE and reversed-phase HPLC as previously described (Bates et al. 2000, 2002). The purification of uniformly ^{15}N-labeled rmMBP (rmMBP, 176 residues including an LEH_6 tag) was based on a modification of the procedure described above. A starter culture of *E. coli* BL21-CodonPlus(DE3)-RP cells (Stratagene, La Jolla, CA), transformed with a pET-22b(+) plasmid containing the murine 18.5 kDa MBP clone with a C-terminal LEH_6 tag (Bates et al. 2000) was grown in Luria-Burtani (LB) media overnight, and used to inoculate a starter culture in fresh LB broth, which was grown until $OD_{600} = 0.7$. The *E. coli* cells in the starter culture were harvested by gentle centrifugation and immediately resuspended in pre-warmed M9 minimal media supplemented with $^{15}NH_4Cl$ as the sole nitrogen source. The cultures were grown in M9 media to $OD_{600} = 0.6$, and protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cultures were allowed to grow for an additional four hours. The cells were recovered by centrifugation (3,000 rpm, 20 min), and frozen at −80°C. The cell pellets were resuspended in lysis buffer (6 M urea, 500 mM NaCl, 100 mM $NaPO_4$, 10 mM Tris, pH 8.0, 1% (v/v) Tween-20) and lysed via sonication (eight cycles of a 10 s pulse and 30 s cooling). The sonicated lysate was cleared by centrifugation (14,000*g* for 35 min), and passed through a 0.45 μm syringe filter (Millipore, Bellerica, MA).

The crude cell extract was then passed twice through a metal chelation (Ni^{2+}-NTA) column (Qiagen), with the flow-through from the initial run used to load the column for the second pass; this step was designed to maximise recovery of the labelled protein. Column fractions were collected and analysed by SDS-PAGE to assess protein content. Fractions containing detectable amounts of rmMBP were pooled. The pooled metal chelation column fractions were subsequently dialysed overnight (6 M urea, 80 mM glycine, pH 10.0), then applied to a cation-exchange column (GE Healthcare, Piscataway, NJ) and eluted with a 0–0.2 M NaCl gradient. The fractions containing purified rmMBP were pooled, step-dialysed through increasingly lower NaCl concentrations with a final exchange into Milli-Q water (Millipore), and lyophilised. The purified, lyophilised rmMBP was stored at −80°C until used. A yield of approximately 10–12 mg of purified, labelled rmMBP was consistently obtained per litre of M9 culture. Protein concentrations were determined either by difference weighing, or by measuring the absorbance at 280 nm using an extinction coefficient of 0.627 L g^{-1} cm^{-1}, a method that has previously been calibrated by amino acid analysis (Bates et al. 2000).

Circular dichroic (CD) spectroscopy

Circular dichroic spectroscopy was used to assess qualitatively the relative proportion of secondary structure of rmMBP formed in various solvent conditions, and to evaluate the effects of varying physical parameters such as temperature and pH on the stability and proportion of secondary structure in the protein. The various conditions investigated are described in the following subsections. In all samples, the concentration of rmMBP was 0.2 mg/mL. All CD spectroscopic measurements were performed using a Jasco J-810 spectrapolarimeter (Japan Scientific, Tokyo), equipped with a recirculating water bath. Measurements were taken at a 100 nm min^{-1} rate, at 0.1 nm intervals, over a range of 180–260 nm. Samples (0.3 mL volume) were placed in a 0.1 cm path-length quartz cuvette, except for the temperature series, which used a 1 cm path-length quartz cuvette. Four successive scans were recorded, the sample blank was subtracted, and the scans were averaged. The data averaging and smoothing (using an inverse square algorithm) operations were accomplished with the SigmaPlot (SPSS, Chicago, IL) computer program.

CD spectroscopy—influence of solvent conditions

Initial investigations focussed on the influence of different solvent conditions on the proportion of secondary structure stabilisation in rmMBP. Although MBP is mainly disordered in aqueous solution, it adopts an increased proportion of α-helix in the presence of detergent, lipid, or other membrane-mimetic solvents (see Introduction), showing that segments of the protein have the propensity for forming this secondary structure motif. In this study, solvent conditions that could be amenable for future NMR experiments were selected: KCl, TMAO, TFE, MeOH, the ionic detergent SDS, and the lysolipid DPC. Protein samples (0.2 mg/mL rmMBP) were made up from a single stock solution in the following concentrations or v/v percentages: (1) either KCl, DPC, or SDS 1, 10, 50, 100, and 150 mM; (2) TMAO 25, 100, 500, and 1,000 mM; and (3) either TFE or MeOH 1, 10, 20, 30, 40, 60, and 90% (v/v). The pH was 6.5 for all of these studies, and remained stable over the lifetime of the samples.

CD spectroscopy—influence of temperature

Based on the results of the solvent concentration experiments, the most promising concentrations of each solvent

were chosen by selecting stabilised, non-precipitating samples that used the least amount of solvent as possible. Then CD spectra were collected from 278 to 343 K, in 5 K increments, on rmMBP (0.2 mg/mL) dissolved in either 100 mM KCl, 30% TFE, or with 50 mM DPC.

CD spectroscopy—influence of pH

To refine the NMR sample conditions further, the behaviour of rmMBP at different pH values was assessed. From previous dynamic light scattering experiments (Liebes et al. 1975; Hill et al. 2002), it was known that rmMBP (and natural 18.5 kDa MBP purified from bovine brain) tends to self-associate in solutions that are basic (i.e., pH > 8.0). For this reason, only acidic conditions were evaluated here. Samples of 0.2 mg/mL rmMBP dissolved in either 100 mM KCl, 30% TFE, or 50 mM DPC were adjusted to pH 2.5 (20 mM glycine), pH 4.5 (20 mM acetate), or pH 6.5 (20 mM potassium phosphate), and evaluated using CD as described above.

Solution NMR spectroscopy

The NMR spectra were recorded under varying conditions of temperature and solvent concentration (see individual figure captions for details) to assess qualitatively the effects of these parameters on rmMBP conformation. Samples were prepared by dissolving 8–12 mg of lyophilised rmMBP into either: (1) KCl (10–200 mM) containing 10% D_2O, (2) TFE-d_2 (10–100% v/v), or (3) DPC-d_{38} (50–250 mM in 20 mM potassium phosphate buffer) containing 10% D_2O, to a final volume of 350 µL. This procedure yielded rmMBP concentrations between 1.2 and 1.8 mM, which were introduced into D_2O-matched Shigemi NMR tubes (Shigemi, Allison Park, PA). In all samples, the pH of the protein-containing solution was 6.5, and 0.005% NaN_3 was added as a bactericide and fungicide.

The ^1H–^{15}N HSQC spectra were recorded on a Bruker Avance spectrometer operating at a proton Larmor frequency of 600.13 MHz. All experiments were recorded with 16 transients, 2048 × 256 points ($F_2 \times F_1$); the spectral widths were 11 and 70 ppm for F_2 and F_1, respectively. The carrier frequencies were set at 4.7 ppm (F_2) and 119 ppm (F_1), with echo-anti-echo quadrature detection used in F_2. The spectra were apodised using a shifted ($\pi/6$), squared sinusoidal bell function, and zero-filled (F_2, 1024 points) and linear-predicted (F_2, 16 prediction coefficients), prior to Fourier transformation. The program TopSpin 2.0 (Bruker BioSpin GmbH, Rheinstetten, Germany) was used for spectral processing. The ^1H chemical shifts were referenced to the methyl signal of DSS (2,2-dimethylsilapentane-5-sulphonic acid) in an external sample tube. The ^{15}N shifts were indirectly referenced using the IUPAC recommended ratio of 0.101329118 ($\gamma^{15}N/\gamma^{1}H$) (Markley et al. 1998).

Results and discussion

CD spectroscopy—selection of a "physiologically relevant" solvent condition

Several criteria were used to select the initial slate of solvent conditions (salt, alcohol, detergent, or lipid) that could serve as potential candidates for NMR sample preparation of rmMBP, i.e., provide a stabilising solution environment for rmMBP to allow it adopt a more-ordered secondary structure type than random coil: (1) the molecule should not form a complex with MBP that would cause a significant increase in the rotational correlation time of rmMBP; (2) the molecule must be readily available in perdeuterated form; (3) the molecule should somehow stabilise rmMBP so that the sample would not change (e.g., no aggregation or precipitation) for the relatively long measurement times needed for NMR structural studies (i.e., days to weeks); and (4) the molecule should be able to satisfy the above criteria at a sufficiently low concentration. Six solvent conditions were selected for further investigation: KCl and TMAO (to study the effects of charge and other stabilisation), TFE and MeOH (to study the effects of reducing the dielectric constant of the protein's environment), and SDS and DPC (which form micelles, thereby mimicking its natural membrane environment). The initial trial was to determine the effects on rmMBP of the selected solvent conditions at various concentrations, the results of which are shown in Fig. 1.

CD spectroscopy—aqueous solution with salts or osmolytes

First, KCl was selected because it is known to influence the overall stability of rmMBP in solution by providing a charge stabilisation effect (Bates et al. 2003b) on an extended basis (i.e., months). This knowledge is based on our experience in storage and handling of this protein, as well as experiments using fluorescence spectroscopy (Libich et al. 2002b) and dynamic light scattering (Hill et al. 2002). Our binding studies of the rmMBP–CaM interaction were conducted in a NaCl-containing buffer (Libich et al. 2003), and we considered that KCl would serve as a suitable ionic environment for future protein–protein interaction studies. Since the resonance assignments of CaM were already available recorded in 100 mM KCl (Ikura et al. 1991), we selected this salt concentration for further experiments. Indeed, there was very little observable difference in rmMBP's conformation between 1 and 150 mM KCl as

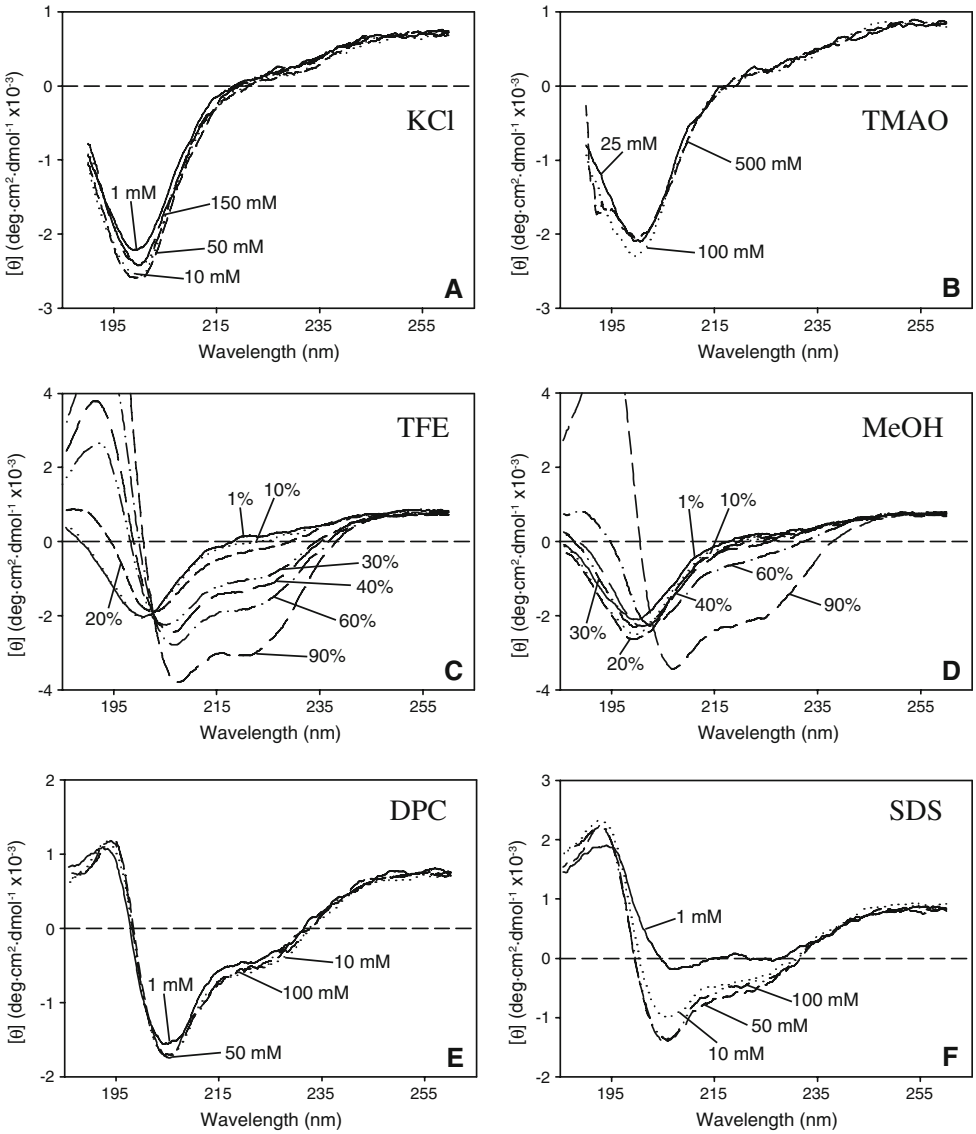

Fig. 1 Circular dichroic spectra of rmMBP dissolved in various solvent conditions, to investigate protein stabilisation by charge, altered dielectric constant, or detergent/lysolipid interaction. Initial conditions examined by CD, for subsequent NMR of rmMBP, included: **a** KCl in water at concentrations of 1, 10, 50, and 150 mM; **b** TMAO at concentrations of 25, 100, and 500 mM; **c** TFE at concentrations of 1, 10, 20, 30, 40, 60, and 90% (vol/vol); **d** MeOH at concentrations of 1, 10, 20, 30, 40, 60, and 90% (vol/vol); **e** DPC at concentrations of 1, 10, 50, and 100 mM; and **f** SDS at concentrations of 1, 10, 50, and 100 mM. Due to the overlap of the traces, labels have been added to all the panels for clarity. Since KCl has a strong absorption in the far-ultraviolet region (**a**), the traces have been trimmed below 190 nm. Furthermore, 1,000 mM TMAO (**b**) also has a very strong absorbance in the far-ultraviolet region, and this trace was excluded from the panel

judged by CD spectroscopy (Fig. 1a)—the spectra obtained at 1, 10, 50, and 150 mM KCl were almost superimposable, consistent with previous CD studies of rmMBP (Ishiyama et al. 2001; Hill et al. 2002).

Osmolytes such as TMAO have been used as natural stabilisers of protein conformations in many studies (Bolen et al. 2001; Bolen 2001; Uversky et al. 2001; Kumar et al. 2007), including in NMR spectroscopy (Bennion et al. 2004a, b; Doan-Nguyen et al. 2007; Xie et al. 2007). Similarly to KCl, TMAO (Fig. 1b) had little influence on the proportion of secondary structure stabilised in rmMBP, as previously reported by us (Hill et al. 2002). Furthermore, we considered that the high concentrations of TMAO (1–2 M) that would be needed to have an effect on the stability of rmMBP, without a concomitant increase in degree of ordered secondary structure, precluded its usefulness as a solvent for solution NMR studies, and it was rejected for further investigation.

CD spectroscopy—solutions of membrane-mimetic alcohols

Alcohols and other organic solvents (ethanol, methanol, propanol, chloroform, acetone, dimethylsulphoxide, hexa-

fluoroacetone, hexafluoroisopropyl alcohol, and TFE) may be considered to be membrane-mimetic solvents for proteins in solution due to their proposed mechanism of action (Buck 1998; Roccatano et al. 2002; Otzen et al. 2007; Povey et al. 2007), and have been employed in numerous peptide and protein studies. By reducing the dielectric constant around the peptide or protein, alcohols allow the preferential formation of inter-residue hydrogen bonds which stabilise secondary structure elements such as α-helices (Roccatano et al. 2002). Membrane–protein interfaces also stabilise inter-residue hydrogen bond networks by the exclusion of bulk solvent (reducing the dielectric constant) (Otzen et al. 2007). It is by this analogy that alcohols such as TFE are referred to as membrane-mimetic solvents. Moreover, alcohols and other organic solvents do not form large complexes with rmMBP that would restrict its tumbling in solution.

Here, TFE (Fig. 1c) and MeOH (Fig. 1d) were evaluated at 1, 10, 30, 40, 60, and 90% (v/v in H_2O). A previous CD spectroscopic study by Liebes et al. (1995) of MBP dissolved in TFE demonstrated three 'transitions' of increasing α-helical content in MBP at approximately 30, 60, and 90% TFE (Liebes et al. 1975). These findings are echoed in Fig. 1c, which clearly shows increasing α-helical content (as ascertained by the troughs at roughly 209 and 220 nm) between the 20–30%, 40–60%, and 60–90% traces. It is also apparent that TFE is much more effective at stabilising secondary structure in rmMBP than MeOH (compare Fig. 1d with Fig. 1c, respectively), so MeOH was rejected as a potential solvent for solution NMR studies of rmMBP. Examination of the CD traces in Fig. 1c suggests that at a relatively low concentration of 30%, TFE is able to stabilise a substantial portion of α-helical structure in rmMBP and lies at the first transition (compared with the 20% trace). For these reasons, 30% TFE was chosen as a potential candidate solvent for solution NMR studies of rmMBP.

CD spectroscopy—micelle-forming detergents and lysolipids

Small micelle-forming detergents and lysolipids such as SDS and DPC are frequently used for NMR studies of membrane-associated peptides and small proteins (Mendz et al. 1988; Lee et al. 2003; Raussens et al. 2003). Here, the CD spectra of rmMBP in DPC (Fig. 1e) and SDS (Fig. 1f) were recorded at detergent/lipid concentrations of 1, 10, 50, and 100 mM. At concentrations as low as 10 mM, DPC seems to have an effect similar to 100 mM DPC (Fig. 1e). Briefly reviewing the literature, DPC concentrations in the range of 40–100 mM are typically used in solution NMR investigations (Weers et al. 1998; Wang et al. 1998; Hwang et al. 2002; Ding et al. 2006); therefore, DPC was chosen as a potential solvent for subsequent NMR studies of rmMBP.

The strongly ionic detergent SDS (Fig. 1f) at 100 mM has a very similar (in terms of proportion of secondary structure) effect to that of 100 mM DPC, and there seems to be very little difference between 50 and 100 mM SDS traces. Thus, SDS could also be considered as a potential solvent for subsequent NMR investigations, and indeed has been used for proteins such as alpha-synuclein (Eliezer et al. 2001). However, because of our a priori knowledge that rmMBP exhibits erratic electrophoretic mobility in SDS-PAGE (Campagnoni et al. 1974; Libich et al. 2002a), and that complexes of unpredictable shapes are formed by other proteins in the presence of SDS (Samso et al. 1995; Westerhuis et al. 2000), we concluded that SDS would thus be unsuitable for solution NMR for our purposes, and decided to exclude it from subsequent investigations.

We also rejected other lipids and lipid combinations with which to reconstitute rmMBP into a membrane environment for solution NMR. The protein aggregates large unilamellar vesicles (LUVs) to form multibilayers with MBP in between, in a physiological environment (Jo et al. 1995; Boggs et al. 1997), so structural studies of rmMBP reconstituted in these types of systems requires solid-state NMR spectroscopy (Zhong et al. 2007). Lipid bicelles (as opposed to micelles such as those formed by SDS or DPC) represent an alternative type of bilayer structure for solution NMR of membrane-associated proteins (Sanders et al. 1994, 1998). A systematic evaluation of the association of numerous proteins (including unfractionated MBP) with bicelles has been published (Sanders et al. 1995). However, we have found using dynamic light scattering that rmMBP also aggregated bicelles of dihexanoylphosphatidylcholine:dioleoylphosphatidylcholine at increasing protein:lipid ratios (Farès and Harauz, unpublished data), and chose not to pursue this approach further. It has long been known that MBP gains an increased proportion of α-helix in the presence of non-vesicle forming lipids such as GM_1 or PI_4P, and these protein–lipid complexes remain soluble for CD spectroscopy (Beniac et al. 2000; Ishiyama et al. 2001, 2002). However, it was judged that these conditions would also form a complex which would be too large (i.e., their rotational correlation times would be too long) to produce sufficiently narrow linewidths in NMR spectra, and they were not considered further.

CD spectroscopy—effects of temperature

The three conditions selected from the first solvent concentration trials (100 mM KCl, 30% TFE, and 50 mM DPC) were next evaluated at different temperatures (Fig. 2). The objective of this set of CD experiments was to define the range of temperatures that would provide the best stability for rmMBP, as judged qualitatively by tracking the movement of the major observable CD transitions in each set of traces.

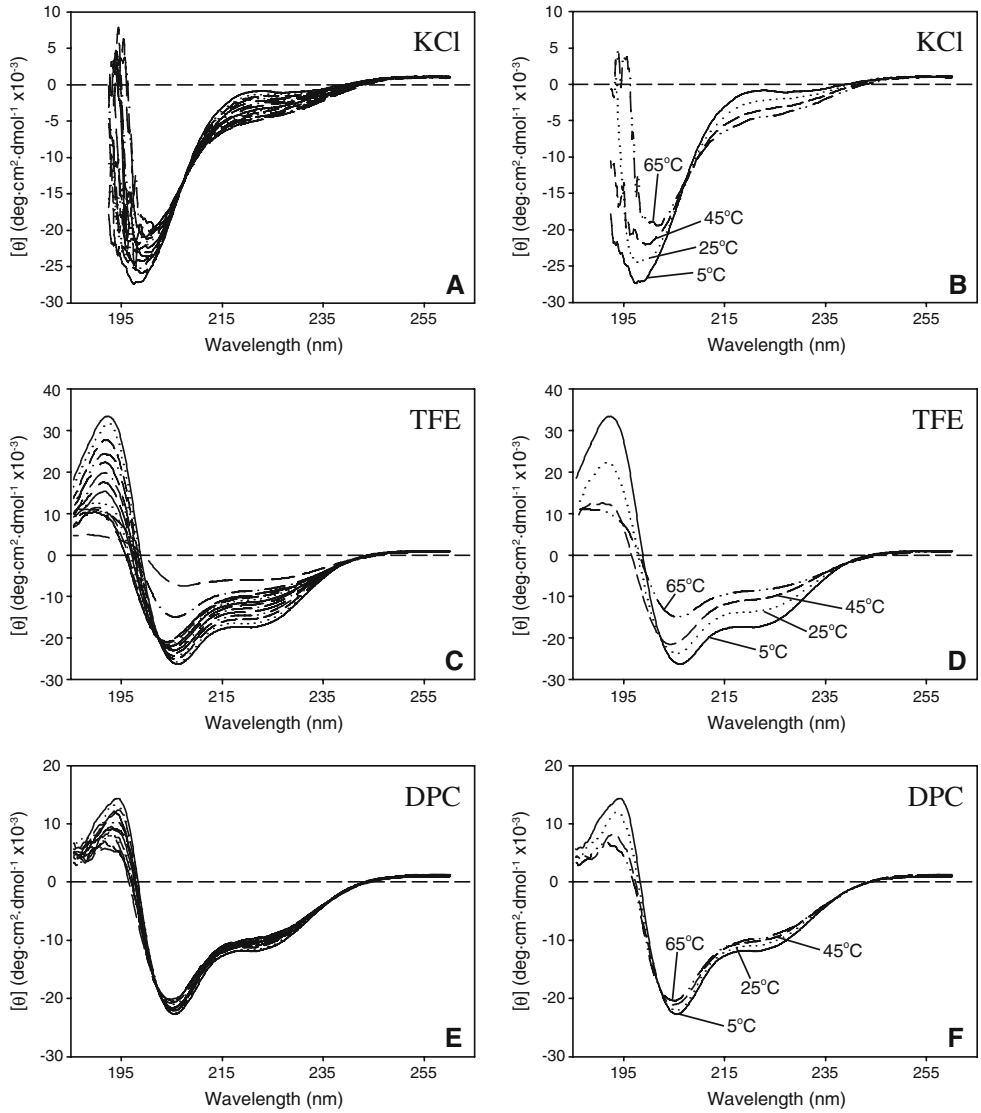

Fig. 2 Temperature dependence of the conformation of rmMBP. Three conditions from the solvent investigation (Fig. 1) were selected as possible solution NMR sample conditions and evaluated by CD. The relative stability of rmMBP dissolved in 100 mM KCl (**a**, **b**), 30% TFE (**c**, **d**), and 50 mM DPC (**e**, **f**), was investigated as a function of temperature. Panels **a**, **c**, and **e** show the complete range of temperatures (5–70°C) in the series. Panels **b**, **d**, and **f** each show four sample traces at temperatures 5, 25, 45, and 65°C which exemplify the changes that rmMBP undergoes upon temperature increases. For this experiment, samples (0.3 mL volume) were placed in a 1 cm path-length quartz cuvette (water-jacketed flow cell). For the experiments shown in Figs. 1 and 3, a 0.1 cm path-length quartz cuvette was used. Thus, although the concentration of rmMBP was the same in all three studies, a factor of 10 in recorded intensity is apparent here due to the different volumes of the two types of cuvettes

For rmMBP dissolved in 100 mM KCl (Fig. 2a, b), the transition at 198 nm was monitored, and for rmMBP dissolved in either 30% TFE (Fig. 2c, d), or 50 mM DPC (Fig. 2e, f), the characteristic α-helical transitions at 209 and 222 nm were monitored. Generally, increasing temperature reduced the effectiveness of the solvent as a stabilising agent if rmMBP was dissolved in either 100 mM KCl or 30% TFE, presumably due to increased molecular motion at increased temperatures (Fig. 2b, d, respectively). There was very little effect on the α-helical content of rmMBP upon increasing temperature for the protein dissolved in 50 mM DPC, as ascertained by CD spectroscopy. Thus, selection of the best temperatures suitable for NMR studies of rmMBP in these three conditions required the qualitative analysis of NMR spectra (see below).

CD spectroscopy—buffer selection

It has been previously shown using dynamic light scattering that rmMBP may form small sub-populations of aggregates at basic pH (Liebes et al. 1975; Hill et al. 2002). Although these complexes make up less than 1% of the overall

conformer ensemble, and may be diminished using salt (e.g., NaCl), the investigation of pH values suitable for solution NMR spectroscopy of rmMBP was limited to acidic conditions. Furthermore, in acidic pH (3–5) conditions, the exchange rate of the amide proton with water is at a minimum; this exchange is increased as the sample pH is increased, and thus can reduce peak intensity, but is still minimal near neutral pH (Bai et al. 1993; Rule et al. 2006).

The three conditions selected above for further investigation from the concentration and temperature trials (100 mM KCl, 30% TFE, and 50 mM DPC) were re-evaluated in 20 mM buffered solutions at pH 2.5, 4.5, and 6.5 (Fig. 3). As a comparison, rmMBP in the absence of any of the discussed stabilising agents (i.e., rmMBP in H_2O) was also examined at each of the three pH conditions (Fig. 3a). These CD spectra further illustrate the influence of these particular stabilising agents on the secondary structure of rmMBP, since rmMBP clearly reverts to a higher proportion of random coil as the pH is decreased. Interestingly, in the presence of any of the selected stabilising agents, either 100 mM KCl (Fig. 3b), 30% TFE (Fig. 3c), or 50 mM DPC (Fig. 3d), there is very little influence of pH on the secondary structure of rmMBP, particularly when contrasted with rmMBP dissolved in water (Fig. 3a). This observation further illustrates the stabilisation effect of the solvent, and underscores the importance of environmental influence on the conformation of rmMBP. Since there was no perceived advantage (based on the CD results) to preparing samples at more acidic pH, and in the interests of sample simplicity, it was decided to exclude a buffer for subsequent solution NMR spectroscopy, and all successive NMR experiments were conducted at pH 6.5.

NMR spectroscopic evaluation of rmMBP sample conditions

Circular dichroic spectroscopy is an ideal technique for sampling many combinations of potential solvent and physical conditions prior to performing solution NMR studies, since it requires very small amounts of protein, and is comparatively fast and efficient. The CD experiments described above provided a good initial screen for appropriate conditions, which were next studied further by collecting ^{1}H–^{15}N HSQC spectra of rmMBP at different solvent concentrations and temperatures. These data were qualitatively assessed by the dispersion of the cross-peaks, and peak shape (i.e., were the peaks sufficiently narrow to be resolved on an HSQC spectrum?). The results of these trials are shown in Figs. 4, 5, and 6.

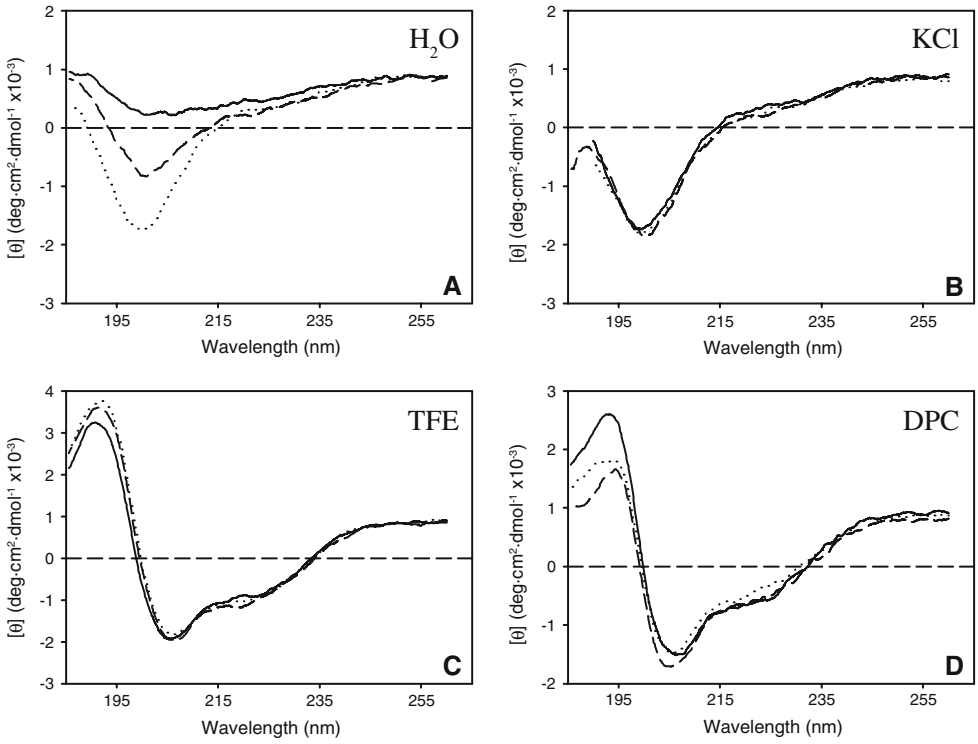

Fig. 3 Effects of pH on the conformation of rmMBP. The CD spectra for three conditions selected as potential candidates for rmMBP NMR sample preparation. Samples (**b**) 100 mM KCl, (**c**) 30% TFE, and (**d**) 50 mM DPC were qualitatively compared with the stability changes observed for rmMBP dissolved in (**a**) H_2O as a function of pH. Each solution condition was examined at pH 2.5 (20 mM glycine buffer, *solid line*), pH 4.5 (20 mM acetate buffer, *dashed line*), and pH 6.5 (phosphate buffer, *dotted line*)

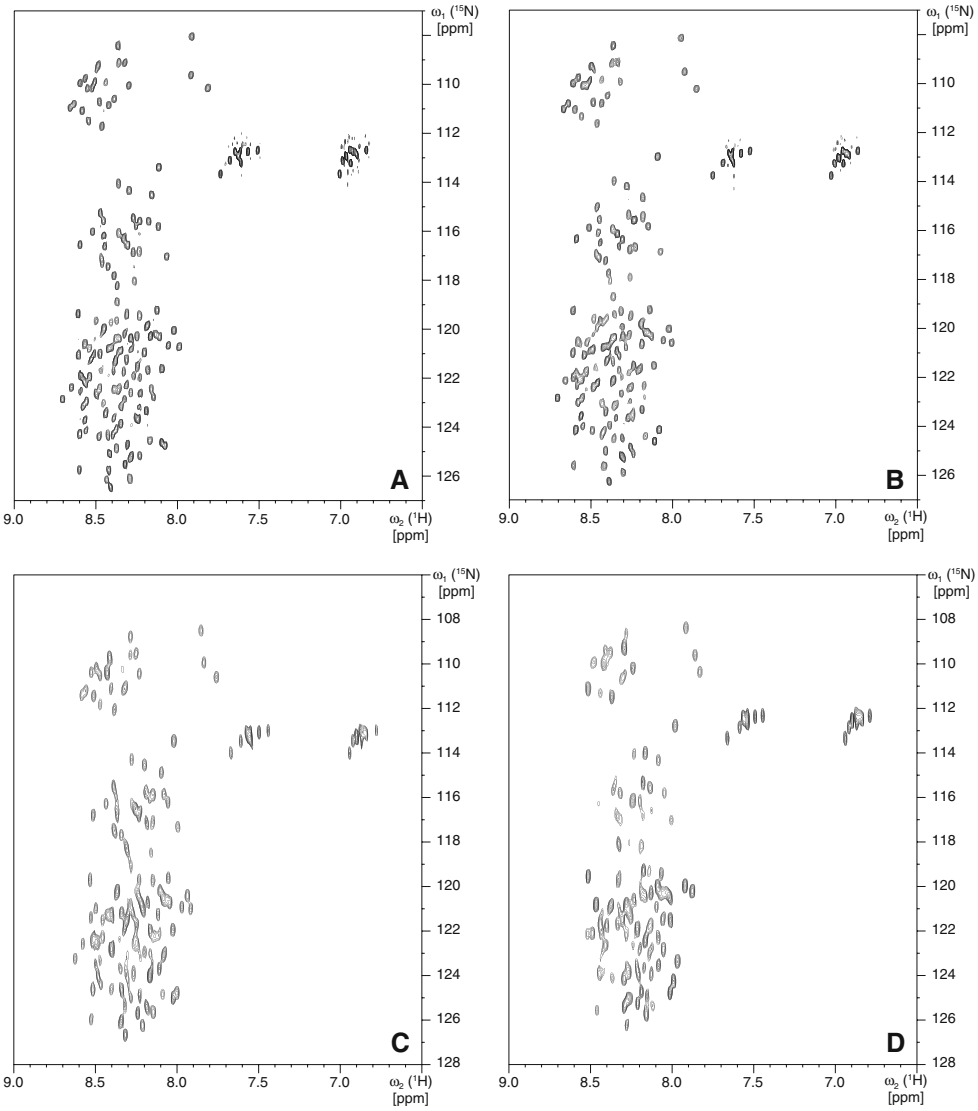

Fig. 4 The ^{1}H–^{15}N HSQC spectra of rmMBP in KCl. **a**, **b** Evaluation of ionic strength. Uniformly ^{15}N-labelled rmMBP dissolved in (**a**) 10 mM KCl, or **b** 200 mM KCl. Each sample was at pH 6.5 and contained 10% D_2O and 0.005% NaN_3; the temperature was 277 K. Protein concentration was 1.47 mM in each sample. The acquisition and processing parameters for each spectrum were identical for qualitative comparison. **c**, **d** Evaluation of temperature. Uniformly ^{15}N-labelled rmMBP (1.26 mM) dissolved in 100 mM KCl, 10% D_2O, and 0.005% NaN_3. Spectra were recorded at (**c**) 277 K, or (**d**) 300 K. The acquisition and processing parameters for each set of spectra (**a**, **b**) or (**c**, **d**) were identical, allowing for qualitative comparison

NMR spectroscopy of rmMBP in KCl

Similar to the results from the CD concentration experiments for KCl, the NMR spectra of rmMBP dissolved in either low (10 mM KCl, Fig. 4a) or high (200 mM KCl, Fig. 4b) salt concentrations indicated that KCl was a simple yet effective stabilising agent for rmMBP. There were very few macroscopic differences observed between the two spectra. Since previous rmMBP-CaM binding studies had been conducted in buffered salt solutions (Libich et al. 2003), we chose 100 mM KCl as one of the solvent conditions for resonance assignments of rmMBP.

The effects of temperature on rmMBP dissolved in 100 mM KCl were also evaluated using two-dimensional NMR spectroscopy; the results of the low (277 K, Fig. 4c) and high (300 K, Fig. 4d) temperatures are shown. Although the spectra are very similar in appearance, it was judged that 277 K would be a more suitable temperature for resonance assignments of rmMBP because of the slightly reduced line widths. Furthermore, cooling the sample was considered to aid in preservation and stability over the long periods necessary to record a set of three-dimensional heteronuclear correlation spectra used for resonance assignments (Cavanagh et al. 1996; Rule et al. 2006).

NMR spectroscopy of rmMBP in TFE

The results of the concentration trials are shown for rmMBP dissolved in either 30% TFE-d_2 (Fig. 5a), or 100% TFE-d_2 (Fig. 5b). It is clear that although the CD results indicated that rmMBP adopts a significant proportion of α-helix at increasing TFE-d_2 concentrations, this greater degree of order does not translate to suitable NMR conditions. The HSQC spectrum of rmMBP in 100% TFE-d_2 displayed far fewer than the expected number of cross-peaks, and the resonances that were resolved tended to be very broad, possibly indicating precipitation and/or aggregation. In contrast, the dispersion and peak width observed in the

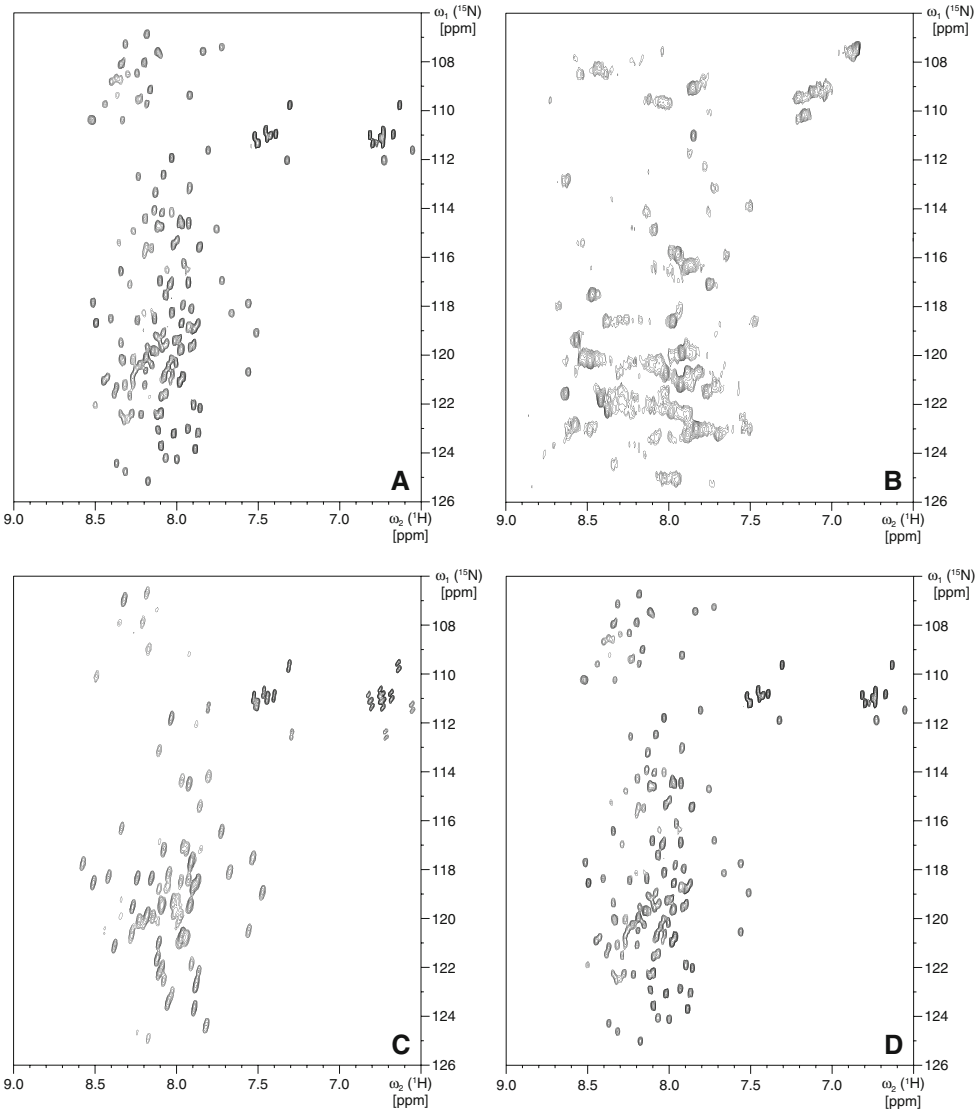

Fig. 5 The ^{1}H–^{15}N HSQC spectra of rmMBP in TFE-d_2. **a**, **b** Evaluation of solvent concentration. Uniformly ^{15}N-labelled rmMBP dissolved in (**a**) 30% TFE-d_2, or (**b**) 100% TFE-d_2. The protein concentrations were (**a**) 1.78 mM, and (**b**) 1.51 mM. The samples also contained 0.005% NaN_3; the pH was 6.5, and the temperature was set to 300 K. The acquisition and processing parameters for each spectrum were identical for qualitative comparison. **c**, **d** Evaluation of temperature. Uniformly ^{15}N-labelled rmMBP (1.78 mM) dissolved in 30% TFE-d_2 and 0.005% NaN_3. Spectra were recorded at (**a**) 277 K, or (**b**) 300 K. The acquisition and processing parameters for each spectrum were identical, allowing for qualitative comparison

spectra of rmMBP in 30% TFE-d_2 were sufficient for resonance assignments. Thus, 30% TFE-d_2 was selected as a solvent condition for future resonance assignments.

The comparison of HSQC spectra of rmMBP at low (277 K, Fig. 5c) and high (300 K, Fig. 5d) temperatures suggested that temperatures at ambient or above are the most suitable for rmMBP dissolved in 30% TFE-d_2. Many cross-peaks were unresolved in the spectra recorded at 277 K, whereas nearly all expected resonances were resolved at the 300 K condition.

Since long-term stability of rmMBP was a concern, samples of rmMBP in these two solvent conditions were checked after several months using both a pH meter and comparison of ^{1}H–^{15}N HSQC spectra (results not shown). Samples of rmMBP dissolved in either 100 mM KCl or 30% TFE-d_2 were found to have maintained a pH of 6.5, and did not exhibit any changes in the observed peak positions, indicating excellent sample stability with no evidence of aggregation or degradation.

NMR spectroscopy of rmMBP in DPC micelles

Examination of HSQC spectra of rmMBP dissolved in moderate (50 mM, Fig. 6a) and high (250 mM, Fig. 6b) concentrations of the perdeuterated lysolipid DPC-d_{38} show a striking difference. Although the HSQC spectrum of rmMBP recorded with 50 mM DPC-d_{38} seems better resolved than the spectrum with 250 mM DPC-d_{38} (in terms of line widths and peak intensity), approximately two-thirds of the expected cross-peaks remain unresolved under both conditions. This phenomenon is presumably due to immobilisation of the portions of rmMBP that associate with the DPC-d_{38} micelles (Raussens et al. 2003). On the basis of our SDSL/EPR studies, we would predict these segments to be primarily in the N-terminus (Bates et al. 2003a), as well as the central amphipathic α-helix (Bates et al. 2004; Farès et al. 2006). Similarly, the HSQC spectra of rmMBP dissolved in 50 mM DPC-d_{38} at 298 K (Fig. 6c) and 318 K (Fig. 6d) displayed a temperature-dependence that was not

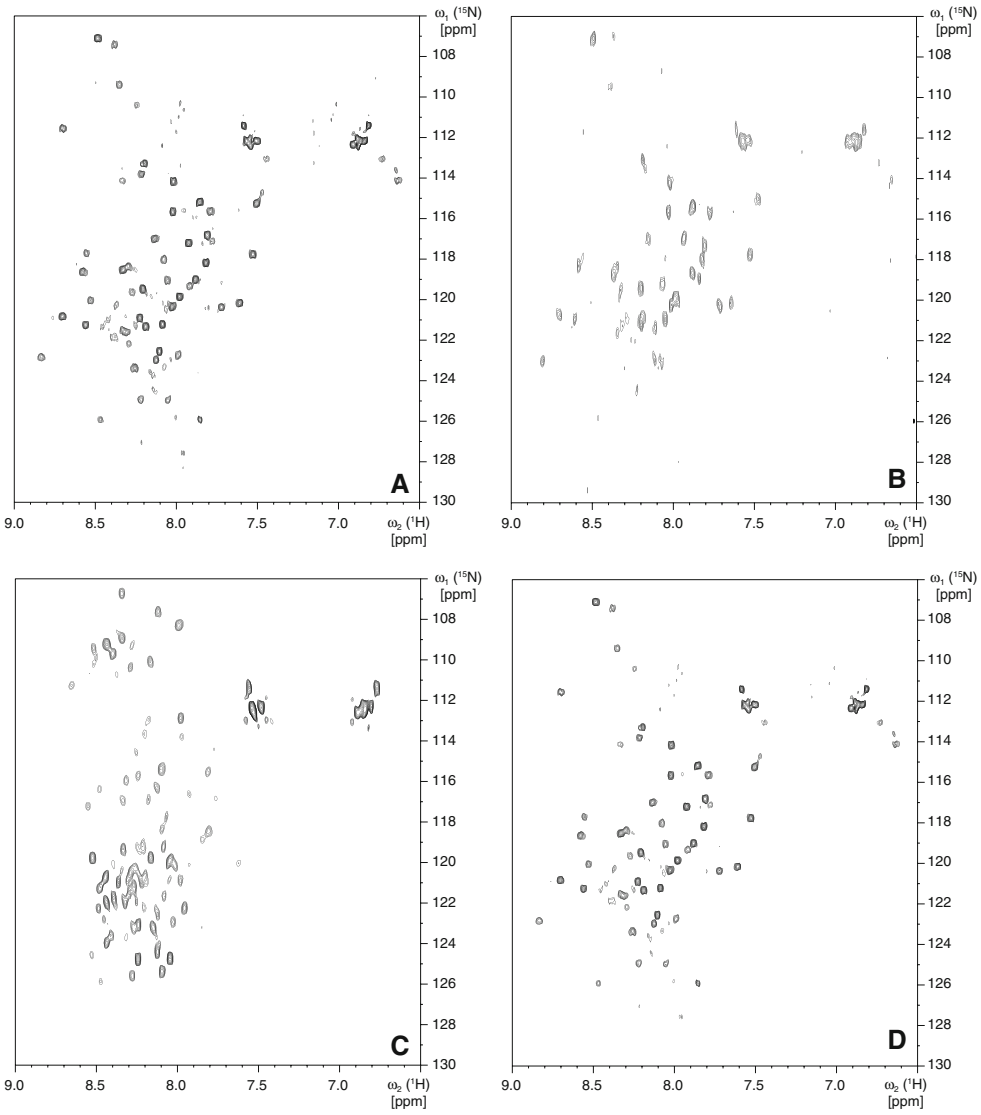

Fig. 6 The ^{1}H–^{15}N HSQC spectra of rmMBP in DPC-d_{38}. **a**, **b** Evaluation of lipid concentration. Uniformly ^{15}N-labelled rmMBP dissolved in (**a**) 50 mM DPC-d_{38} at a protein concentration of 1.30 mM, or (**b**) 250 mM DPC-d_{38} at a protein concentration of 1.20 mM. The samples also contained 50 mM $NaPO_4$, pH 6.5, 10% D_2O, and 0.005% NaN_3. The temperature was set to 318 K. The acquisition and processing parameters for each spectrum were identical for qualitative comparison. **c**, **d** Evaluation of temperature. Uniformly ^{15}N-labelled rmMBP (1.30 mM) dissolved in 50 mM DPC-d_{38}, 50 mM $NaPO_4$, pH 6.5, 10% D_2O, and 0.005% NaN_3. Spectra were recorded at (**c**) 300 K, or (**d**) 318 K. The acquisition and processing parameters for each spectrum were identical, allowing for qualitative comparison

revealed in the CD studies. Further experiments such as dynamic light scattering or pulsed field gradient NMR may help define the specific association of rmMBP with the DPC micelle(s) and may be useful for GM_1 and PI_4P comparative analysis (Dyson et al. 2004; Receveur-Bréchot et al. 2006; Rule et al. 2006). All in all, since DPC micelles did not satisfy one of the four criteria defined in the Introduction (no large complex formation) for a suitable solution NMR spectroscopic condition for backbone resonance assignments of rmMBP, it was rejected for further use here.

Effects of divalent cations

Previous studies have suggested that MBP may bind zinc as a physiologically relevant functional interaction (Riccio et al. 1995; Tsang et al. 1997). This divalent cation has been identified as an important component of the myelin sheath, and stabilises the association of MBP with the myelin membrane (Earl et al. 1988; Nuzzo et al. 2002; Haas et al. 2007). The binding of zinc to rmMBP was assessed here using solution NMR (Fig. 7), with the primary focus of observing secondary structure that may be stabilised upon zinc association (similar to the EF-hand motif of CaM) (Porumb et al. 1997). Interestingly, rmMBP with 10 mM zinc added seemed to be destabilised at lower temperatures compared to the 100 mM condition. At 277 K (Fig. 7a) there seemed to be an attenuation of certain resonances compared with the spectrum recorded at 300 K (Fig. 7b). This observation raises interesting questions about the association of Zn^{2+} with rmMBP, since similar results are not observed with other divalent cations (Cavatorta et al. 1994; Berlet et al. 1994; Riccio et al. 1995). Specifically, the effects of the addition of 10 mM Ca^{2+} were evaluated, and no differences between the Ca^{2+} containing and Ca^{2+} free rmMBP spectra were observed (Libich et al. 2008). The preliminary results observed here are consistent

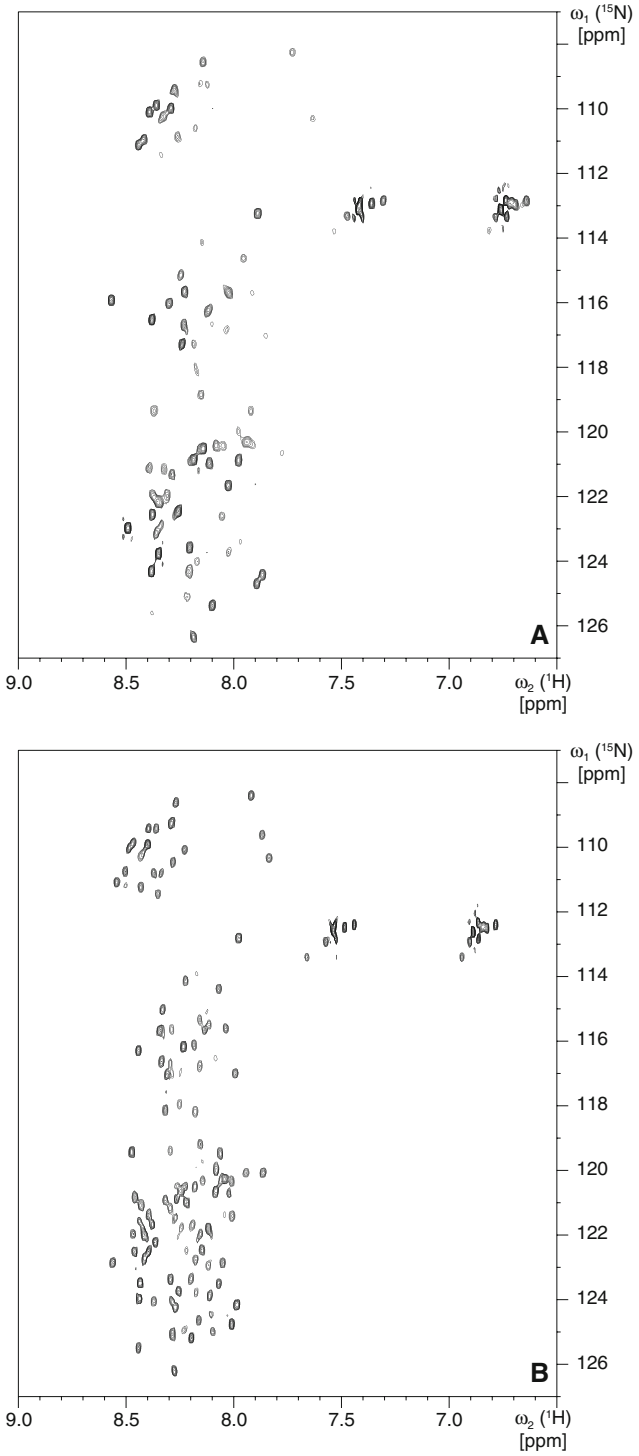

Fig. 7 The ^{1}H–^{15}N HSQC spectra of rmMBP in 10 mM Zn^{2+}. Uniformly ^{15}N-labelled rmMBP (1.47 mM) dissolved in 100 mM KCl, 10 mM Zn^{2+}, pH 6.5, 10% D_2O, 0.005% NaN_3. Spectra were recorded at (**a**) 277 K, or (**b**) 298 K. The acquisition and processing parameters for each spectrum were identical, allowing for qualitative comparison

with Zn^{2+} stabilisation of other IDPs (Uversky et al. 2000, 2002; Yi et al. 2007), and the phenomenon warrants further investigation, but a limiting factor at the present time is generating an rmMBP sample with the His_6-tag removed, that can be overexpressed in *E. coli* and purified in suitable amounts by alternate forms of chromatography. In any event, Zn^{2+} does not cause rmMBP to adopt a folded, tertiary structure—the conformational effects are more subtle.

Concluding remarks

The optimal solution NMR conditions for resonance assignments of rmMBP are (1) 100 mM KCl, pH 6.5, recorded at 277 K (Figs. 2a, b, and 4c), and (2) 30% TFE (v/v), pH 6.5, recorded at 300 K (Figs. 1c, 2c, and 5c). These two conditions represent the best combination of sample stability, dispersion, resolution, and sample resilience as judged from both CD and NMR characterisation experiments, while satisfying the initial search criteria. Furthermore, rmMBP dissolved in either of these solvents was stable for extended periods of time (i.e., >months). For future CaM-binding studies, assignment of rmMBP in 100 mM KCl was also advantageous since it allowed for the use of, and comparison to, the extensive literature of CaM NMR studies (Ikura et al. 1990; Libich et al. 2008), without having to reassign CaM dissolved in a different solvent, and thus eliminating an experimental variable. On the basis of the preliminary investigations described in detail in this study, we have performed and reported NMR assignments of 18.5 kDa rmMBP in both 30% TFE-d_2 (BMRB entry 6100) (Libich et al. 2004), and in 100 mM KCl (BMRB entry 15131) (Libich et al. 2007).

The studies described here also illustrate the importance of using multiple techniques to assess sample conditions. For example, the CD spectra of rmMBP dissolved in DPC (Figs. 1e, 2e, and 3d) suggested that it would be an ideal solution NMR solvent. Yet, NMR investigations (Fig. 6) revealed that there was a potential protein-micelle complex formation which contributed to approximately two-thirds of the expected resonances not being observed. Although potentially interesting from a membrane association/functionality perspective of MBP, due to the attenuation of some resonances, the lysolipid DPC was not suitable for backbone assignments. Nonetheless, either this detergent or others (Krueger-Koplin et al. 2004) could represent useful conditions for examining the lipid-binding sites of peptide fragments of the protein (e.g., Farès et al. 2006). Another sample preparation condition for solution NMR spectroscopy, that may be worthy of further investigation, is reconstituting rmMBP into reverse micelles (Valdez et al. 2001; Peterson et al. 2005; Van Horn et al. 2008), which represents a higher level of experimental complexity.

All isoforms of myelin basic protein are intrinsically disordered, with a variety of conformations that are environment-specific (Harauz et al. 2004). The most physiologically

relevant environment for the classic 18.5 kDa MBP isoform would be a semi-solid, membrane-associated sample, requiring EPR (Bates et al. 2003a) or emerging techniques in solid-state NMR spectroscopy (Zhong et al. 2007). Here, we have defined suitable environmental conditions for high-resolution solution NMR spectroscopic experiments which can be used to discern domains of greatest order and/or protein interaction sites (Ahmed et al. 2007; Polverini et al. 2008; Libich et al. 2008), to define conformational ensembles (Marsh et al. 2007; Mittag et al. 2007), to compare different splice isoforms (e.g., 14–21.5 kDa), and to investigate the effects of post-translational modifications such as deimination and phosphorylation (Beniac et al. 2000; Bates et al. 2003a; Musse et al. 2006; Polverini et al. 2008).

Acknowledgments This work was supported by the Canadian Institutes of Health Research (Operating Grant MOP 74468), and the Natural Sciences and Engineering Research Council of Canada (Operating Grant RG121541). DSL was the recipient of an Ontario Graduate Scholarship. The University of Guelph NMR Centre benefited from funding by the Canada Foundation for Innovation, and the Ontario Innovation Trust. The authors are grateful to Dr. Vladimir Bamm, Ms. Valerie Robertson (Guelph NMR Center), Dr. Martine Monette (Bruker BioSpin Canada), and Dr. Vladimir Ladizhansky (Physics, Guelph) for many helpful discussions, advice, and support.

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