**INTRODUCTION**

The prion diseases or transmissible spongiform encephalopathies are a group of closely related neurodegenerative disorders characterized by spongiform degeneration of the brain. They include scrapie of sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease affecting deer and elk and the human disorders Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease, fatal familial insomnia (FFI) and variant CJD (vCJD) (Collinge, 2001). A unique and defining feature of these diseases in humans is that they can be inherited, arise idio-pathically or be acquired through infection (Collinge, 2001). According to the protein-only hypothesis (Griffith, 1967), the central event in the pathogenesis of prion disease is the conversion of the normal, cellular form of the prion protein (PrPC) into an aberrantly folded, disease-associated form known as PrPSc (Prusiner, 1982, 1998).

Inherited forms of prion disease are caused by mutations in the PrP gene (PRNP) located on human chromosome 20 (Collinge, 2001). Many pathogenic mutations in PrP have now been described, along with two polymorphisms: methionine (M) or valine (V) at codon 129 (M129V) and glutamine or lysine at codon 219 (E219K) (Owen et al., 1990; Petraroli & Pocchiari, 1996). These polymorphisms, although not pathogenic, have a major effect on susceptibility to, and phenotypic expression of, prion disease. Heterozygosity at either of these codons is associated with resistance to sporadic CJD (Palmer et al., 1991; Shibuya et al., 1998). In addition, differences in codon 129 status result in phenotypic variation within pedigrees also carrying a pathogenic mutation in PrP (Dlouhy et al., 1992; Goldfarb et al., 1992; Poulter et al., 1992; Monari et al., 1994; Hainfellner et al., 1999) and homozygosity at this codon is a significant risk factor for acquired prion diseases, such as kuru, vCJD and iatrogenic CJD (Collinge et al., 1991, 1996a; Zeidler et al., 1997; Hill et al., 1999; Lee et al., 2001; Mead et al., 2003).

Additionally, codon 129 genotype has a strong interaction with prion-strain propagation, certain prion strains only occurring in individuals with specific PRNP codon 129 genotypes (Collinge et al., 1996b; Wadsworth et al., 1999; Hill et al., 2003). Most notably, vCJD occurs exclusively in PRNP codon 129 methionine homozygotes. The protective effect of PRNP codon 129 heterozygosity has been attributed to the greater efficiency of protein–protein interactions between homologous species (Palmer et al., 1991), known to be important in prion transmission (Prusiner et al., 1990; Collinge et al., 1995). The methionine/valine variation is, however, relatively conservative with respect to the volume and hydrophobicity of the side chains (Grantham, 1974), and nuclear magnetic resonance (NMR) studies comparing the three-dimensional structures of the two forms in the PrPC conformation failed to find any difference in structure or global stability (Hoszu et al., 2004). However, it is also now clear that this polymorphism has a key effect on prion-strain propagation and transmission barriers (Wadsworth et al., 2004) and may act by restricting the repertoire of thermodynamically permissible PrPSc conformers: the conformational-selection model of prion-transmission barriers (Collinge, 1999, 2001; Hill & Collinge, 2003; Wadsworth et al., 2004).

From these observations, it seems likely that the influence of residue 129 on prion disease results from its effect on the physical properties of the PrPSc isofrom or its precursors or on the kinetics of their formation. Whilst there is no reliable,
Given that it is a β-sheet form of PrP that is involved in the formation of prion aggregates in vivo, we investigated the behaviour of this β-PrP species to see whether the switch between V and M at codon 129 had any influence on its physical properties. Here, we present an analysis of the PrP codon 129 polymorphism and its affect on several aspects of the behaviour of recombinant human PrP, comprising residues 91–231. By using a variety of techniques, the stability, structural plasticity, aggregation properties, metal binding, protease resistance and amyloid formation of the M129 and V129 forms of human PrP have been investigated.

METHODS

Protein production and conversion to β-PrP. Recombinant prion protein (residues 91–231), containing either methionine or valine at codon 129 (M129 or V129), was produced as described previously (Hoszna et al., 2004); the conversion of recombinant α-PrP to β-PrP was carried out at pH 4.0 and under reducing conditions (Jackson et al., 1999). Total amount of protein was calculated by UV absorption using a calculated molar absorption coefficient of the function for the unfolded and native states, respectively. Data were fitted to the equation

\[ F_1 = F_0 - \left(\frac{F_{\text{amp}} \times [L]}{K_{\text{d(app)}}} + [L]\right) \]  

where \( F_1 \) is the fluorescence at a given concentration of ligand, \( [L] \), \( F_0 \) is the starting fluorescence and \( F_{\text{amp}} \) is the fluorescence amplitude. Apparent dissociation constants were converted to actual dissociation constants \( [K_{\text{d(real)}}] \) by using the equation

\[ K_{\text{d(real)}} = K_{\text{d(app)}} \times \frac{[K_{\text{d1}}]}{[G]} \times \frac{[K_{\text{d2}}]}{[G]} \]  

where \([G]\) is the concentration of glycine used in the experiment and \( K_{\text{d1}} \) and \( K_{\text{d2}} \) are the serial dissociation constants for the binding of copper(II) ions to glycine (Dawson et al., 1986).

Protease resistance. Aliquots (10 μl; 1 mg ml\(^{-1}\)) of protein of the appropriate codon 129 status and predominant fold (either V or β) were incubated with proteinase K (PK) at 0-1, 1, 10 or 100 μg ml\(^{-1}\) final concentration. Reactions were terminated by the addition of 2× SDS loading buffer [125 mM Tris/HCl (pH 6.8), 20% (v/v) glycerol, 4% (v/v) SDS, 4% (v/v) 2-mercaptoethanol, 0.02% (v/v) bromophenol blue] containing 8 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF; Pefabloc SC). Samples were heated to 100 °C for 10 min, subjected to SDS-PAGE and stained by using Coomassie brilliant blue.

Formation of amyloid fibrils. Fibril formation was performed and analysed by using protocols adapted from those of Baskakov et al. (2002). Briefly, recombinant α-PrP of both M- and V129 status was transferred into 20 mM sodium acetate (pH 3.7) and denatured by the addition of 10 M urea. The protein was then dialysed into 1×3 M urea, 1 M GuHCl, 20 mM sodium acetate (pH 6.0). To stimulate assembly of monomeric PrP into fibrils, 800 μM protein (0.8 mg ml\(^{-1}\)) was aliquoted into a 1×5 ml microtube and shaken at 37 °C and 600 r.p.m. An identical reaction was also performed with the concentration of V129 PrP (1.6 mg ml\(^{-1}\)). Monitoring of fibril formation was carried out by determination of thioflavin T fluorescence. A 10 μl sample was removed from the shaking aliquots and diluted 1:20 into 5 mM sodium acetate (pH 5.5). Thioflavin T was then added to a final concentration of 50 μM and fluorescence was measured on a Jasco FP-750 spectrofluorimeter (excitation 450 nm, emission 485 nm, 5 nm band widths). Each data point was generated from three independent samples. All experiments were performed at 20 °C.

An automated method for monitoring amyloid formation was also used. Recombinant protein was prepared in a manner similar to that described above and pipetted into a flat-bottomed 96-well plate at a concentration of 10 μM. The wells also contained thioflavin T at 10 μM and four 1 mm diameter ceramic spheres to assist with agitation. The plates were incubated at 37 °C in a Tecan Spectrofluor Plus with constant agitation. Readings were taken every 600 s and a mean of six wells was used for each data point.

To determine the lag time for fibril formation, data were fitted to an empirical function described by Nielsen et al. (2001):

\[ F_1 + F_t \left/ \left[1 + \exp\left\{-(t - t_m)/t_i\right\}\right]\right] \]  

where \( F_1 \) is the initial fluorescence, \( F_t \) is the final fluorescence, \( t \) is time, \( t_m \) is the time taken to half maximal fluorescence and \( t_i \) is the reciprocal of the propagation rate during the rise phase \([1/K_{\text{apparent}}]\). Lag time is defined as \( t_m - 2t \).
RESULTS

Structure and stability of α-PrP and β-PrP

To examine the impact of the codon 129 polymorphism on both the structure and stability of different conformational isoforms of PrP, recombinant PrP91–231 was examined by using CD. Spectra in the far-UV range (190–250 nm) were recorded and compared qualitatively (Figs 1a, 2a). The M and V polymorphs exhibited virtually identical spectra in both α-PrP and β-PrP conformations, a finding consistent with data from NMR studies of the two variants (Hosszu et al., 2004). To assess whether the polymorphism has any impact on the stability of either conformation, the proteins were subjected to equilibrium unfolding. Again, no significant difference was observed between M129- and V129-containing proteins in either the α form (Fig. 1b) or the β form (Fig. 2b). The free energy of folding in water was calculated for all four proteins, with values for α-PrP of 6.62 kcal mol⁻¹ (M129) and 6.52 kcal mol⁻¹ (V129). The stability of β-PrP was low (M129, 2.36 kcal mol⁻¹; V129, 2.73 kcal mol⁻¹), consistent with the observation that it has characteristics of a molten-globule folding material (Jackson et al., 1999).

Conversion efficiency

The lack of significant differences between the NMR solution structure, secondary-structure content or the stability of the
two polymorphic variants in either conformation suggests that the polymorphism may act by affecting the efficiency of conversion between isoforms. To investigate this, both polymorphs were converted to $\beta$-PrP as described previously (Jackson et al., 1999). The yield for conversion to $\beta$-PrP was calculated as a percentage of the total starting material remaining in the converted sample following clarification by ultracentrifugation. No significant difference ($t$-test, $P=0.28$) was found between the two as regards their propensity to form the $\beta$-sheet conformer, with conversion yields of $76\pm9.6\%$ (M129) and $69\pm12.8\%$ (V129).

**Chelation of copper(II) ions**

The binding of copper to the two polymorphic variants of PrP was examined at glycine concentrations of 200 $\mu$M (squares) and 1 mM (circles) (Fig. 3). At 200 $\mu$M glycine, the apparent dissociation constant was $2.0\times10^{-6}$ M for V129 PrP (■) and $2.5\times10^{-6}$ M for M129 PrP (□). These increased to $48\times10^{-6}$ and $58\times10^{-6}$ M for M129 (○) and V129 (●), respectively, at 1 mM glycine. Once the presence of glycine had been accounted for, the $K_d$ values calculated at pH 7-5 were roughly equivalent for both M and V polymorphs (875 and 750 pM, respectively).

**Protease resistance**

Both M and V variants of $\alpha$-PrP digested for 1 h at pH 8-0 with a variety of concentrations of PK exhibited similar levels of protease resistance and fragmentation patterns. Both forms were digested completely by treatment with 10 $\mu$g PK ml$^{-1}$ for 1 h. $\beta$-PrP was digested at pH 4-0. The optimum pH for PK activity is around pH 8-0; therefore, to control for the reduction in PK activity at low pH, $\alpha$-PrP was also digested at pH 4-0. Both M and V polymorphs of $\beta$-PrP were more resistant to PK treatment than their $\alpha$-PrP counterparts and no difference in fragment pattern or resistance to digestion between the M and V polymorphs could be discerned (Fig. 4).

**Fibril formation**

By using a discontinuous, manual method of determining amyloid formation, M129 fibrilized with a mean lag time of 38 h, as determined from five independent experiments (Fig. 5). The thiazole dye thioflavin T was used to report the formation of amyloid. The increase in thioflavin T fluorescence correlated well with the abundance of regular fibrils that could be visualized by negative-stain electron microscopy (data not shown).

In stark contrast to the M polymorph, V129 did not form any detectable amyloid material after a period exceeding 200 h. This was also true when the reaction was performed with twice the protein concentration (Fig. 5).

To ensure reproducibility, three experiments were carried out from one batch of proteins, with the remaining two experiments carried out with protein from independent protein preparations. From the latter preparations, samples of the bacterial cultures used for protein purification were used to prepare DNA for sequencing (data not shown), which positively identified the polymorphisms.

An alternative continuous assay for amyloid formation was also used to investigate the oligomerization of the M and V forms of the protein. A key element of prion-fibrilization

![Fig. 3. Binding of copper(II) ions to the M and V forms of recombinant human PrP. The binding of copper ions was monitored by the quenching of intrinsic tryptophan fluorescence. Results are shown for M129 (open symbols) and V129 (filled symbols) at 200 $\mu$M glycine (squares) and 1 mM glycine (circles). The lines superimposed upon the data represent a fit to equation (2), as defined in Methods.](image)

![Fig. 4. Protease resistance of $\alpha$- and $\beta$-conformational isoforms of M129 and V129 human PrP. Using identical conditions for digestion, soluble $\beta$-PrP has increased resistance to proteinase K (PK) when compared with $\alpha$-PrP. The M and V polymorphs have indistinguishable levels of resistance and display similar patterns of cleavage products. The concentrations of PK indicated are the final concentrations in the digestion reactions. Digestion was carried out at 37 °C for 1 h.](image)
Amyloid formation from PrP polymorphs

**Fig. 5.** Discontinuous monitoring of amyloid formation by the M129 and V129 forms of recombinant human PrP. The formation of amyloid fibrils as reported by thioflavin T fluorescence is shown as a function of time. Fibrillogenesis of M129 PrP (●) occurs after a mean lag time of 38 h, whereas V129 PrP at the same concentration (▲) and twice the concentration (△) shows no evidence of fibril formation, even after 200 h incubation. The data shown are the mean of three reactions ±SD. Five such datasets were collected to calculate the mean lag time described in Results. The line superimposed upon the data represents a best fit to equation (4), as defined in Methods.

**DISCUSSION**

This study of the physical behaviour of the methionine and the valine forms of human prion protein was stimulated by the dramatic effect of the codon 129 variation on both susceptibility to, and phenotype of, human prion disease. This effect can be explained by the principle of molecular complementarity combined with a gene-dosage effect (Palmer et al., 1991). That is, if there is a spontaneous conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> and only identical molecules can be recruited in the propagation process, then a homozygote will have a double dose of available substrate; therefore, the probability of initiation and the rate of propagation will be enhanced. Moreover, recent work has demonstrated that BSE and vCJD prion infection in transgenic mice can result in the propagation of distinct molecular and neuropathological phenotypes dependent on host PrP residue 129 status (Asante et al., 2002; Wadsworth et al., 2004). These data were interpreted as revealing a critical role for codon 129 in governing the thermodynamic balance between human PrP<sup>Sc</sup> conformations that determine strain type and gave rise to a conformational-selection model of prion-transmission barriers (Collinge, 1999, 2001).

Residue 129 polymorphism could have an indirect effect upon prion propagation by altering the biological function of PrP<sup>C</sup>. Although the precise function of PrP<sup>C</sup> remains enigmatic, it is known to bind copper(II) ions with high affinity, but we found no difference in the ability of M129 or V129 to bind copper. Our previous work has shown that the M/V variation has no measurable effect on the global structure or dynamics of the normal cellular conformer (PrP<sup>C</sup>) and thus we concluded that the powerful effect of residue 129 on prion-strain selection and propagation is likely to be mediated by its effect on the conformation of PrP<sup>Sc</sup>, its precursors or on the kinetics of their formation (Hosszu et al., 2004).

**Fig. 6.** Automated, continuous monitoring of amyloid formation. The formation of amyloid fibrils as reported by thioflavin T fluorescence is shown as a function of time. Fibrillogenesis of M129 PrP (filled circles) occurs after a mean lag time of 6·8 h, whereas V129 PrP at the same concentration (open circles) displays a mean lag time of 14·5 h. The data shown are the mean reading from six reactions ±SD. Three such datasets were collected to calculate the mean lag time described in Results. The line superimposed upon the data represents a best fit to equation (4), as defined in Methods.
This finding prompted us to examine the effect of the polymorphism on an alternative β-sheet-dominated fold of human PrP, known as β-PrP (Jackson et al., 1999). At low ionic strength, the β-PrP species is a soluble monomer composed almost entirely of β-sheet, as determined by both CD and Fourier-transform infrared spectroscopy. NMR measurements show this species to be molten globule-like with respect to the dynamics of the side chains (Jackson et al., 1999). At higher ionic strength or on prolonged incubation under acidic conditions, this conformer assembles into fibrils with partial resistance to digestion with PK, characteristic determinants of PrPSc. The data that we present here show that there was no difference in the ability of the M and V forms to convert from the α-helical PrPC conformation to the β-PrP isoform. Further, an examination of CD spectra, equilibrium unfolding and protease resistance of β-PrP in the M and V forms failed to reveal any difference in global conformation, stability or accessibility to enzymic cleavage.

However, the polymorphic forms showed a striking difference in their propensity to form ordered amyloid fibrils. By using the existing protocols originally developed by Baskakov et al. (2002), the M129 version of partially denatured α-PrP formed fibrils readily, with a mean lag time of 38 h as measured by thioflavin T fluorescence; a finding that was in very close agreement with previous work (Baskakov, 2004). In stark contrast, V129 did not form amyloid, even after more than 200 h incubation at twice the protein concentration. This finding appeared to conflict with a previous report (Baskakov et al., 2005) that the valine polymorphism polymerized with a shorter lag time, with M129 being more prone to form unstructured oligomers (Tahiri-Alaoui et al., 2004). This discrepancy could be due to the presence of the polyhistidine tag that the authors did not remove before study. An alternative explanation could be that the precise nature of the assay conditions may select for the more rapid assembly of one polymorph over another.

To determine whether lag times were dependent upon the type of assay and conditions used, an alternative continuous system of measuring thioflavin T fluorescence was employed. Although this system reported different absolute lag times, the V129 polymorph still consistently displayed an increased lag phase compared with M129. Thus, we conclude that the human M/V polymorphism acts by influencing the kinetics of amyloid formation.

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REFERENCES


