1. Introduction

Creutzfeldt-Jacob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep are fatal neurodegenerative diseases. The infectious agent of these diseases is devoid of nucleic acid and is almost entirely composed of the prion protein, PrP^{Sc}, which is a misfolded form of a cellular prion protein PrP^{C} (1). The misfolding and folding intermediates of PrP are therefore of significant interest to those studying the mechanism of Prion replication. The mature form of the benign mammalian prion protein is a ~208 residue glycoprotein that possesses two N-glycosylation sites and one disulfide bridge. After transportation through the secretory pathway, PrP^{C} is tethered to the cell surface via a GPI anchor at the C-terminus (1). Three dimensional NMR solution structures of PrP^{C} from a number of mammalian species have been reported which show that PrP^{C} consists of two structurally distinct domains. The N-terminal domain (residues 23 to 125) is highly disordered and is notable for its ability to bind Cu^{2+} ions. The N-terminus contains a highly conserved octa-repeating sequence, PHGGGWGQ between residues 57 and 90. The C-terminal domain (residues 126-231) is predominantly helical, containing 3 α-helices (residues 144-154, 175-193 and 200-219) as well as two short anti-parallel β-strands (residues 128-131 and 161-164).

Studies by Baskakov and others have shown that PrP (with its disulphide bond intact) can fold in one of three forms. Firstly the α-helix rich, soluble monomeric form, PrP^{C}, is rapidly formed under near neutral non-denaturing conditions. Secondly a high molecular weight multimer that forms amyloid fibres is favoured under neutral pH, moderately denaturing conditions and agitation. The third form is a soluble species favoured at low pH (~4) under moderately denaturing conditions with a high salt content. This latter soluble species has a high β-sheet content as indicated by CD while light scattering experiments suggest that this oligomer contains 8-15 monomers or higher molecular mass species. Structural details, on a per-residue basis, are lacking for the pH 4 folding intermediate, despite its importance in influencing the susceptibilities to prion propagation. We therefore aim to characterise the pH 4-intermediate using NMR methods. Chemical shift dispersion in 3D ^{15}N-HSQC-TOCSY experiments will indicate the extent of folding of PrP. ^{15}N transverse relaxation measurements will provide information on the flexibility of the pH 4 intermediate main-chain and ^{1}H translational diffusion will indicate its molecular size.

2. 2D ^{15}N-HSQC Spectroscopy of mPrP(23-231)

Chemical shift dispersion in NMR is a powerful method of determining the extent of structure in proteins on a per-residue basis. For this reason 2D ^{15}N-HSQC was used to characterize the folding of PrP under various conditions. The 2D ^{15}N-HSQC spectrum of native mPrP(23-231) (Figure 1A) possesses amide ^{1}H signals in the ^{1}H dimension from ~6.5-9.5 ppm, characteristic of a folded protein. However, for conditions promoting formation of the β-intermediate i.e. 3.5 M Urea, 150 mM NaCl at pH 4.1 (Figure 1B), ^{1}H signals lie between 7.8 and 8.7 ppm. This contraction in the chemical shift dispersion is typical of a completely unfolded protein (40-42). The 0.9 ppm ^{1}H dispersion range for amides in the 2D^{15}N-HSQC of mPrP(23-231) was very similar to that of the acid denatured species (Figure 1C), although the chemical shifts of the amide resonances are not the same.

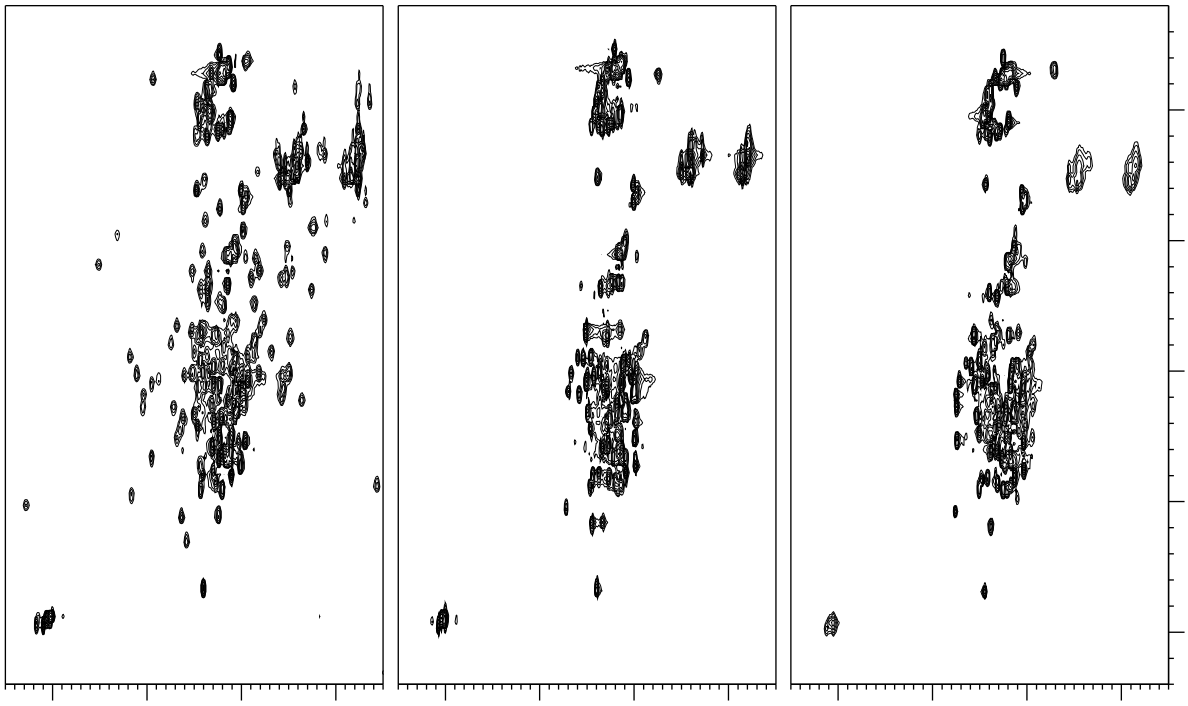


Figure 1. 2D ^{15}N-HSQC spectra of mouse PrP(23-231) (A) pH 5.21, 20 mM sodium acetate (B) pH 4.11, 3.5 M Urea, 150 mM NaCl, 20 mM sodium acetate (C) pH 1.65, 3.5 M Urea, 150 mM NaCl, 20 mM sodium acetate. Concentration of His tagged PrP(23-231) ~5 mg/ml, spectra were recorded at 30 ^{o}C.

3. Circular Dichroism Spectroscopy of mPrP(23-231)

An aliquot from each of the three NMR samples was prepared for UV-CD spectroscopy. The CD spectrum of native mPrP(23-231), Figure 2A, is typical of a protein possessing α-helices. However, the CD spectrum taken from the NMR sample of mPrP(23-231) under β-intermediate forming conditions, is characteristic of a β-sheet species with a strong negative CD band at ~217 nm (Figure 2B). The acid denatured spectrum, Figure 2C, shows less ellipticity suggesting a more random conformation.

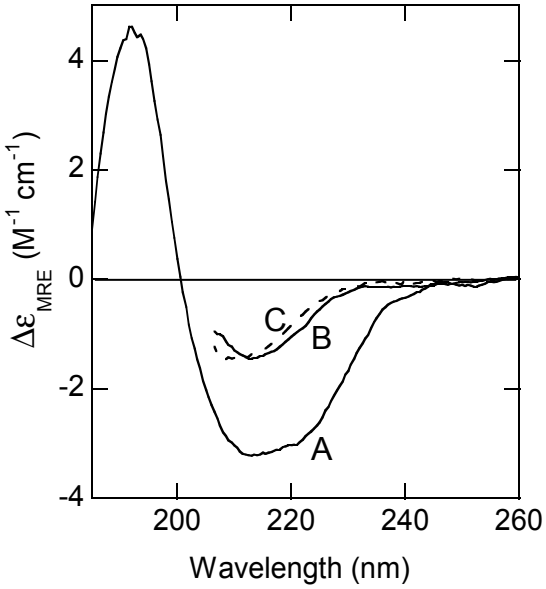


Figure 2. UV-CD spectra of mPrP(23-231): (A) pH 5.1, 20 mM sodium acetate (B) pH 3.9, 3.5 M Urea, 150 mM NaCl, 20 mM sodium acetate (C) pH 1.65, 3.5 M Urea, 150 mM NaCl, 20 mM sodium acetate (dashed line). Concentration of His tagged PrP(23-231) samples are 0.4 mg/ml, spectra were recorded at 25 ºC with a 0.02 cm path length.

4. pH 4 Intermediate created at 37 °C

A recent study has indicated that the pH 4 intermediate can be produced in the absence of chemical denaturant by simply raising the temperature to 37 °C (24,25). Figure 3A shows the 2D ^{15}N-HSQC of mPrP(23-231) at pH 3.6 incubated for ~3 hours at 37 °C. Incubation of the NMR sample for a further 30 hours caused no further change in the 2D ^{15}N-HSQC spectra. A CD spectrum of this NMR sample was then obtained at 37 °C. The incubation at 37 °C causes a loss of helical content and the appearance of a strong band at ~217 nm (Figure 3B). After incubation at 37 °C, both the 2D ^{15}N-HSQC and CD spectra (Figure 3A,B) have a strong resemblance to the corresponding spectra of the urea induced pH 4 intermediate shown in Figures 1 and 2; although there is not a direct correspondence between amide resonances obtained at 30 °C in urea and those obtained at 37 °C in water. It was possible to obtain estimates of the amount of secondary structure from the CD spectra of the pH 4 intermediate at 37 °C using deconvolution algorithms. Anyalysis with the CDSSTR algorithms suggested 31% β-sheet present and only 3 % α-helix. However deconvolution analysis with the K2D secondary structure estimation program, 31 % β-sheet was indicated but as significantly higher estimate of the α-helical content at 16 %.

A 'head' count of the number of observable ^{15}N amides resonances in the pH 4 intermediate suggest a loss of some signals; from ~150 native full-length PrP (207 amino acids minus prolines and octarepeat degeneracy of signals) to half that in the pH 4 intermediate. Using the 2D ^{15}N HSQC alone it was not clear if this loss of signals was due to signal overlap in the less dispersed spectra or the possibility that only a portion of the PrP was detected by the 2D ^{15}N HSQC in the pH 4 intermediate. The formation of a high molecular weight soluble polymeric species would become largely undetectable in the 2D ^{15}N-HSQC experiment due to their increase line-widths. The retention of signals attributable to the Trp side-chain and Gly residues hinted at the possibility that only the N-terminal half of the protein was being detected. (Note, the octa-repeats within the N-terminus contain the majority of Trp and Gly residues within PrP). A truncated version of full-length PrP lacks nearly all the unstructured N-terminal residues of native PrP^C. Figure 3 presents a comparison the the pH 4 intermediate for full-length PrP(23-231) to that of PrP(113-213). There is a dramatic change in the appearance of the 2D ^{15}N HSCQ spectrum for the truncated PrP(112-231) pH 4 intermediate, with only a few residues (~16 amide signals) being detected. This observation strongly suggests that the 2D ^{15}N HSQC of full-length PrP for the pH 4 intermediate only detects N-terminal residues (23–121).

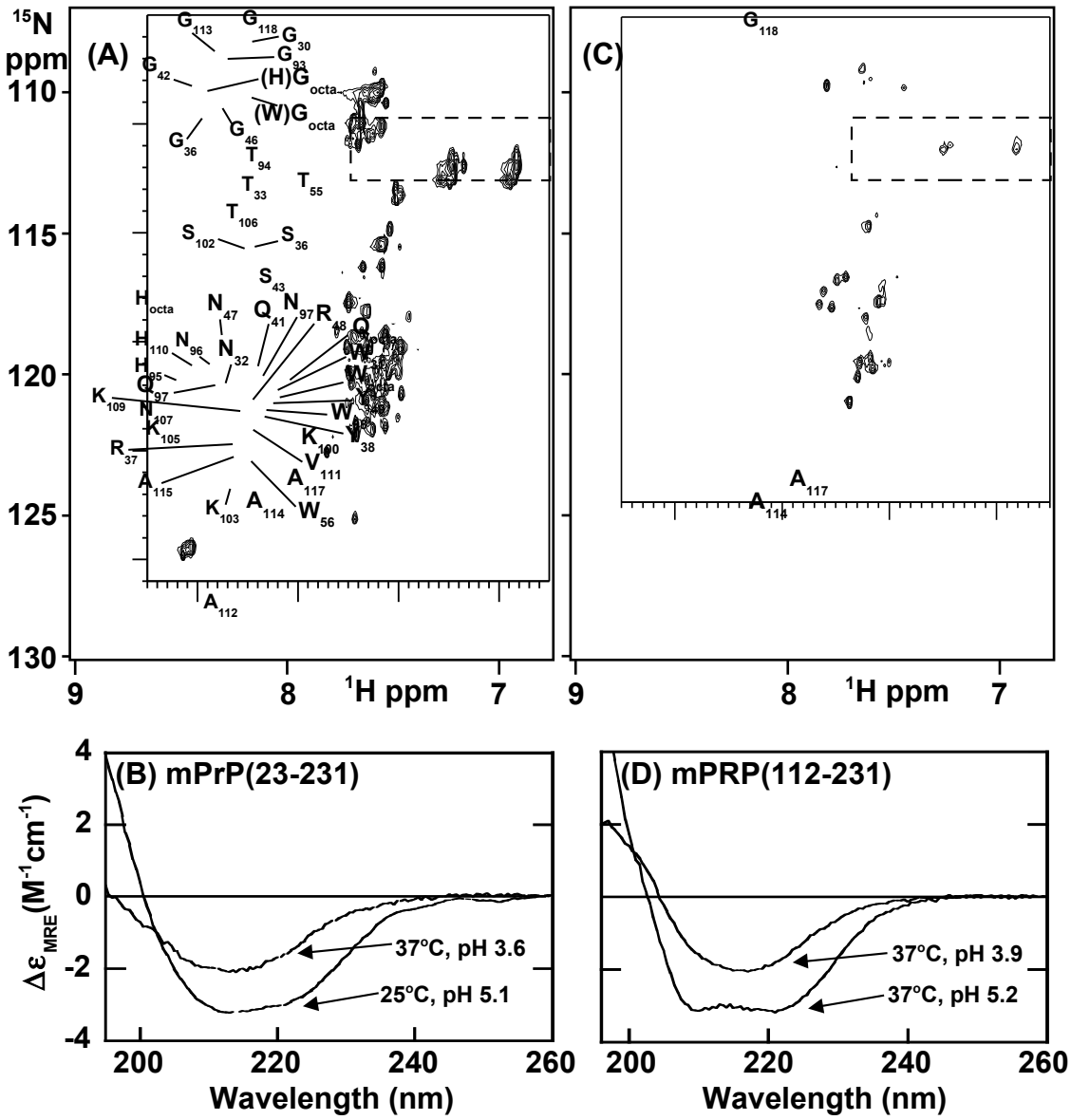
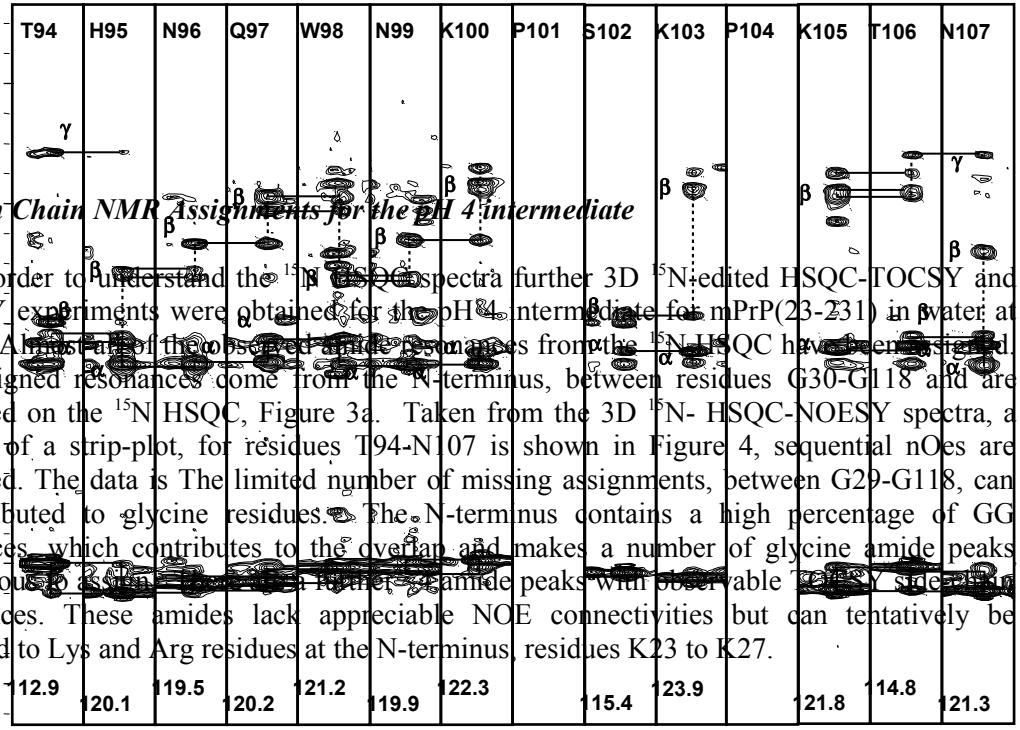


Figure 3. **2D ^{15}N-HSQC and UV-CD spectra of two mPrP constructs at pH ~4.** (A) ^{15}N HSQC recorded after 2 hours incubation at 37 ºC of his-tag-free mPrP(23-231) at pH 3.6, 20 mM sodium acetate. [PrP(23-231) ~5 mg/ml]. Peaks are assigned based on 3D ^{15}N-edited HSQC-TOCSY and –NOESY data. Unlabelled peaks had no NOE connectivities. The dashed box surrounds Q/N side-chain amine resonances. (B) CD spectra of mPrP(23-231), pH 3.6 at 37 ºC and pH 5.1 at 25 ºC . (C) ^{15}N HSQC, recorded after 18 hours incubation at 37 ºC of PrP(113-231) ~3 mg/ml pH 3.9. Resonances that directly overlap peaks in (A) are highlighted and (D) CD spectra of mPrP(113-231) at, pH 3.9 and pH 5.2 at 37 ºC.



6. Main Chain NMR Assignments for the pH 4 intermediate

In order to understand the ^{15}N HSQC spectra further 3D ^{15}N-edited HSQC-TOCSY and NOESY experiments were obtained for the pH 4 intermediate for mPrP(23-231) in water at 37 °C. Almost all of the observed amide resonances from the ^{15}N HSQC have been assigned. All assigned resonances come from the N-terminus, between residues G30-G118 and are indicated on the ^{15}N HSQC, Figure 3a. Taken from the 3D ^{15}N- HSQC-NOESY spectra, a section of a strip-plot, for residues T94-N107 is shown in Figure 4, sequential nOes are indicated. The data is The limited number of missing assignments, between G29-G118, can be attributed to glycine residues. The N-terminus contains a high percentage of GG sequences, which contributes to the overlap and makes a number of glycine amide peaks ambiguous to assign. There are a number of amide peaks with observable TOCSY side-chain resonances. These amides lack appreciable NOE connectivities but can tentatively be assigned to Lys and Arg residues at the N-terminus, residues K23 to K27.

Figure 4: 3D ^{15}N-HSQC-NOESY strip-plot of mPrP(23-231) at pH 4.0 at 37 °C. Residues T94 to N107 are shown, side-chain assignments also observed in 3D TOCSY are indicated, as are sequential NOE's.

Chemical shift values have minimal deviations from 'random-coil'. For example, there are no Hα deviations that exceed 0.15 ppm and the overwhelming majority have deviation less than 0.1 ppm. This indicates that residues 23-118 are indeed unstructured in the pH 4 intermediate (43,44). Hα chemical shift assignments and deviations from random coil are available as supplementary material. We note that signals up to residue 115 are quite intense, resonances for A117 and G118 have strongly attenuated signals. Residues beyond 118 are not observed, due to the increased line widths of these signals.

Figure 4 O' Sullivan/Jones/Viles

The ^{15}N HSQC spectrum for mPrP(113-231) (Figure 3C) shows a large reduction in the number of observed amide resonances (~16 amide signals observed), residues 114-118 directly overlay with residues from mPrP(23-231).

7. ^{15}N Transverse relaxation times of mPrP(23-231)

We then went on to characterize the molecular dynamics of the soluble PrP pH 4 intermediate using ^{15}N transverse (T_2) relaxation measurements. R_2 relaxation is sensitive to the overall rotational correlational time (τ_c) of a molecule. The presence of additional main-chain flexibility on a nano-to-pico second timescale can reduce R_2 values considerably.

R_2 relaxation values for amide resonances of the native mouse PrP(23-231) are comparable to values reported for full-length Syrian hamster PrP (2) as would be expected. For example, typical ^{15}N R_2 relaxation values for amide resonances in helix A, B and C were 12-19 s^{-1} for full-length Syrian hamster PrP at 30 °C (2). In this study, amides in the same region of mouse PrP(23-231) gave comparable R_2 values, e.g. 13.2, 16.7 and 18.7 s^{-1} for Y156, K203 and R207 respectively. In addition, amides from the unstructured tail (residues 29-120) gave R_2 values of ~4 s^{-1} (2). Similar values are obtained here for mouse PrP(23-231), for example, A112 has an R_2 of 3.0 s^{-1}.

R_2 values for individual resonances for the pH 4 intermediate were nearly all below 4 s^{-1}; typically 3.6 s^{-1}. These R_2 values are considerably smaller than those of a folded protein ~200 amino acids in size. It is clear R_2 values are dominated by local pico-second timescale motions indicating a high degree of flexibility of the N-terminal half of the protein, residues 23-114.

8. Translational diffusion measurements of mPrP(23-231)

Translational diffusion measurements can indicate the hydro-dynamic radius or molecular size of a protein. The techniques measures the bulk property of the protein rather than local internal motions. Using translational diffusion to indicate the molecular size of the β-intermediate has some advantages over previous methods such as size exclusion chromatography, as the studies are performed in solution under conditions similar to that used for the CD measurements. Figure 5 shows the ^{1}H spectra for native PrP acquired at different gradient strengths used to calculate the translational diffusion for PrP(23-231) in the 3 states; native, β-intermediate and acid denatured. The translational diffusion coefficient (D_t) of native PrP(23-231) in 20 mM sodium acetate at pH 5.23 and 30 °C was $0.78\pm0.01\times 10^{-6}$ cm^2 s^{-1}. This value is identical to the D_t value reported by workers for Syrian hamster PrP(29-231) and is typical for a monomeric protein of this size (2).

The D_t for mPrP(23-231) in 3.5 M Urea, 150 mM NaCl at pH 4.4 (the β-intermediate form) was $0.81\pm0.02 \times 10^{-6}$ cm^2 s^{-1}. This is a value corrected for the increased viscosity due to the presence of 3.5 M Urea. The close similarity of the β-intermediate D_t value to that of native mPrP(23-231) indicated that the protein in its β-intermediate form is also predominantly monomeric. Uncorrected, the D_t ($0.66\pm0.02 \times 10^{-6}$ cm^2 s^{-1}) of the β-intermediate is comparable to that for dimeric PrP.

The D_t for mPrP(23-231) in 3.5 M Urea, 150 mM NaCl at pH 1.65 (the acid denatured form) was $0.50\pm0.01 \times 10^{-6}$ cm^2 s^{-1}. The D_t for the acid denatured form, after correcting for the increased viscosity of the solution due to the presence of urea, was $0.61\pm0.01 \times 10^{-6}$ cm^2 s^{-1}. The decreased D_t of the acid denatured mPrP(23-231) compared to the other 2 forms may indicate that it has aggregated into an oligomer. It is possible to calculate the increase in frictional coefficient, *f,* upon going from a monomer to an oligomer of *n* subunits (46). The experimental D_t ratio of the acid denatured form of mPrP(23-231) to that of native mPrP(23-231) in this study is 0.78. This ratio is very similar to a D_t ratio of 0.75 expected for a theoretical monomer dimer model. It is also similar to an experimental D_t ratio of 0.72 for the monomer/dimer system of ubiquitin and of the dimeric cytokine MCP-1 proteins. Thus the translational diffusion data indicates that the native and β-intermediate forms of mPrP(23-231) are predominantly monomeric while the D_t of the acid denatured form implies a dimer.

The translational diffusion coefficient for the PrP sample obtained for the pH 4 intermediate at 37 ºC but without urea present, exhibits a D_t of $0.62\pm0.02 \times 10^{-6}$ cm^2s^{-1}. This value did not need to be corrected for the viscosity of urea as the denaturant was not present. The pH 4 intermediate, produced under these conditions, gives a translation diffusion coefficient close to that expected for a dimer of PrP. However correction for the increased temperature (37 ºC) gave a value of 0.54×10^{-6} cm^2 s^{-1}. This value might suggest a larger molecule close to a trimer in size. Certainly a higher molecular weight oligomer is not suggested.

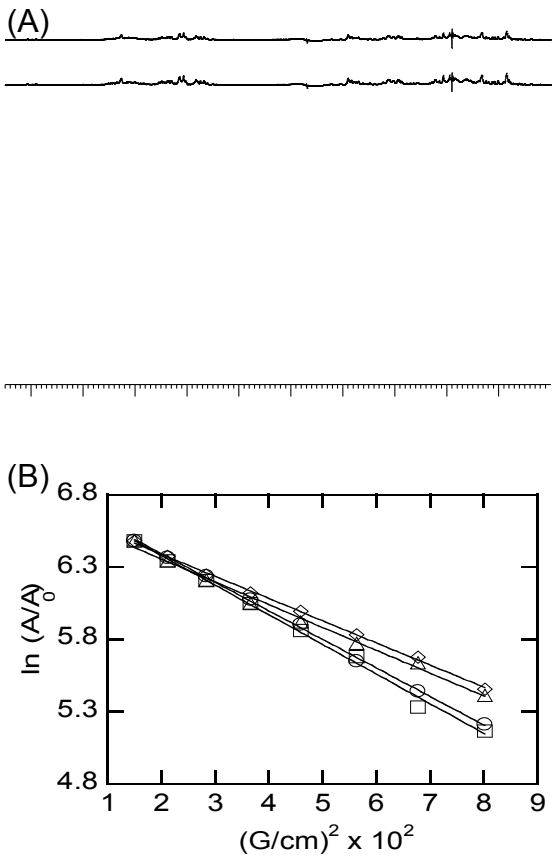


Figure 5. Translational diffusion measurements of mPrP(23-231) in its native, β-intermediate and acid denatured forms. (A) 1D ^{1}H NMR spectra of native mPrP(23-231) (6 mg/ml) using stimulated echo with increasing field gradient strength between 28.3 and 12.2 G/cm. (B) The natural log of the peak intensity for the aromatic region of the spectra was plotted against the corresponding square of the gradient strengths. Least square straight line fit to: (i) native state pH 5.2 (circle), (ii) pH 4.40, 3.5 M Urea, 150 mM NaCl (square) (iii) pH 1.64, 3.5 M Urea, 150 mM NaCl (diamond), (iv) pH 3.67 after incubation at 37 ^{o}C (triangle).

10. Discussion

For the first time the nature of the prion protein pH 4 β-intermediate, has been characterized on a per-residue basis. The NMR chemical shifts and assignments of the β-intermeadiate indicate that the majority of prion protein molecules within a pH 4 oligomer possess a long flexible tail incorporating residues 23-118. The exact nature of the C-terminal half of PrP remains elusive as large line-widths have rendered these residues undetectable by NMR. However deconvolution of the CD spectra indicate a high proportion of β-sheet for this region 31-46% β-sheet for PrP(113-231). The variability in the secondary structure estimates is perhaps due to the nature of the oligomer with ill defined extended conformations in a molten globule state, which fits poorly to standard secondary structure elements. Protein oligomers have a tendency to form extended conformations that produce CD spectra with a negative CD band at ~217 nm similar to that observed for β-sheet proteins. It is therefore not clear if either an extended β-like polypeptide chain, or a more ordered hydrogen bonded network, found in cross β-sheets of amyloids, is present in the beta intermediate. It is clear that the formation of the soluble pH 4 oligomer is largely reversible, returning the pH to 5.5 results in the refolding of the monomeric native species.

The studies described here have been carried out with the native disulphide bond intact. It is generally believed the PrP^{Sc} exists with the native disulphide bond present (3). A model of amyloids of PrP^{Sc} based predominantly on EM data suggests that much of helix B and C remains intact in the fibrils while residues 89-174 forms a cross-β structure in a β-helix conformation (4). FTIR spectroscopy suggests that some of the α-helical structure is retained in the pH 4 oligomeric species (5). Estimates of α-helix content from the CD spectra, (although variable) between 3 and 21 %) suggest there is little α-helix content for the pH 4 intermediate. It remains to be established if helices remain in the pH 7 amyloid species.

The translational diffusion measurements suggest that the β-intermediate is a trimer in size. This is a surprise as a number of studies using guanidine hydrochloride, urea or elevated temperature as partial denaturants have indicated that the pH 4 β-intermediate is oligomeric. Investigations using size exclusion chromatography and dynamic light scattering measurements suggest that this β-intermediate is polymeric (6,7,8). Multi-angle laser light scattering experiments suggest that this oligomeric species contains 8-15 monomers (24) small angle X-ray scattering has also indicated a large oligomer (9). Our own 2D ^{15}N-HSQC spectra indicate a complete loss of signal from the C-terminus of PrP suggesting a molecule of high molecular weight to rendering the signal too broad for detection. However we do note that a loss of signal might also be due to exchange broadening due to slow milli-micro second motions of the main-chain of the C-terminal domain in a molten globule state.

Characterizing folding intermediates of the prion protein is an important goal towards understanding the mechanism of prion replication. Recently it has been shown that the rate of formation of the pH 4 β-intermediate is influenced by polymorphism at residue 129. Met129 has a higher propensity to form the pH 4 β-intermediate than Val129 (10). Interestingly, to date only individuals homozygous for methionine (Met/Met) have contracted variant CJD. It is clear that the soluble pH 4 β-intermediate contains appreciable amounts of unstructured polypeptide chain (residues 23-118) with a high degree of flexibility. Interestingly, in the pH 4 intermediate the amyloidogenic residues, 90-126, which are required for prion replication, remain unstructured.

Acknowledgements

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Abbreviations

BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jacob disease; GPI, glycosyl-phosphatidylinositol; huPrP human PrP; mPrP mouse PrP; PrP, prion protein; PrP^{C}, cellular isoform of PrP; PrP^{Sc}, scrapie isoform of PrP.

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