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Raman Optical Activity Demonstrates Poly(L-proline) II Helix in the N-terminal Region of the Ovine Prion Protein: Implications for Function and Misfunction

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The aqueous solution structure of the full-length recombinant ovine prion protein PrP²⁵-²³³, together with that of the N-terminal truncated version PrP⁹⁴-²³³, have been studied using vibrational Raman optical activity (ROA) and ultraviolet circular dichroism (UVCD). A sharp positive band at \( \nu = 1315 \text{ cm}^{-1} \) characteristic of poly(L-proline) II (PPII) helix that is present in the ROA spectrum of the full-length protein is absent from that of the truncated protein, together with bands characteristic of \( \beta \)-turns. Although it is not possible similarly to identify PPII helix in the full-length protein directly from its UVCD spectrum, subtraction of the UVCD spectrum of PrP⁹⁴-²³³ from that of PrP²⁵-²³³ yields a difference UVCD spectrum also characteristic of PPII structure and very similar to the UVCD spectrum of murine PrP²⁵-¹¹³. These results provide confirmation that a major conformational element in the N-terminal region is PPII helix, but in addition show that the PPII structure is interspersed with \( \beta \)-turns and that little PPII structure is present in PrP⁹⁴-²³³. A principal component analysis of the ROA data indicates that the \( \alpha \)-helix and \( \beta \)-sheet content, located in the structured C-terminal domain, of the full-length and truncated proteins are similar. The flexibility imparted by the high PPII content of the N-terminal domain region may be an essential factor in the function and possibly also the misfunction of prion proteins.

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Introduction

Prion proteins are associated with a variety of neurodegenerative diseases, known as transmissible spongiform encephalopathies (TSE), characterised by the presence of spongiform degeneration and astrocytic gliosis.¹ Despite much effort, an understanding of some key aspects of TSE disease remains elusive, such as the existence of an infectious form along with genetic and sporadic forms, something that has not been recognised in any other class of neurodegenerative disease. The basic current model involves conversion of a ubiquitous cellular form of the prion protein (PrP⁰) into a scrapie (amyloid fibril) form (PrPSc), the prion protein being both target and infectious agent. PrP⁰ has a high proportion of \( \alpha \)-helical structure, is soluble in detergents and is susceptible to proteolysis. Conversely, PrPSc has a high \( \beta \)-sheet content, is insoluble in detergents and is partially resistant to proteolysis. Further understanding of TSE disease requires more detailed knowledge of the solution structures of prion proteins and the conformational changes associated with the possible different transformation pathways of PrP⁰ into PrPSc that are involved in the infectious, genetic and sporadic forms.²

After expression and post-translational processing, PrP⁰ has between 208 and 220 amino acid residues depending on mammalian species, two N-glycan linkage sites of variable occupancy and a C-terminal glycosyl-phosphatidylinositol (GPI) membrane anchor. Structural analysis by Fourier
transmit infrared (FTIR) and ultraviolet circular dichroism (UVCD) spectroscopy indicated that PrPSc contains ~40% \( \alpha \)-helix and little \( \beta \)-sheet, whilst PrPSc contains ~30% \( \alpha \)-helix and ~45% \( \beta \)-sheet.\(^5\)\(^6\) Although there have been numerous NMR studies on the solution structure of PrPSc,\(^5\) X-ray crystallographic studies have been hampered by difficulties in crystallising the protein. The NMR structures of full-length recombinant prion proteins of different mammalian species all revealed a structured C-terminal globular domain containing mainly \( \alpha \)-helical sequences in the form of a compact three-helix bundle, and a long flexibly disordered N-terminal domain of 80–100 amino acid residues, which includes a metal ion binding octapeptide repeat motif.\(^5\)\(^6\) Recently, however, the first crystal structure of ovine PrP, has been solved\(^7\) and found to be very similar to the solution NMR structures of other mammalian species. For crystallisation, only the folded C-terminal domain 128–233 was present. This structure of ovine PrP\(^{128-233}\) is represented pictorially in Figure 1.

An increasing amount of research is focusing on the contribution of the disordered N-terminal region to PrP function. Previously, the role of this region in the disease process tended to be overlooked mainly because a long N-terminal segment of PrPSc is lost on \emph{in vitro} proteolytic digestion of PrPSc to the core structure PrP27-30 (meaning a protein of between 27 kDa and 30 kDa). In addition, transgenic mice that are devoid of much of the N-terminal region still support prion replication,\(^8\)\(^9\) indicating that the N-terminal region is not required \emph{a priori} for the conversion process. However, immunologic studies of PrPSc suggest that the conformational change that occurs during conversion of PrPSc into PrPSc is mostly in the region from residues ~94–149 or perhaps 179 with the major changes localised largely to a part of the disordered N-terminal region bounded by residues ~94 and ~124,\(^10\)\(^11\) and Donne et al.\(^12\) have suggested that the N-terminal region could feature in the conversion of PrPSc to PrPSc by template-assisted formation of \( \beta \)-structure. Furthermore, recent evidence suggests that the presence of the N-terminal region could play a key role in determining the conformation of PrPSc and in modulating cross-species disease transmission and TSE pathogenesis.\(^13\) Thus, the impact of the N-terminal region on the structure of mature length PrP is clearly of considerable importance in TSE disease.

Many reports of structural characterisation of the octapeptide repeat region of the N-terminal domain of PrPSc complexed with copper ions exist, including a recent crystal structure of copper complexed to a pentapeptide derived from the prion N terminus.\(^14\) Conversely, there is scant information on the structure of this domain in isolation, primarily because the detailed characterisation of non-regular peptide sequences in proteins is difficult using standard solution phase structural techniques such as NMR and UVCD. However, it has recently been shown that Raman optical activity (ROA), which measures vibrational optical activity by means of a small difference in the intensity of Raman scattering from chiral molecules in right- and left-circularly polarised incident laser light,\(^15\)\(^16\) is able to provide new information about the structure of disordered sequences in intact proteins in aqueous solution.\(^17\) This work suggests that an important conformational element in such sequences is poly(\( \epsilon \)-proline) II (PPII) helix. Although originally defined for the conformation adopted by polymers of \( \epsilon \)-proline, PPII helix can be supported by amino acid sequences other than those based on \( \epsilon \)-proline and has been recognised as a common structural motif within loops in the X-ray crystal structures of many proteins.\(^20\) It consists of a left-handed helix with 3-fold rotational symmetry in which the \( \phi, \psi \) angles of the constituent residues have values around \( -78^\circ, +146^\circ \), corresponding to a region of the Ramachandran surface to one side of the \( \beta \)-region. The extended nature of the PPII helix precludes intrachain hydrogen bonds, the structure being stabilised instead by main-chain hydrogen bonding with water molecules and side-chains.\(^21\)

PPII helix currently attracts much interest as a major conformational element of disordered polypeptides and unfolded proteins in aqueous solution.\(^22\) PPII structure can be distinguished from random coil in polypeptides by UVCD,\(^22\)\(^23\)

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**Figure 1.** The tertiary structure of the globular, C-terminal domain of the ovine prion protein as determined by X-ray crystallography.\(^7\) The structure was rendered using Molmol and Povray.
vibrational circular dichroism (VCD), FTIR and Raman but these techniques are less sensitive than ROA to PPII structure when there are a number of other conformational elements present as in proteins. Although the lack of main-chain hydrogen bonding makes NMR determinations of PPII structure difficult, a recent NMR study, complemented by UVCD, of a peptide sequence containing seven alanine residues provides strong evidence for a predominantly PPII conformation in aqueous solution. A subsequent ROA study of a similar peptide firmly demonstrated the ability of ROA to identify PPII structure. PPII helix may be important in the regulatory multiple weak interactions that are increasingly being recognised as associated with intrinsically unstructured peptide sequences within proteins. Furthermore, proteins that are completely unfolded in their native state may contain a large amount of PPII helix, which imparts a rheomorphic (flowing shape) character to their structure and which appears to be important for their function.

The presence of significant amounts of PPII structure in the N-terminal disordered region of the prion protein has already been suggested from UVCD studies of synthetic polypeptides based on the octarepeat region. Subsequent UVCD studies of the N-terminal peptide PrP showed that it can also form a PPII structure in aqueous buffer, with the discovery of 4-hydroxyproline in recombinant PrP from Chinese hamster ovary cells and PrP from terminally infected mouse brains providing proof for the formation of PPII helix in PrP in vivo. In addition, NMR measurements of peptides derived from the octarepeat region of PrP suggested that certain residues adopt loop and β-turn structures.

Here, we report an ROA study of the aqueous solution structures, especially the characterisation of PPII helix, of recombinant ovine prion protein residues 25–233, the sequence of which is given in Figure 2, and the N-terminal truncated protein comprising residues 94–233, in order to obtain new information about the fold of these biomolecules. This information is compared with that derived from UVCD spectra of the same proteins and a C-terminally truncated construct.

Results and Discussion

Figure 3 shows the back-scattered Raman and ROA spectra of ovine PrP and PrP in aqueous solution. Despite the high backgrounds to the parent Raman spectra of the two samples, ROA spectra of reasonable quality were obtained by summing the spectra acquired in several different measurement runs. An account of most of the ROA assignments given in what follows can be found.

PPII helix

A prominent feature in the ROA spectrum of PrP (Figure 3, top) is the narrow positive band observed at ~1315 cm⁻¹ within the extended amide III region (~1230–1350 cm⁻¹) where normal vibrational modes made up of various combinations of the in-plane N–H deformation (in-phase), the C–N stretch and Cα–H deformations

Figure 2. The amino acid sequence of the ovine prion protein. The initial 24 amino acid residues and final 23 amino acid residues comprise the N-terminal and C-terminal signal peptides, respectively. The full-length prion protein starts at lysine 25 (in bold); the N-terminally truncated protein starts at glycine 94 (bold and italic). Both constructs end at the mature C terminus, alanine 233.

Figure 3. Back-scattered Raman (I° + I°) and ROA (I° – I°) spectra in sodium acetate buffer (pH 5.5), of ovine PrP and PrP (top pair) and PrP (bottom pair). The high fluorescence backgrounds have been subtracted from the Raman spectra.
occur. A similar positive band at \( \sim 1319 \text{ cm}^{-1} \) dominates the ROA spectra of disordered poly (L-lysine) and poly(L-glutamic acid). Since these disordered polypeptides are thought to contain substantial amounts of the PPII-helical conformation, perhaps in the form of short segments interspersed with residues having other conformations, this ROA band has been assigned to a vibrational mode of the PPII helix. Similar positive bands are seen in this region in the ROA spectra of some \( \beta \)-sheet proteins where they have been assigned to PPII-helical elements. This assignment has been reinforced recently by an ROA study of the aqueous solution structures of a series of alanine oligopeptides, including AcOOAAAAAAAOO-NH\(_2\), the ROA spectrum of which is dominated by a positive ROA band at \( \sim 1319 \text{ cm}^{-1} \). This peptide is similar to the one shown by Shi et al. to adopt a predominantly PPII conformation. PrP\(_{25-233}\) therefore appears to contain a significant number of residues in the PPII conformation. Since the positive \( \sim 1315 \text{ cm}^{-1} \) band is absent from the ROA spectrum of PrP\(_{94-233}\) (Figure 3, bottom), we deduce that most of the PPII sequences are located in the N-terminal 23–89 fragment. Also missing from the PrP\(_{94-233}\) ROA spectrum are the negative and positive bands at \( \sim 1257 \text{ cm}^{-1} \) and \( \sim 1296 \text{ cm}^{-1} \), respectively, assigned to \( \beta \)-turns, from which we deduce that the 23–89 fragment also contains \( \beta \)-turns. These results are consistent with the previous suggestions of both PPII and \( \beta \)-turns in the N-terminal region.

The UVCD spectra of ovine PrP\(_{25-233}\) and PrP\(_{94-233}\) are displayed in Figure 4(a). Both show characteristic minima at \( \sim 222 \text{ nm} \) and 208 nm, indicating a predominance of \( \alpha \)-helix in the structures. Although the UVCD spectra are qualitatively very similar, they are quantitatively different. These differences arise either from differences in the folded domains of each protein or from the presence of the N-terminal region in PrP\(_{25-233}\). Since NMR determinations of PrP globular structures indicated only subtle differences in these regions when the N-terminal region was incorporated, we believe that the differences in the UVCD spectra reflect the contributions made to the spectra by the N-terminal region.

Subtraction of the UVCD spectrum of PrP\(_{94-233}\) from that of PrP\(_{25-233}\) yields a difference spectrum (Figure 4(b)) with a strong negative minimum at \( \sim 195 \text{ nm} \) and a weak positive maximum at \( \sim 220-230 \text{ nm} \) characteristic of PPII-helix, which, in agreement with the conclusions from the ROA data, may be ascribed to PPII structure in the 25–93 fragment. This spectrum is similar to those previously determined for the octapeptide repeat region and for a peptide spanning residues 40–57. To further confirm the similarities between these spectra, in Figure 4(b) we also present the UVCD spectrum of a polypeptide derived from murine PrP spanning residues 25–113, which is very similar to the subtracted spectrum and to previous spectra of N-terminal sequences. It also has a maximum at 220–230 nm and a minimum at \( \sim 200 \text{ nm} \) characteristic of PPII helix. Unfortunately, this polypeptide was only sparingly soluble in physiological buffers and was therefore not amenable to ROA analysis.

Useful ROA signatures of \( \alpha \)-helix include positive extended amide III bands at \( \sim 1300–1310 \text{ cm}^{-1} \) and \( \sim 1340 \text{ cm}^{-1} \) characteristic of unhydrated and hydrated versions, respectively, and an amide I ROA couplet centred at \( \sim 1650 \text{ cm}^{-1} \) that is negative at low wave number and positive at high. Useful \( \beta \)-sheet ROA signatures include negative bands at \( \sim 1220 \text{ cm}^{-1} \) and \( \sim 1245 \text{ cm}^{-1} \) possibly associated with hydrated and unhydrated variants, respectively, and a similar amide I ROA couplet to that generated by \( \alpha \)-helix but shifted by \( \sim 10 \text{ cm}^{-1} \) to higher wave number.

We are developing a pattern recognition program, based on principal component analysis (PCA), to identify protein structural types from ROA band patterns. From the ROA spectral data,
PCA calculates a set of sub-spectra that serve as basis functions, the algebraic combination of which with appropriate expansion coefficients can be used to reconstruct any member of the original set of experimental ROA spectra. Figure 5 shows a plot of the coefficients for our current set of 80 polypeptide, protein and virus ROA spectra for the two most important basis functions. This map provides a two-dimensional representation of the structural relationships among the members of the set. The protein positions are colour-coded with respect to the seven different structural types listed in the Figure and which provide a useful initial classification that will be refined in later work to give quantitative estimates of structural elements such as helix, sheet, loops and turns. The spectra separate into clusters corresponding to different dominant types of protein structural elements, with increasing \( \alpha \)-helix content to the left, increasing \( \beta \)-sheet content to the right, and increasing disordered or irregular structure from bottom to top. PrP\textsubscript{25-233} and PrP\textsubscript{94-233} lie close together near the centre of the alpha beta region, which reinforces the impression from visual inspection of their ROA spectra of a significant amount of \( \alpha \)-helix. This also suggests little difference in the amounts of \( \alpha \)-helix and \( \beta \)-sheet in both full-length and truncated proteins. However, PrP\textsubscript{94-233} lies below PrP\textsubscript{25-233}, consistent with the absence of the disordered N-terminal region. Disordered poly(L-lysine), poly(L-glutamic acid) and the AcOOAAAAAAAOO-NH\textsubscript{2} peptide lie towards the top of the Figure.

The similar amount of \( \alpha \)-helix in PrP\textsubscript{25-233} and PrP\textsubscript{94-233} revealed by ROA is consistent with the NMR solution structures, which indicate almost identical amounts of \( \alpha \)-helix in full-length and truncated prion proteins (for example, PDB structures 1qlz and 1qml for the resolved sequence 128–231 in human PrP\textsubscript{25-233} and PrP\textsubscript{94-233} contain 51.9% and 51.0% \( \alpha \)-helix, respectively). However, our ROA data reflect a higher \( \beta \)-sheet content than that expected from the solution NMR structures (\( \sim \)3–9% in sequence 125–228). This may be due to oligomerisation at the high concentrations of ovine PrP\textsubscript{25-233} and PrP\textsubscript{94-233} used (\( \sim \)40 mg/ml). Analytical ultracentrifugation showed our samples to be monomeric at \( \sim \)3 mg/ml (data not shown) but we anticipate the formation of dimers and oligomers at higher concentrations.

On the basis of recent work on model \( \beta \)-sheet polypeptides and proteins,\textsuperscript{40} the positive ROA band at \( \sim 1557 \text{ cm}^{-1} \) (amide II) and negative ROA band at \( \sim 1616 \text{ cm}^{-1} \) (amide I) in the spectra of PrP\textsubscript{25-233} and PrP\textsubscript{94-233} suggest the presence of multi-stranded \( \beta \)-sheet that is more flat and uniform than that found in typical \( \beta \)-sheet proteins. Again this may reflect some oligomerisation.

**Implications for prion function and aggregation**

Sequence analysis revealed that the N-terminal region of PrP\textsuperscript{C} is highly conserved between species,\textsuperscript{42,43} which suggests that it has an important
structural and functional role. The results of some studies indicate that this functional role involves copper metabolism: four Cu ions bind to the protein in the highly conserved group of octapeptide repeats, having the consensus sequence PHGGGWWQ and spanning residues 54–95 in the ovine prion protein, with a fifth taken up in the flexible region between the octarepeats and the globular C-terminal region. Presumably the rheomorphic character of the PPII-rich N-terminal region of the apoprotein, together with the ability of the four glycine residues within each octarepeat to support turns and extended structure as well as PPII, enables the appropriate sequences to readily adapt to the fixed Cu(II)-bound conformation of the holoprotein. This conformational adaptability may also be important in binding of PrP to other ligands within the intra- or extracellular environments. Various binding partners for the N-terminal region of PrP have been suggested, including polypeptides, the laminin receptor and nucleic acids. It is therefore likely that an extended, rather flexible structure in this region is important in promoting binding and facilitating molecular interactions.

An ROA study revealed that PPII helix is formed at the expense of hydrated z-helix in the prefibrillar amyloidogenic intermediate of human lysozyme, which prompted the suggestion that PPII helix could be a critical conformational element in some amyloid diseases. Elimination of water molecules between extended PPII chains having hydrated C=O and N-H groups to form b-sheet hydrogen bonds is a favourable thermodynamic process. Since PPII chains are close in conformation to b-strands, they would be expected to readily undergo this type of aggregation with each other and with established b-sheet. A more recent study using conventional techniques has corroborated this idea, but without explicitly identifying the extended polypeptide chains as PPII-type structure. A subsequent ROA study of the brain proteins z-synuclein and tau, which have a propensity to form the fibrils associated with Parkinson’s and Alzheimer’s disease, respectively, revealed that these natively unfolded proteins consist largely of PPII helical sequences. However, although disorder of the PPII type may be essential for the formation of regular fibrils, the presence of a large amount of PPII structure does not necessarily impart a fibrillogenic character since not all PPII-rich non-regular protein structures form amyloid fibrils. For example, the casein milk proteins and the brain proteins b- and g-synuclein show little propensity for amyloid fibril formation and are not associated with disease, yet their natively unfolded structures are based largely on PPII sequences as in z-synuclein, which is highly amyloidogenic.

A complete understanding of the fibrilligenic propensity of a particular sequence, PPII or otherwise, requires knowledge of the various physicochemical properties of the constituent residues. In particular, a combination of high net charge and low mean hydrophobicity has been shown to be an important prerequisite for protein sequences to remain natively unfolded.

The potential importance of PPII in amyloid disease has been reinforced by recent demonstrations of the presence of significant amounts of this conformation in the Alzheimer amyloid peptide Aβ(12–28) by UVCD and in Aβ(1–28) by FTIR and Raman. The latter study also identified the presence of b-strand.

There have been some recent attempts to use spectroscopic data to quantify the propensities of different residues to adopt the PPII conformation. The 25–93 sequence of the disordered N-terminal region of PrPc has a high proportion of proline (ten) and glycine (31) residues, many of which occur in the metal-binding group of octarepeats. It appears that glycine is quite favourable for PPII formation, perhaps due to backbone solvation. The 25–93 sequence of the PrPc propensivity of proline itself, the 25–93 sequence is expected to have a high average PPII propensity.

The physicochemical properties of the constituent residues of the disordered N-terminal region are highly relevant to the prion problem. Since PrPc formation, and prion propagation, are possible with deletions in PrPc up to residue ~110, these residues are evidently not playing a critical role in PrP misfolding. In addition, since the protease-resistant core structure of PrPc formed from full-length PrPC contains only residues ~90–233, most of the N-terminal sequence appears not to participate in the compact folded domain of PrPc. It therefore appears that, despite the high PPII content revealed by the present study, the 25–93 sequence of the disordered N-terminal region of PrPc is not intrinsically fibrillogenic. Due to the presence of four positively charged residues in the extreme N-terminal sequence KKRPKP, the N-terminal end carries a high net charge and also has a low mean hydrophobicity. Although the mean hydrophobicity of the rest of the 25–93 sequence is only average, ~59% of the sequence is made up of proline and glycine residues, which have very low b-sheet forming propensities. The 25–93 sequence of the disordered N-terminal region therefore appears to be well protected against association into b-sheet structures.

Comparison of the solution NMR structures of full-length and truncated prion proteins revealed that the N-terminal region has a small but significant stabilising influence on certain sequences of the structured C-terminal domain. The first indication of this was the detection of a stabilisation of helical structure associated with residues 190–196 within helix 2 of the hamster prion protein that was suggested to arise from transient tertiary interactions between the N-terminal residues and helix 2 residues.

Subsequent studies of human prion protein revealed a similar stabilisation of this segment of helix 2, but also revealed some stabilisation in residues 222–229 and 219–225 within helix 3, with similar observations for the bovine prion protein. However, the implied
increase of helix population amounts only to a few percent, as confirmed by our ROA results. So although not directly participating in the β-fibril formation responsible for prion disease, it appears from these NMR studies that there is scope for the disordered N-terminal region to modulate the fibrillogenesis itself, together with the infectivity and species barriers that characterise prion disease. Certainly, this was born out in recent investigations of cross-species disease transmission in vivo and in vitro.

**Conclusions**

ROA has revealed that a major conformational element in the “disordered” N-terminal region of the full-length ovine prion protein PrP25-233 is PPII helix, which was confirmed by UVCD, and that some β-turns are also present. ROA further revealed that little PPII structure is present in the truncated protein PrP94-233 and that the amounts of α-helix in the full-length and truncated proteins are very similar. The presence of a large amount of PPII structure in the N-terminal region is consistent with the flexibility required for its ability to bind metal ions and for its apparent ability to modulate properties of the full-length protein via interactions with the structured C-terminal region. Changes in environmental factors, or of the physicochemical properties of critical residues associated with genetic variants, could readily modify subtle characteristics such as fibrillogenic propensity of particular sequences within the N-terminal region itself or within regions of the C-terminal domain that interact with this region. Hence, the PPII-rich character of the N-terminal region appears to be an essential factor in the function, and possibly a modulating factor in the misfunction, of prion proteins.

**Materials and Methods**

**Materials**

Recombinant prion proteins were produced as described. Briefly, PrP proteins were expressed from the pTrc plasmid (Quiagen) either without (PrP25-233) or with (PrP94-233) the hexHis tag in *Escherichia coli* with IPTG. Protein production was induced by the addition of IPTG and cells were harvested by centrifugation. Cells were lysed using lysozyme and inclusion bodies harvested by centrifugation after removal of DNA by treatment with sodium deoxycholate and DNase. Protein from inclusion bodies was solubilised in 8 M urea and PrP purified by immobilised metal ion affinity chromatography followed by cation exchange chromatography. The single disulphide bond was remade by incubation with fivefold molar excess of copper ions followed by extensive dialysis against 50 mM sodium acetate to refold the protein. Final fractions were checked by SDS-PAGE, UVCD and electrospray mass spectrometry (ESMS). Concentrations were determined by UV absorbance.

To produce fractions of sufficient concentration for ROA spectroscopy, protein was concentrated in filtration concentration devices of progressively smaller size (centriprep, centricon, microcon; Amicon). Final concentration was done by removal of solvent under vacuum. The fidelity of the protein was checked after concentration by removal of a 1 μl aliquot, dilution and analysis by UVCD and ESMS.

DNA encoding the murine PrP sequence 25–113 was cloned from a template of the full-length protein and inserted into the pTrc expression plasmid (Quiagen). Transformed *E. coli* were used for expression of the protein, which was isolated as described above. Yields of this polypeptide were poor and the final fraction was only sparingly soluble in 50 mM sodium acetate (pH 5.5).

**ROA spectroscopy**

The instrument used for the Raman and ROA measurements has a back-scattering configuration, which is essential for ROA studies of aqueous solutions of biopolymers, and employs a single-grating spectograph fitted with a back-thinned CCD camera as detector and an edge filter to block the Rayleigh line. ROA is measured by synchronising the Raman spectral acquisition with an electro-optic modulator to switch the polarisation state of the incident argon-ion laser beam between right and left circular at a suitable rate. The spectra are displayed in analog-to-digital counter units as a function of the Stokes wave number shift with respect to the exciting laser wave number. The ROA spectra are presented as circular intensity differences $I^R - I^L$ and the parent Raman spectra as circular intensity sums $I^R + I^L$, where $I^R$ and $I^L$ are the Raman-scattered intensities in right- and left-circularly polarised incident light, respectively.

The PrP25-233 and PrP94-233 solutions were studied at concentrations ~40 mg/ml in ~50 mM acetate buffer (pH 5.5), at ambient temperature (~20 °C). The solutions were filtered through a 0.22 μm Millipore filters into quartz microfluorescence cells, which were centrifuged gently prior to mounting in the ROA instrument. Residual visible fluorescence from traces of impurities, which can give large backgrounds in Raman spectra, was quenched by leaving the samples to equilibrate in the laser beam for a few hours before acquiring ROA data. The experimental conditions were as follows: laser wavelength, 514.5 nm; laser power at the sample, ~700 mW; spectral resolution, ~10 cm$^{-1}$; acquisition time, ~50 hours in total for the prion proteins.

**UVCD spectroscopy**

The UVCD spectra were recorded by use of a Jasco J-710 spectropolarimeter. All samples were dissolved in sodium acetate buffer (pH 5.5). Spectra of PrP25-233 (0.62 mg/ml) and PrP94-233 (0.64 mg/ml) were recorded in a cell of path length 0.01 cm. The spectrum of murine PrP25-105 (0.03 mg/ml) was recorded in a cell of path length 0.2 cm. The spectra were averaged over three individual spectra, each being the sum of at least 20 scans. Concentrations were determined by UV absorbance at 280 nm by use of a Beckman DU650 spectrophotometer.

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References

38. Yoshida, H., Matsushima, N., Kumaki, Y., Nakata, M.

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