

Biochemical and structural studies of the prion protein polymorphism

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Abstract A hallmark event in transmissible spongiform encephalopathies is the conversion of the physiological prion protein into the disease-associated isoform. A natural polymorphism at codon 129 of the human prion gene, resulting in either methionine or valine, has profound influence on susceptibility and phenotypic expression of the disease in humans. In this study, we investigated the local propensity of synthetic peptides, corresponding to the region of the polymorphism and containing either methionine or valine, to adopt a β -sheet-rich structure similar to the pathological protein. Circular dichroism studies showed that the methionine-containing peptide has a greater propensity to adopt a β -sheet conformation in a variety of experimental conditions. The higher β -sheet tendency of this peptide was also associated with an increased ability to aggregate into amyloid-like fibrils. These results suggest that methionine at position 129 of the prion protein increases its susceptibility to switch to the abnormal conformation, in comparison with the presence of valine at the same position. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Prion; Transmissible spongiform encephalopathy; Protein structure; Creutzfeldt–Jakob disease; Circular dichroism

1. Introduction

Transmissible spongiform encephalopathies (TSE), also known as prion diseases, are a group of devastating degenerative disorders of the brain that affect humans and animals. The group includes Creutzfeldt–Jakob disease (CJD), kuru, Gerstmann–Straussler syndrome (GSS) and fatal familial insomnia (FFI) in humans as well as scrapie and bovine spongiform encephalopathy in animals. TSE can have diverse origins, namely infectious, familial and sporadic [1]. Although the aetiology and pathogenesis are not fully understood, the disease is associated with an abnormally shaped form of a natural protein named prion protein (PrP^C). The formation

of the disease-associated isoform (PrP^{Sc}) appears to be the key step in the pathogenesis and involves changes in the secondary structure of the protein [1,2]. PrP^{Sc} is the only known component of the infectious agent and has the capability to self-replicate at the expense of PrP^C [2]. No chemical differences have been detected to distinguish the two PrP isoforms, but they can be experimentally differentiated by four properties [2,3]: (a) PrP^{Sc} is partially resistant to proteolytic degradation, whereas PrP^C is sensitive to proteolysis; (b) PrP^C is a soluble protein, while PrP^{Sc} is stubbornly insoluble and becomes deposited in infected brain parenchyma; (c) PrP^C is mainly composed of α -helical conformation, while PrP^{Sc} is rich in β -sheet secondary structure; (d) PrP^{Sc} is able to replicate itself in vitro and in vivo by converting PrP^C into PrP^{Sc} [4,5].

The human prion protein is a product of a single host gene located on the short arm of chromosome 20 that contains two exons. The sequence of the translated product is 253 amino acids long, contains five amino-terminal octapeptide repeats, two glycosylated sites and one disulphide bridge [1,3]. In addition, there are two signal sequences in the amino- and carboxy-terminal parts, which are removed during processing and a glycosylphosphatidylinositol anchor attaches the protein to the outer surface of the cell membrane [6]. The three-dimensional structure of several fragments as well as the full-length recombinant PrP have been obtained from different species [7–11]. The nuclear magnetic resonance (NMR) structure of the mature recombinant human prion protein, as well as the protein from several other species, predicts a structured globular domain extending from residues 125 to 228 and an N-terminal flexible and disordered region. The globular domain contains three α -helices comprising the residues 144–154, 173–194, and 200–228 and an anti-parallel β -sheet consisting of two short strands comprising the residues 128–131 and 161–164 [9]. Based on immunological, biochemical, and molecular modeling studies, it has been proposed that the fragment 90–145 of PrP is the main region of the protein that switches to the β -sheet conformation upon the formation of PrP^{Sc} [12–20].

Model peptides have been used extensively to study the contributions of different fragments of PrP to its properties and to elucidate the mechanism of PrP conversion [20]. Peptides spanning the PrP sequence 109–141 can reproduce in vitro some of the characteristics of the transition between PrP^C and PrP^{Sc} [21], including a random to β -sheet conformational change, which result in acquisition of protease resistance and aggregation into rod-shaped fibrils [21]. In addition, the interaction of this fragment with PrP^C induces the normal

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Abbreviations: TSE, transmissible spongiform encephalopathies; CJD, Creutzfeldt–Jakob disease; GSS, Gerstmann–Straussler syndrome; FFI, fatal familial insomnia; PrP^C, cellular prion protein; PrP^{Sc}, scrapie prion protein; CD, circular dichroism; TFE, trifluoroethanol; ThT, thioflavine T

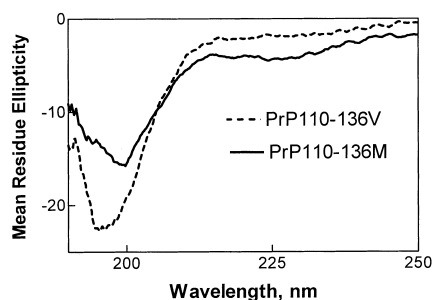


Fig. 1. CD spectra of PrP109–136 peptides bearing Met or Val at position 129. 50 μ g of each peptide was dissolved in 350 μ l of 10 mM Tris, pH 7.4 and immediately the CD spectrum was recorded as described in Section 2. The spectrum of buffer was subtracted and CD data converted to mean residue ellipticity. Solid line, PrP109–136M; dotted line, PrP109–136V.

protein to acquire some properties of the pathological isoform, including protease resistance and insolubility [22]. Moreover, several peptides around the PrP sequence 100–150 inhibit the interaction between the two PrP isoforms resulting in prevention of the *in vitro* conversion of PrP^C into PrP^{Sc} in a cell-free system [15,18]. Finally, the fragment 106–126 of the human protein causes nerve cell death by apoptosis and induces hypertrophy and proliferation of astrocytes and activation of microglial cells *in vitro* [17,23]. These data suggest that the central PrP fragment (around residues 106–140) might be the nidus at which conformational change is initiated during the conversion of PrP^C into PrP^{Sc}.

Several mutations in the prion gene have been associated with inherited forms of TSE [1–3]. In humans there is a polymorphism at codon 129, where an ATG or GTG results in either a methionine (Met) or a valine (Val) at that position. A large body of evidence indicates that this polymorphism alone or in conjunction with mutations in the prion gene modulates disease susceptibility and phenotypic expression of human TSE [24–30]. However, the molecular mechanism by which the polymorphism affects disease pathogenesis is unknown.

The aim of this study was to evaluate the influence of polymorphism at position 129 on the properties of the protein and its propensity to undergo conformational changes, using model peptides containing the human PrP109–136 sequence and bearing either Met or Val at position 129.

2. Materials and methods

2.1. Peptide synthesis

PrP fragments spanning the sequence 109–136 of human PrP and bearing Met (PrP109–136M, sequence MKHMAGAAA-GAVVGGLGGYMLGSAMSR) or Val (PrP109–136M, sequence MKHMAGAAAAGAVVGGLGGYVLGSAMSR) at position 129 were synthesised in solid phase at Neosystem Inc. Peptides were purified by HPLC and purity (>95%) was evaluated by peptide sequencing and laser desorption mass spectrometry. Stock solutions of the peptides were prepared in water/0.1% trifluoroacetic acid and stored lyophilised in aliquots at -70°C . Concentration of the stock solution was estimated by amino acid analysis.

2.2. Circular dichroism (CD)

Spectra of solutions containing PrP synthetic peptides (50 μ M), prepared in the buffers described in Figs. 1–3, were recorded in a Jasco J-810 spectropolarimeter at 25°C using a 0.1 cm path-length cell. Spectra of five scans were accumulated at 1 nm intervals over the wavelength range 190–260 nm and buffer spectrum obtained under identical conditions was subtracted. Results are expressed as molar

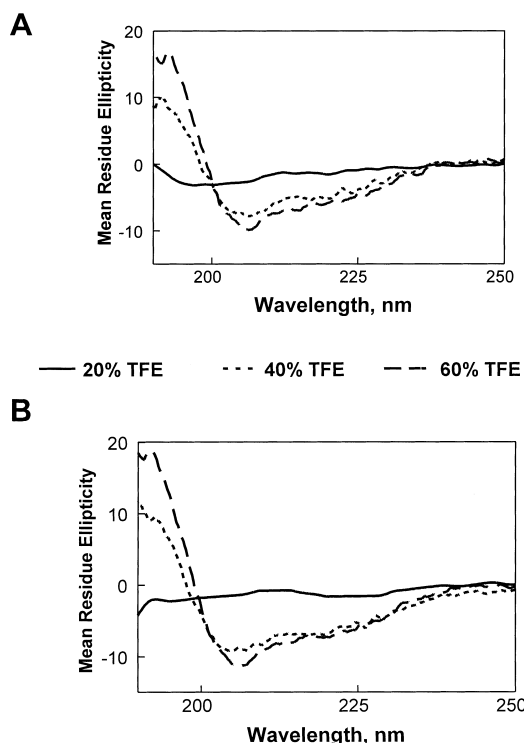


Fig. 2. Effect of TFE on the CD spectra of PrP fragments. 50 μ g of PrP109–136M (A) or PrP109–136V (B) was dissolved in 350 μ l of 10 mM Tris, pH 7.5 containing either 20% (solid line), 40% (dotted line) or 60% (dashed line) of TFE. Spectra were recorded as described in Section 2 and the buffer subtracted to obtain the final spectra that were converted to mean residue ellipticity using the Jasco software.

ellipticity in units of $\text{deg cm}^2 \text{dmol}^{-1}$, and data were analysed by the Jasco Secondary Structure Estimation software (version 1.00, 1998) that uses the Yang algorithm [31] to obtain the percentages of different secondary structure motifs.

2.3. Studies of amyloid formation *in vitro*

Aliquots of PrP synthetic peptides at different concentrations in 0.1 M Tris, pH 7.4, were incubated for various times at 37°C . Amyloid formation was quantitatively assessed by a fluorometric assay based on the fluorescence emission of thioflavine T (ThT), as previously described [32]. Additional characterisation of the fibrillogenesis was performed by electron microscopy after negative staining [33]. Samples were placed on carbon formvar-coated 300 mesh nickel grids and stained for 60 s with 2% uranyl acetate. Grids were analysed using a Zeiss EM 10 electron microscope at 80 kV.

3. Results

3.1. Influence of polymorphism on peptide conformation

Secondary structural studies of peptides containing the sequence 109–136 of human PrP and bearing either Met or Val at position 129 were carried out under a variety of conditions, using CD. Studies in aqueous solutions at neutral pH showed significantly different CD spectra in the far UV depending upon the amino acid at position 129 (Fig. 1). Analysis of the spectra to obtain the estimated content of different secondary structural motifs (Tables 1 and 2) revealed that both peptides consisted of a mixture of β -sheet, β -turn and unordered structures, with no evident α -helical conformation. However, the relative content of β -sheet and random coil was significantly different, because PrP109–136M has a higher

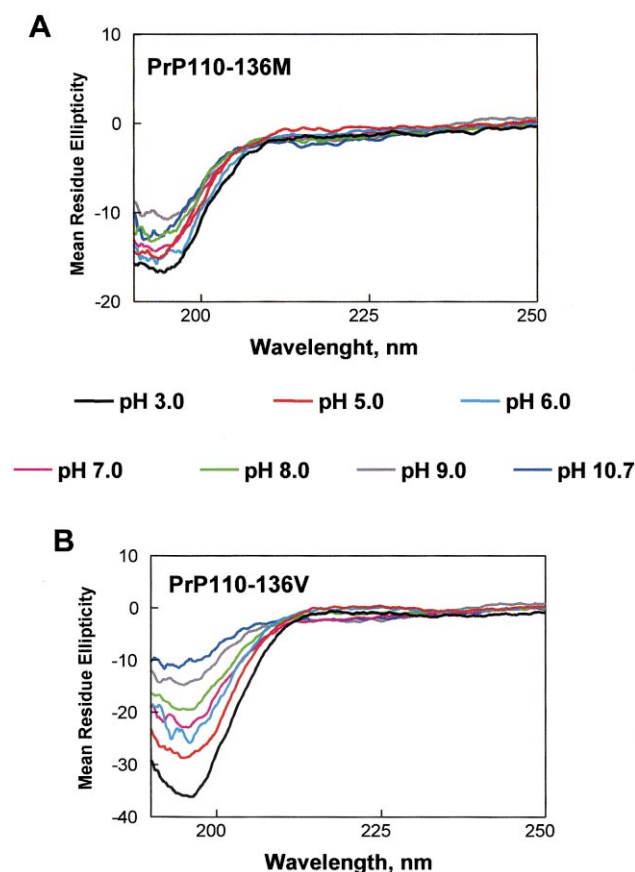


Fig. 3. Effect of pH on the CD spectra of PrP fragments. 50 μ g of PrP109–136M (A) or PrP109–136V (B) was dissolved in 350 μ l of various buffers adjusted to different pHs. The buffers used were the following: 10 mM sodium citrate at pH 3.0; 10 mM sodium acetate at pH 5.0; 10 mM MES at pH 6.0; 10 mM sodium phosphate at pH 7.0; 10 mM Tris at pH 8.0 and pH 9.0; and 10 mM carbonate-bicarbonate buffer at pH 11.0. Spectra were recorded as described in Section 2 and the contribution of each buffer was subtracted.

β -sheet and a lower random coil level than the homologous Val-containing peptide. Short peptides usually adopt a mainly unordered structure in aqueous solution and it is a common practice to add some organic solvents (in particular trifluoroethanol (TFE)) to try to mimic the environment on the interior of a protein molecule and thus evaluate the putative structure of the fragment in the context of the entire protein [34]. In the presence of 20% TFE both peptides become more structured (Fig. 2), which is reflected in the decrease in the

random coil content (Tables 1 and 2). However, while PrP109–136M increased its β -sheet content by 17% to reach 43.8% of this structural motif (Table 1), the content of β -sheet in the PrP109–136V remained unchanged at around 11% (Table 2). The increase in structure of the latter peptide is in the form of α -helix, reaching 12% of this motif at 20% TFE (Table 2). At higher concentrations of TFE both peptides behave similarly (Fig. 2, Tables 1 and 2), which is not surprising because high quantities of TFE will induce secondary structure in any peptide, regardless of the sequence.

Structural studies at different pH in aqueous solvents showed that whereas the CD spectrum of PrP109–136M does not change between pH 3.0 and 10.7 (Fig. 3A), the spectrum of the Val-containing fragment varied significantly in the same pH range (Fig. 3B). At acid pH, there is virtually no β -sheet structure in PrP109–136V, while the content of this motif increased to 28.6% at pH 10.7, reaching a similar pattern as the Met-containing peptide (Tables 1 and 2). These data suggest that a low pH, similar to the conditions in which the conversion may occur intracellularly in vivo, determines a large difference in the propensity of each peptide to adopt a conformation closer to the normal or to the pathological PrP isoform.

3.2. Influence of polymorphism on peptide aggregation

As reported earlier, PrP fragments containing the sequence 109–141 and 106–126 form amyloid fibrils in vitro, a phenomenon that has also been observed in the brain of a great proportion of TSE cases [35]. In order to evaluate the effect of the polymorphism at codon 129 on the ability of the peptides to make amyloid, fibrillogenesis studies were carried out in vitro. Both PrP109–136M and PrP109–136V were able to form amyloid fibrils after incubation at 37°C in 0.1 M Tris, pH 7.4, as evaluated by the fluorescence emission of the amyloid binding dye ThT (Fig. 4) and by electron microscopy (Fig. 5). The amount of fibrils formed was dependent upon the concentration of the peptide (Fig. 4A) and the time of incubation (Fig. 4B). Under both experimental conditions, the amount of amyloid obtained with the Met-containing peptide was dramatically higher than for the Val-containing analogue. For example, after 3 days of incubation at a concentration of 3 mg/ml, PrP109–136M formed five-fold more amyloid than PrP109–136V (Fig. 4B). This conclusion was qualitatively supported by the electron microscopic analysis of the aggregates (Fig. 5). Morphologically, the fibrils consisted of unbranched filaments with a diameter of 5–10 nm and more than 200 nm in length, similar to amyloid fibrils extracted from the brain of people affected by Alzheimer's

Table 1
Secondary structure of PrP109–136M in various conditions

Condition	α -Helix (%)	β -Sheet (%)	β -Turn (%)	Random (%)
Buffer	0	26.7	21.4	51.9
20% TFE	0	43.8	7.8	48.3
40% TFE	19.2	43.1	0	37.7
60% TFE	37.2	21.9	0	41.0
pH 3.0	0	22.8	25.3	51.9
pH 5.0	0	18.0	29.8	52.3
pH 6.0	0	20.6	27.0	52.5
pH 7.0	0	24.9	23.4	51.7
pH 8.0	0.5	27.7	23.5	48.3
pH 9.0	2.3	25.2	24.9	47.6
pH 10.7	1.4	28.6	22.2	47.8

Table 2
Secondary structure of PrP109–136V in various conditions

Condition	α -Helix (%)	β -Sheet (%)	β -Turn (%)	Random (%)
Buffer	0	10.7	27.2	62.2
20% TFE	12.0	11.0	30.4	46.7
40% TFE	17.5	42.6	0	39.9
60% TFE	36.0	22.5	0	41.5
pH 3.0	0	2.7	36.7	60.6
pH 5.0	0	0	37.8	62.4
pH 6.0	0	0	37.2	62.8
pH 7.0	0	10.9	30.2	58.8
pH 8.0	0	18.2	28.6	53.3
pH 9.0	2.2	23.5	26.1	48.2
pH 10.7	2.0	25.6	24.8	47.6

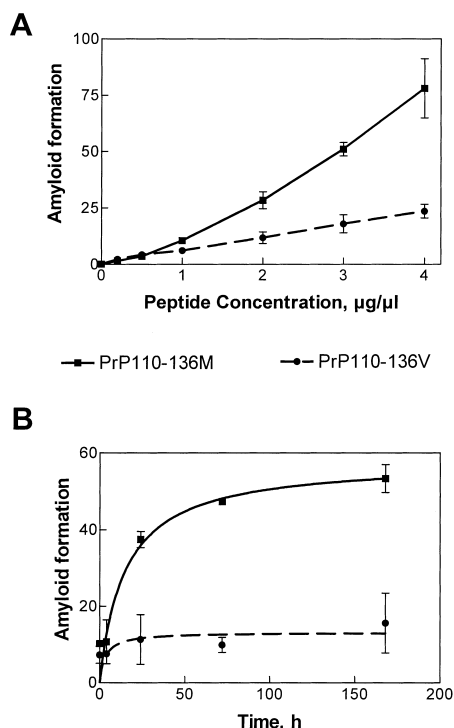


Fig. 4. Amyloid fibrillogenesis of PrP109–136M and PrP109–136V. Aliquots of each peptide were incubated at different concentrations (A) during 5 days at 37°C or a fixed concentration of 3 mg/ml during different times (B). Amyloid formation was measured by the ThT fluorometric assay as described in Section 2 and is expressed as arbitrary fluorescence units. In both graphs, squares represent the PrP109–136M samples and circles the PrP109–136V samples.

disease or GSS [35] and those produced *in vitro* by incubating the human recombinant PrP90–231 [36]. No evident morphological differences were observed between fibrils formed by the two peptides.

4. Discussion

A central event in the pathogenesis of TSE is the conversion of a natural protein isoform (PrP^C) into the β -sheet-rich, aggregated, toxic and protease resistance conformer (PrP^{Sc}). Several epidemiological studies have demonstrated that a natural polymorphism at position 129 (Met/Val) of PrP is the most important risk factor known for human TSE [24,26,28,29,37,38]. Both Met and Val homozygotes are

over-represented, while heterozygous cases are under-represented in CJD [39]. About 40% of the normal population is Met-homozygous, however respectively 78%, 60% and 100% of patients affected by the sporadic, iatrogenic and variant forms of CJD are Met-homozygous [24]. These data suggest that the presence of Met at position 129 confers a higher susceptibility for the protein to be converted into the pathogenic isoform. The polymorphism has also been shown to alter the neuropathological pattern of lesions in sporadic CJD, the glycoform profile of protease-resistant PrP^{Sc} and the duration and severity of the disease [25–27,40]. A study involving 300 patients showed that Met-homozygotes develop a more aggressive phenotype characterised by a short duration of disease (4.5 months), while heterozygotes and Val-homozygotes have a much longer disease duration (14.3 and 16.9 months, respectively) [27]. Val-homozygosity seems to cause damage preferentially in the deep grey matter, while Met-homozygosity seems to target mainly cortical structures [26]. Codon 129 polymorphism also influences the phenotypic expression of mutations elsewhere in the prion gene [38,41–43]. For example, people with a mutation at codon 178 resulting in a change of aspartic acid to asparagine develop either familial CJD or FFI depending on whether the amino acid at codon 129 is Val or Met, respectively [30].

Despite the clear importance of PrP polymorphism at position 129 in the disease propensity and pathogenesis, the molecular mechanism of this effect is unknown. Experimental and computational modeling studies of the three-dimensional structure of PrP have been unable to identify any significant difference between the two isoforms. In addition, no difference was reported on the *in vitro* thermodynamic stability of recombinant PrP bearing either Met or Val at position 129 [44]. Structural studies have shown evidence for hydrogen bonding between Asp178 and Tyr128, which might provide a structural basis for the influence of the polymorphism on the disease phenotype that segregates with the mutation Asp178Asn [45]. In addition, it has been reported that a slightly different conformation of recombinant Met- or Val-containing PrP isoforms was induced upon copper binding [46].

In this study we show that a synthetic peptide containing the human PrP109–136 sequence and bearing Met at position 129 has a higher propensity to adopt a β -sheet structure as observed by CD, than the homologous Val-containing peptide. The higher β -sheet propensity of the former resulted in an increase in aggregation into amyloid-like fibrils. Some of the major biochemical differences between PrP^C and PrP^{Sc} are

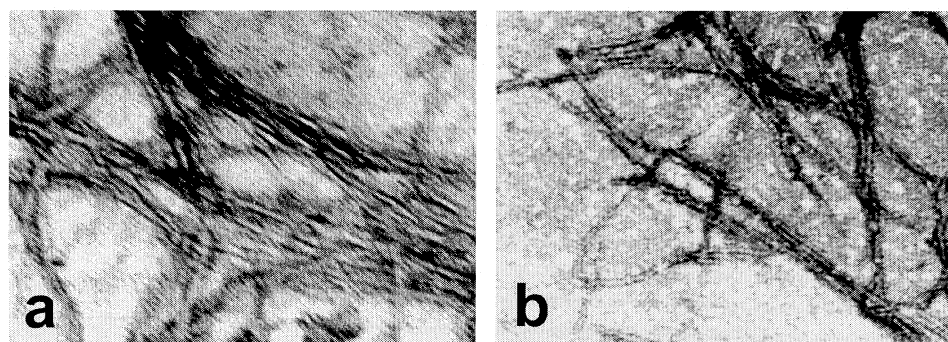


Fig. 5. Electron microscope photographs of amyloid fibrils formed by PrP fragments. 60 µg of PrP109–136M (a) or PrP109–136V (b) was incubated for 5 days in 20 µl of 0.1 M Tris, pH 7.4. Samples were loaded in electron microscope grids and stained with uranyl acetate as described in Section 2. Grids were visualised with 80× magnification.

precisely the greater levels of β -sheet secondary structure resulting in aggregation of the pathological conformer [1,2]. Therefore, our findings suggest that Met at position 129 provides a greater local propensity for the conversion of PrP^C to PrP^{Sc} than Val at that position. This conclusion, which is counter-intuitive, because Val is usually considered a stronger β -sheet-forming amino acid than Met [47,48], has to be evaluated in the context of the entire protein. It is possible that the differences in β -sheet propensity are mainly in the local structure and might not be seen easily in a larger fragment or in the entire protein. Using a similar approach, it was reported previously that synthetic peptides spanning the region 106–136 of human PrP and containing Met or Val at position 129 did not show a significant difference in secondary structure as analysed by CD studies [49]. One possible explanation for the differences between these results and ours is that the extension of the peptide toward the N-terminus in the study by Smith and co-workers (106 versus 109) precludes the formation of β -sheets in the model peptide [49]. This explanation is supported by studies from Nguyen and co-workers [50] who demonstrated that a peptide spanning the sequence 109–122 of Syrian hamster PrP adopts a β -sheet structure, whereas the 104–122 peptide has a random coil structure in aqueous solution. The reason for the dramatically different conformations adopted by these two peptides is unknown, but it is likely to be related to a distinct balance between hydrophobic and hydrophilic amino acids [50]. Indeed, PrP104–122 has one more positively charged residue than PrP109–122, which could minimise intermolecular interactions that are required for β -sheet formation. The same charge difference is also observed between PrP109–136 (used in this study) and PrP106–136 (used in the study by Smith et al. [49]).

According to the NMR solution structure of human PrP^C, residue 129 lies on a short β -strand comprising residues 128–131, which forms an antiparallel β -sheet with a second β -strand spanning residues 161–164 [9]. It has been proposed that this short β -sheet might be a nucleation site around which the loop connecting the β -sheet as well as the first α -helix are transformed into several longer β -strands during PrP^C \rightarrow PrP^{Sc} conformational transition [7]. Based on the results described here, we propose that Met at position 129 induces a higher local propensity to extend the short β -sheet present in the normal protein into a larger sheet, which results in an increase in the rate of PrP conversion to the pathological isoform. This mechanism might explain the association of Met129 polymorphism with an increased risk of developing sporadic and variant CJD.

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