THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 30, Issue of July 23, pp. 31390–31397, 2004

Printed in U.S.A.

Methionine 129 Variant of Human Prion Protein Oligomerizes More Rapidly than the Valine 129 Variant

IMPLICATIONS FOR DISEASE SUSCEPTIBILITY TO CREUTZFELDT-JAKOB DISEASE*[S]

Received for publication, February 17, 2004, and in revised form, May 4, 2004
Published, JBC Papers in Press, May 6, 2004, DOI 10.1074/jbc.M401754200

Abdessamad Tahiri-Alaoui‡§, Andrew C. Gill¶‖, Petra Disterer‡, and William James‡**

From the ‡Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, United Kingdom and the ¶Institute for Animal Health, Compton, Berkshire, Newbury RG20 7NN, United Kingdom

The human PrP gene (*PRNP*) has two common alleles that encode either methionine or valine at codon 129. This polymorphism modulates disease susceptibility and phenotype of human transmissible spongiform encyphalopathies, but the molecular mechanism by which these effects are mediated remains unclear. Here, we compared the misfolding pathway that leads to the formation of β-sheet-rich oligomeric isoforms of the methionine 129 variant of PrP to that of the valine 129 variant. We provide evidence for differences in the folding behavior between the two variants at the early stages of oligomer formation. We show that Met^{129} has a higher propensity to form β-sheet-rich oligomers, whereas Val^{129} has a higher tendency to fold into α-helical-rich monomers. An equimolar mixture of both variants displayed an intermidate folding behavior. We show that the oligomers of both variants are initially a mixture of α- and β-rich conformers that evolve with time to an increasingly homogeneous β-rich form. This maturation process, which involves no further change in proteinase K resistance, occurs more rapidly in the Met^{129} form than the Val^{129} form. Although the involvement of such β-rich oligomers in prion pathogenesis is speculative, the misfolding behavior could, in part, explain the higher susceptibility of individuals that are methionine homozygote to both sporadic and variant Creutzfeldt-Jakob disease.

The transmissible spongiform encephalopathies are a group of fatal, neurodegenerative disorders that affect humans and animals and are believed to be caused by a novel class of infectious pathogen, the prion (1, 2). These diseases have attracted considerable interest not only because of their unique biology but also because of the appearance of new variant of Creutzfeldt-Jakob disease (vCJD)[1] (3), which appears to be caused by dietary exposure to the causative agent of bovine spongiform encephalopathy (4, 5). At the heart of disease pathogenesis lies a poorly understood structural rearrangement of PrP, a host-encoded glycoprotein of the nervous and lymphoid systems. The normal cellular form of the prion protein (PrP^{C}) undergoes a conversion that leads to the accumulation of an abnormal, conformationally altered isoform (PrP^{Sc}). According to the "protein-only" hypothesis of prion propagation, PrP^{Sc} is the principal or sole component of transmissible prions (6). PrP^{Sc} differs from PrP^{C} by increased β-sheet content, increased resistance to proteinase K, and an oligomeric rather than a monomeric state (7).

The human PrP gene (*PRNP*) exists in two major allelic forms that encode either methionine or valine at codon 129, with allele frequencies of 0.63 and 0.37 in western European and American populations, respectively (8, 9). This polymorphism is a key determinant of susceptibility to sporadic (10) and acquired (8, 11) prion diseases and may affect age at onset (12–14). Based on the analysis of 300 sporadic CJD subjects, Parchi *et al.* (14) identified six distinct clinicopathological variants of sCJD, which appeared to be specified largely by the genotype at codon 129 and the physiochemical properties of PrP^{Sc}. More recently, an extensive analysis, which included the metal ion-dependent conformation of PrP^{Sc}, of a large number of sCJD cases showed that PrP^{Sc} types are associated with the residue encoded at codon 129, the duration of illness, and with neuropathological phenotype (15). PrP^{Sc} types 1 and 4 have so far been detected only in Met^{129} homozygotes, type 4 being uniquely associated with vCJD, type 3 in cases containing a Val^{129} allele, and type 2 in any *PRNP* codon 129 genotype (16). The codon 129 polymorphism seemed to modulate the pattern of neuropathology and the extent of lesions in sCJD, as shown in a study that involved 70 patients who died in France between 1994 and 1998 (17). Codon 129 polymorphism also has epistatic effects on the phenotypic effects of mutations elsewhere in the prion gene. For example, the $Asp^{178} \rightarrow Gln$ mutation combined with a methionine at position 129 results in fatal familial insomnia (18). In contrast, the same mutation with a valine encoded at position 129 results in familial CJD (19). Similarly, Val^{129} homozygotes in association with the $Phe^{198} \rightarrow Ser$ mutation predispose patients to the Indiana kindred variant of Gerstmann-Straussler-Scheinker disease (20). Furthermore, an increased prevalence of genotype Val/Val at the polymorphic site 129 has been described in patients with early onset Alzheimer's disease (21) and a shift in cognitive decline toward early age in carriers of *PRNP*129 Val/Val has been reported recently (22).

Despite the clear importance of the polymorphism at codon 129 in the *PRNP* gene and its link to disease susceptibility and pathogenesis, the molecular mechanisms by which these effects

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4.

§ University Research Lecturer supported by the Biotechnology and Biological Sciences Research Council (BBSRC), UK. To whom correspondence should be addressed. Tel.: 44-1865-275545; Fax: 44-1865-285756; E-mail: abdou.tahiri-alaoui@path.ox.ac.uk.

‖ Supported by the BBSRC, UK.

** Fellow of Brasenose College, Oxford, and supported by the BBSRC, UK.

[1] The abbreviations used are: vCJD, variant of CJD; CJD, Creutzfeldt-Jakob disease; HPLC, high performance liquid chromatography; SEC, size exclusion chromatography; RP, reversed-phase; PK, proteinase K.

are mediated remain unclear. The *in vitro*, thermodynamic stability of recombinant PrP is not affected by the Met^{129} → Val mutation or by other substitutions related to inherited human prion diseases (23). Structural studies, however, have shown evidence for hydrogen bonding between residues 128 and 178, which might provide a structural basis for the highly specific influence of polymorphism in position 129 on disease phenotype that segregates with Asp^{178} → Gln (24). Molecular dynamic simulations of low pH-induced conformational conversion of PrP has provided further clues as to the role of residue 129 (25). In these simulations, Met^{129} seems to interact with Val^{122} leading to the recruitment of more N-proximal residues into an expanding β-sheet. As a complementary approach, we have chosen to study the folding properties of the two PrP allelomorphs experimentally by examining the misfolding pathway that leads to the formation of β-sheet-rich oligomeric isoforms (26). We show that PrP-Met^{129} has a higher propensity to form β-sheet-rich oligomers, whereas PrP-Val^{129} has a higher tendency to fold into α-helical-rich monomers. We also provide evidence that the dynamics of maturation of the oligomers differ between the two variants. The maturation process occurs over time at the expense of the α-helical-like monomers at a faster rate for PrP-Met^{129} than for PrP-Val^{129}. Once the oligomers from either allelomorph have been formed they show similar proteinase K resistance that does not change throughout the oligomer maturation process. The observed differences in the misfolding of PrP-Met^{129} and PrP-Val^{129} could explain the high susceptibility of individuals that are methionine homozygote to sporadic as well as variant CJD.

MATERIALS AND METHODS

Cloning of PrP Genes and Protein Purification—Genomic DNA encoding methionine/valine at codon 129 of *PRNP* gene (18) was extracted from the blood of a heterozygote individual using standard phenol-chloroform methods. The fragment of the *PRNP* gene spanning codon 90 through 231 was amplified with the oligonucleotide primers 5′-cgggatcccatgcaaggaggtggcacccacagtcagtggaacaagccg-3 (and 5′-cccaagctcatgctcgatcctctctggtaataggcctgag-3′ in 100 μl of PCR buffer that contained 300 ng of each DNA, 2 units of *Taq* DNA polymerase, 200 μM concentration of each dNTP, 0.2 μM concentration each primer, 3.5 μM $MgCl_2$, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 50 mM KCl. The following PCR conditions were used: an initial denaturation (95 °C, 3 min) followed by 25 cycles of denaturation (95 °C, 1 min), annealing (55 °C, 1 min), extension (72 °C, 1 min), and a final elongation (72 °C, 8 min). Restriction sites BamHI and HindIII, including extra nucleotides for efficient cleavage close to the ends, were introduced in the primers. The PCR fragment was cloned into the pTrcHis2B vector that incorporated a C-terminal His tag (Invitrogen, Paisley, UK) according to the manufacturer's instructions. The identity of human PrP clones was confirmed by sequencing with the BigDye Terminator v3.0 on ABI-Prism 3100 Genetic Analyzer (Applied Biosystems). The clones corresponding to PrP$^{90–231}$ with Met^{129} or Val^{129} were identified by comparison with human PrP clones available in the databases (GenBank™ accession numbers: M13667 and P04156). *Escherchia coli* expression and purification of recombinant human PrP was performed as described previously (27) except that the buffer used to solubilize inclusion bodies did not contain dithiothreitol. Stocks of highly purified proteins were stored in 6 M guanidine hydrochloride, 50 mM Tris-HCl, pH 7.2.

Oligomer Formation by HPLC Gel Filtration and Dialysis Methods—Rapid refolding of proteins into oligomeric isoforms was carried out at concentrations of 10 and 30 mg/ml. PrP-Met^{129} and PrP-Val^{129} were denatured in 6 M guanidine hydrochloride, 50 mM Tris-HCl, pH 7.2, and were injected (100 μl) onto a size exclusion chromatography (SEC) column (TSK®-Gel SWXL G3000 HPLC column, 7.8 × 300 mm, Phenomenex, Macclesfield, UK), equilibrated in 20 mM sodium acetate, 0.2 M NaCl, pH 3.7, 1 M urea, and 0.02% sodium azide (26). This rapid oligomer formation on SEC-HPLC corresponded to time 0 as opposed to time course analysis of oligomer formation during dialysis. This time course analysis of oligomer formation was carried out as follows. 1 ml of 6 M guanidine hydrochloride-denatured proteins (10 or 30 mg/ml) was dialyzed at room temperature against 2 liters of 20 mM sodium acetate, 0.2 M NaCl, pH 3.7, 2 M urea by use of a Slide-A-Lyser dialysis cassette (Perbio Science UK Ltd., Tattenhall, UK) with a 10-kDa cutoff. Aliquots were withdrawn, after carefully shaking the cassette to ensure homogeneity, after 30 min and 2, 4, and 24 h and analyzed by SEC as described above. Protein peaks were manually collected for subsequent analysis. Circular dichroism was carried as described previously (28) to assess the secondary structure of the protein.

Analytical Reversed-phase HPLC—Protein fractions collected during SEC analysis were kept in SEC elution buffer at room temperature and analyzed after 2, 12, 30, 110, 140, 200, and 300 days by analytical reversed-phase (RP) HPLC (Sephasil® C4, 5 μm, 4.6 × 250 mm) (Amersham Biosciences, Little Chalfont, UK). Proteins were eluted with a linear gradient of H_2O + 0.1% trifluoroacetic acid to 95% acetonitrile + 0.09% trifluoroacetic acid over 25 min.

All HPLC separations were performed at room temperature with a flow rate of 1 ml/min by means of a PerkinElmer Life Sciences HPLC system composed of a Binary LC pump 250 and a diode array detector 235C controlled by Total Chrome software version 6.2 (PerkinElmer Life Sciences, Seer Green, UK) through a PE Nelson 600 series link. The eluent was monitored by UV absorption at 280 nm.

Proteinase K Digestion—Recombinant human PrP collected from SEC-HPLC was subjected to proteinase K digestion after 2, 12, and 140 days maturation of the oligomers. The solution was incubated at 37 °C with a ratio of 1:24 PK to protein in 20 mM sodium acetate, 0.2 M NaCl, pH 5.5. Aliquots of the digests were taken after 15, 30 and 60 min and snap-frozen for analysis by SDS-PAGE and mass spectrometry.

On-line Capillary HPLC Nanospray Mass Spectrometry—Mass spectrometry was carried out on a Quattro II tandem quadrupole mass spectrometer (Micromass UK Ltd., Altrincham, UK) equipped with on-line capillary HPLC as detailed in Ref. 29. Briefly, the capillary HPLC was 180 μm inner diameter and was packed with 3.5-μm Jupiter C18 resin (Phenomenex, Macclesfield, UK). A flow rate of 1 μl/min was used, and proteins were eluted with a gradient from 0–70% solvent A to B, where solvent A was 95:5 water:acetonitrile with 0.05% trifluoroacetic acid, and solvent B was 95:5 water:acetonitrile with 0.05% trifluoroacetic acid. The eluent was passed directly to the mass spectrometer, which was operated in continuous flow nanospray mode. Full scan mass spectra (*m/z* 300–2100) were acquired every 5 s.

RESULTS

Characterization of Recombinant Human PrP-Met^{129} *and PrP-Val*^{129}—Recombinant human prion protein variants, HuPrP$^{90–231}$ Met^{129} and HuPrP$^{90–231}$ Val^{129}, were purified to homogeneity by immobilized metal affinity chromatography (see supplementary Fig. 1) followed by RP-HPLC. Purified proteins were analyzed by mass spectrometry to confirm purity and identity. Omitting dithiothreitol from the buffer used to solubilize the inclusion bodies and allowing disulfide bond to form in the oxidizing environment of the immobilized metal affinity chromatography was found to yield a fully oxidized protein (see supplementary Fig. 1) that has characteristics of PrP^{C}, such as a monomeric state, high α-helical content, and proteinase K sensitivity. All the studies presented in this paper were performed on proteins that included a C-terminal His tag.

PrP-Met^{129} *Has an Intrinsically Higher Propensity to Oligomerize than PrP-Val*^{129}—To assess the effect of the codon 129 polymorphic residue on the formation of non-native isoforms, both protein variants were denatured in 6 M guanidine hydrochloride, 50 mM Tris-HCl, pH 7.2, and allowed to fold under conditions favoring the formation of β-oligomer species (26). To dissect the *in vitro* folding pathway of prion protein misfolding we used SEC, dialysis, and CD. The denatured proteins (30 mg/ml) were injected onto a SEC column that had previously been equilibrated in 20 mM sodium acetate, 0.2 M NaCl, pH 3.7, 1 M urea, and 0.02% sodium azide and eluted with the same buffer. The elution profile of the Met^{129} variant shows two major peaks and one minor peak (Fig. 1*A*). Peak I (terminology as given by Baskakov *et al.* (26)) eluted at 5.68 min and corresponded to high molecular weight aggregates (Fig. 1*A*). Peak II eluted at 6.54 min and corresponded to the oligomeric isoform, while peak III, which eluted at 9.08 min, had a similar retention time to monomeric protein (Fig. 1*A*). The elution profile of Val^{129} variant shows the same three peaks but in different proportions, with peak II eluting as a shoulder to peak I (Fig.

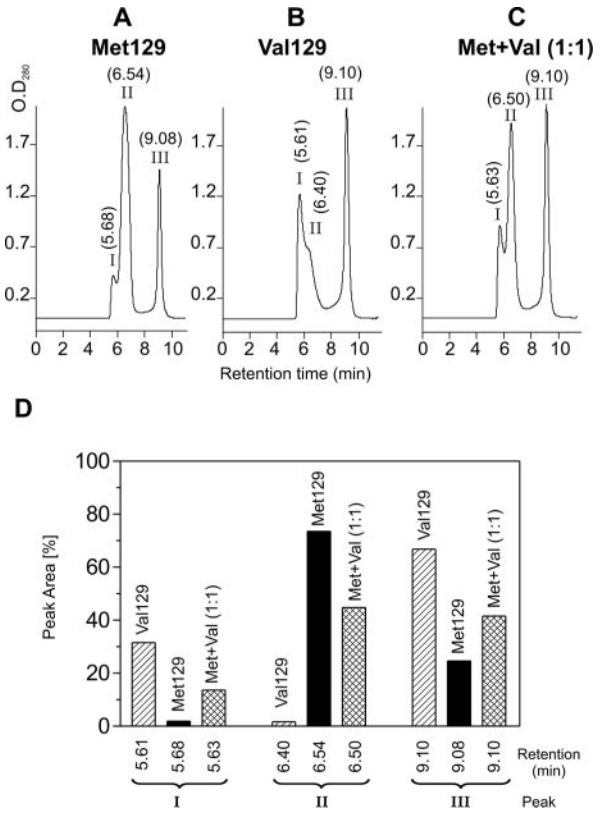

FIG. 1. **Rapid refolding of recHuPrP[90–231] with C-terminal His tag from denatured/unfolded state by SEC-HPLC under conditions that favored oligomers formation.** recHuPrP[90–231] Met[129] (*A*), recHuPrP[90–231] Val[129] (*B*), and an equimolar mixture (1:1) of Met[129] and Val[129] in 6 M guanidine hydrochloride, 50 mM Tris-HCl, pH 7.2, at 30 mg/ml (*C*) were injected onto SEC and eluted with the equilibration buffer (20 mM sodium acetate, 0.2 M NaCl, pH 3.7, 1 M urea, and 0.02% sodium azide). *D*, the areas under the peaks were analyzed using Total Chrome software 6.2 and the data used to construct the plot allowing a direct comparison of the proportions of the peaks eluted from SEC between Met[129] and Val[129] variants.

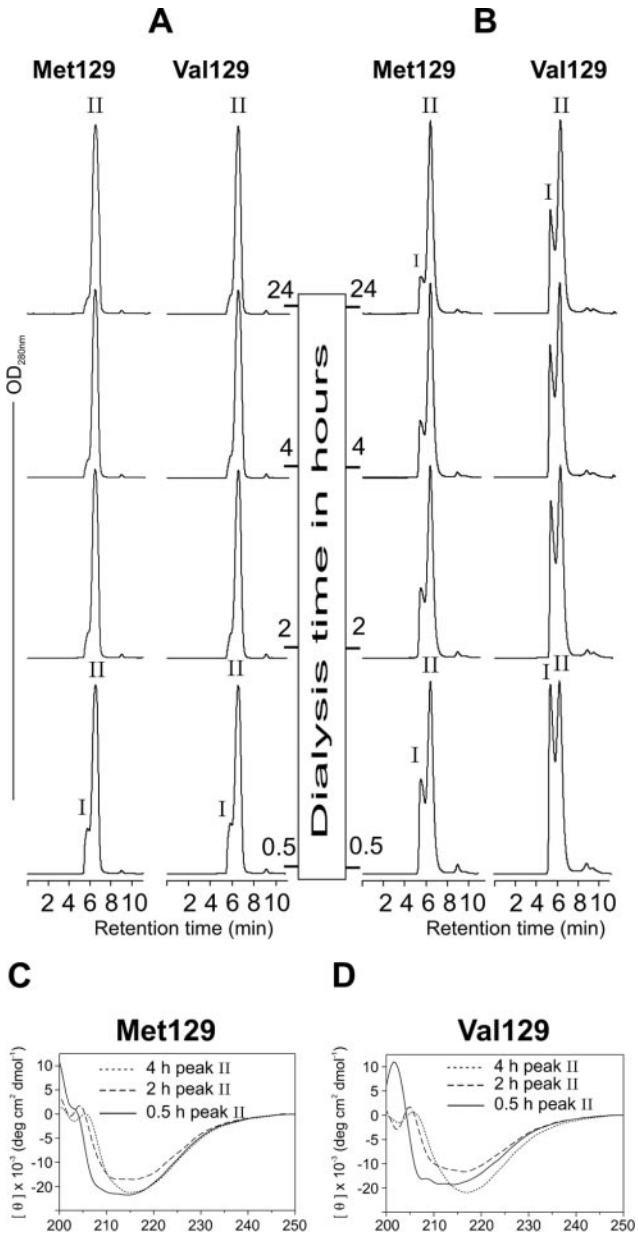

FIG. 2. **SEC profiles of fractions of recHuPrP[90–231] during time course refolding into oligomer isoforms using dialysis method.** *A* and *B* are chromatograms of recHuPrP[90–231] Met[129] and Val[129] at 30 and 10 mg/ml, respectively. Proteins in 6 M guanidine hydrochloride, 50 mM Tris-HCl, pH 7.2, were dialyzed against 20 mM sodium acetate, 0.2 M NaCl, pH 3.7, 2 M urea, and aliquots were withdrawn from the dialysis cassette at the indicated time then injected onto SEC previously equilibrated in 20 mM sodium acetate, 0.2 M NaCl, pH 3.7, 1 M urea, 0.02% sodium azide. *C* and *D* are CD spectra of Met[129] and Val[129], respectively, recorded at the indicated times after manual collection of the oligomer fraction that eluted in peak II from samples in *A*.

1*B*). To mimic a heterozygote situation of an individual that carries both alleles, we have analyzed the folding behavior in a 1:1 mixture of Met[129] and Val[129] (Fig. 1*C*). The elution profile of the equimolar mixture showed three peaks as seen before (Fig. 1*C*). Integration of the peaks in Fig. 1, *A* and *B*, revealed that less than 2% of the Val[129] variant had formed oligomers under these rapid refolding conditions (within the first 10 min of the SEC elution) as compared with more than 70% in the case of Met[129] (Fig. 1*D*). In addition, the percentage of monomeric population that was formed within 10 min was calculated to be about 66% for the Val[129] variant but only 24% for the Met[129] variant (Fig. 1*D*). Integration of the peaks in the equimolar mixture showed that the percentage of oligomeric form was reduced to about 44%, corresponding to ~30% reduction in the amount of oligomers as compared with the situation of the Met[129] alone (Fig. 1*D*). Also the amount of the monomeric population in the mixture 1:1 increased by about 17% when compared with that of the Met[129] alone (Fig. 1*D*). The monomeric forms of Met[129] and Val[129] variants yielded CD spectra with two minima at 208 and 222 nm, indicative of a predominantly α-helical conformation (data not shown). The CD spectra of protein from peak II of PrP-Met[129] and peaks I and II of PrP-Val[129] did not give clear information about the protein conformations but suggested that these fractions were composed of a mixture of α-helical and β-sheet forms.

To investigate the formation and evolution of the oligomers that eluted in peak II in more detail, we used a slower dialysis method of refolding that allowed analysis at different times after initiation of the refolding. Two protein concentrations, 30 and 10 mg/ml, were analyzed in parallel. The composition of the dialysis buffer was the same as that used in SEC elution buffer, except that, to maximize the formation of oligomers, the urea concentration was increased to 2 M (28). After 30-min refolding, and at 30 mg/ml, both allelomorphs adopted predominately oligomeric forms (peak II) with virtually no monomeric protein present (peak III); this elution profile did not change over the course of the experiment (Fig. 2*A*). Similar results were obtained with a mixture (1:1) of Met[129] and Val[129] (data not shown). The CD spectra of oligomers collected at 30 min

and 1 h showed that the population was not dominated by β-sheet-rich structures but contained some α-helical-rich protein (Fig. 2, *C* and *D*). However, by 4 h the CD spectra of the protein contained in peak II from both allelomorphs were typical of proteins possessing high amounts of β-sheet, and the spectra remained constant thereafter.

In contrast, at 10 mg/ml (Fig. 2*B*) the kinetics of oligomerization of PrP-Met^{129} were significantly different from those of the PrP-Val^{129}. The disappearance of aggregated proteins from peak I occurred more rapidly in the Met^{129} variant than in the Val^{129} variant, which suggests that, under oligomer-promoting conditions, the aggregated material from PrP-Met^{129} has a higher propensity to convert into oligomer than aggregated protein from PrP-Val^{129}. To test this, we took aggregated proteins from peak I of PrP-Met^{129} and PP-Val^{129} from SEC at time 0 and monitored their evolution over time by re-injecting them onto the same SEC. Time course comparison between the two allelomorphes clearly demonstrated the higher propensity of the Met^{129} variant to oligomerize than the Val^{129} variant (see supplementary Fig. 2). Virtually all aggregated PrP-Met^{129} converted into oligomeric isoforms over a period of 12 days, whereas a substantial amount of PrP-Val^{129} remained as aggregates. To investigate the behavior of monomeric species in peak III under the same oligomeric-promoting conditions, we compared the rate of oligomerization of the Met^{129} variant to that of the Val^{129} variant collected from SEC at time 0. Time course comparison between the two allelomorphs clearly revealed that monomeric Met^{129} has a higher propensity to oligomerize than monomeric Val^{129}. This difference in oligomerization rates was apparent after 2 days of incubation at room temperature (see supplementary Fig. 2).

From these data we conclude that, under these conditions, the Met^{129} variant has an intrinsically higher propensity to form oligomer isoforms than the Val^{129}. The high oligomerization propensity of the methionine variant becomes even more apparent under the highest protein concentration and rapid refolding conditions. Since this is true for denatured, aggregated, or natively folded monomeric protein, the higher propensity of oligomerization is clearly independent of the starting state of the protein.

The Oligomers of Both Met^{129} and Val^{129} Variants Are Conformationally Heterogeneous and Show Different Kinetics of Maturation—It has been shown that the oligomeric isoforms of mouse and Syrian hamster PrP are formed of populations of structurally heterogeneous proteins (26). RP-HPLC analysis showed that reduced forms of recombinant mouse PrP existed in multiple β-sheet-rich isoforms with distinct retention times (30, 31). Accordingly, we used RP-HPLC to investigate the effect of the residue encoded at codon 129 on the composition of the oligomer population that eluted from SEC in peak II. Aliquots of this fraction were incubated at room temperature in SEC elution buffer (20 mM sodium acetate, 0.2 M NaCl, pH 3.7, 1 M urea, and 0.02% sodium azide) and analyzed at various times (Fig. 3). After 2 days of incubation, the RP chromatogram of PrP-Met^{129} showed two peaks with distinct retention times (Fig. 3*A*); however, for PrP-Val^{129} only one major peak was observed (Fig. 3*B*). A second peak could be seen only after 12 days of incubation. The two peaks were designated IIa and IIb, with shorter and longer retention times, respectively. To rule out the possibility that the two peaks represented unexpected modification to the proteins, we analyzed them by mass spectrometry (Fig. 4). The deconvoluted mass spectra of PrP-Met^{129} IIa and PrP-Met^{129} IIb demonstrate that they represent proteins of the same molecular mass (Fig. 4, *A* and *B*). Similarly, the deconvoluted mass spectra of PrP-Val^{129} IIa and PrP-Val^{129} IIb showed that they also represented proteins with similar molecular masses (Fig. 4, *C* and *D*). From these analyses we infer that no covalent differences are evident and that the two peaks observed by RP-HPLC represent different conformations, with different amounts of hydrophobic residues exposed at the protein surface.

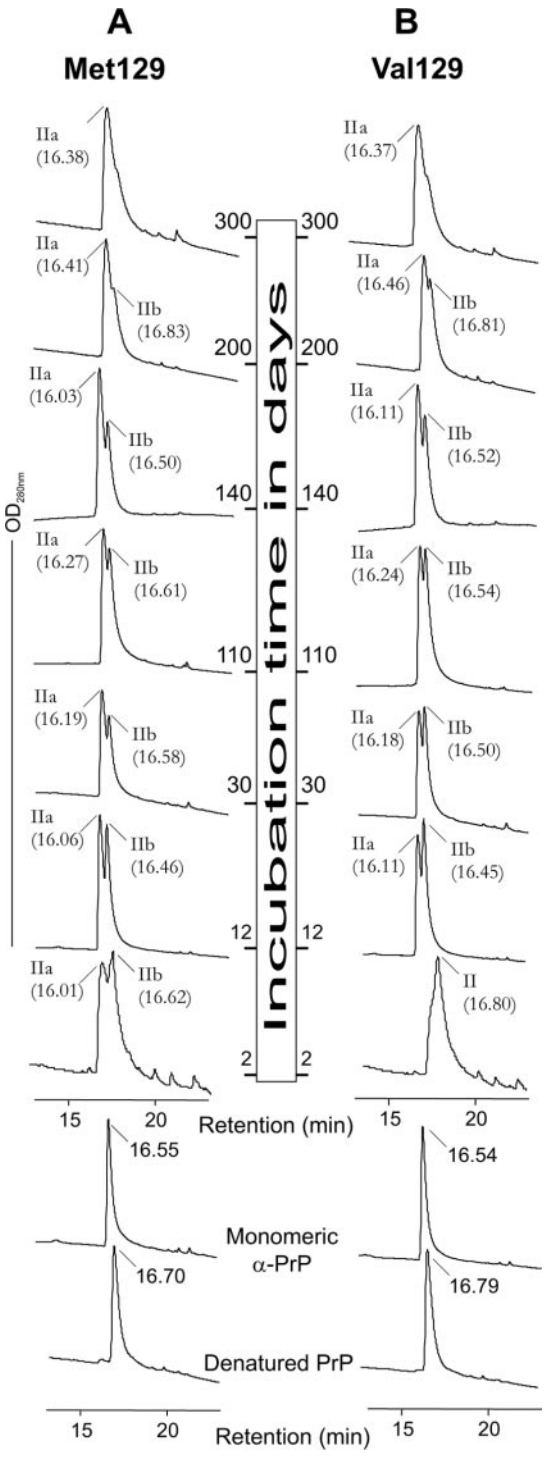

FIG. 3. **Time course analysis by RP-HPLC of oligomeric recHuPrP$^{90-231}$.** Aliquots of recHuPrP$^{90-231}$ Met^{129} (*A*) and recHuPrP$^{90-231}$ Val^{129} (*B*) that were refolded by dialysis into oligomer isoforms for 4 h then fractionated by SEC (see Fig. 2*A*) were incubated at room temperature in SEC elution buffer (20 mM sodium acetate, 0.2 M NaCl, pH 3.7, 1 M urea, 0.02% sodium azide) and anlalyzed by reversed-phase HPLC at the indicated times. The elution was done with a linear gradient of H_2O + 0.1% trifluoroacetic acid to 95% acetonitrile + 0.09% trifluoroacetic acid over 25 min. For direct comparison of retention times, monomeric α-helical and denatured (6 M guanidine hydrochloride) PrPs were analyzed by RP-HPLC under the same conditions.

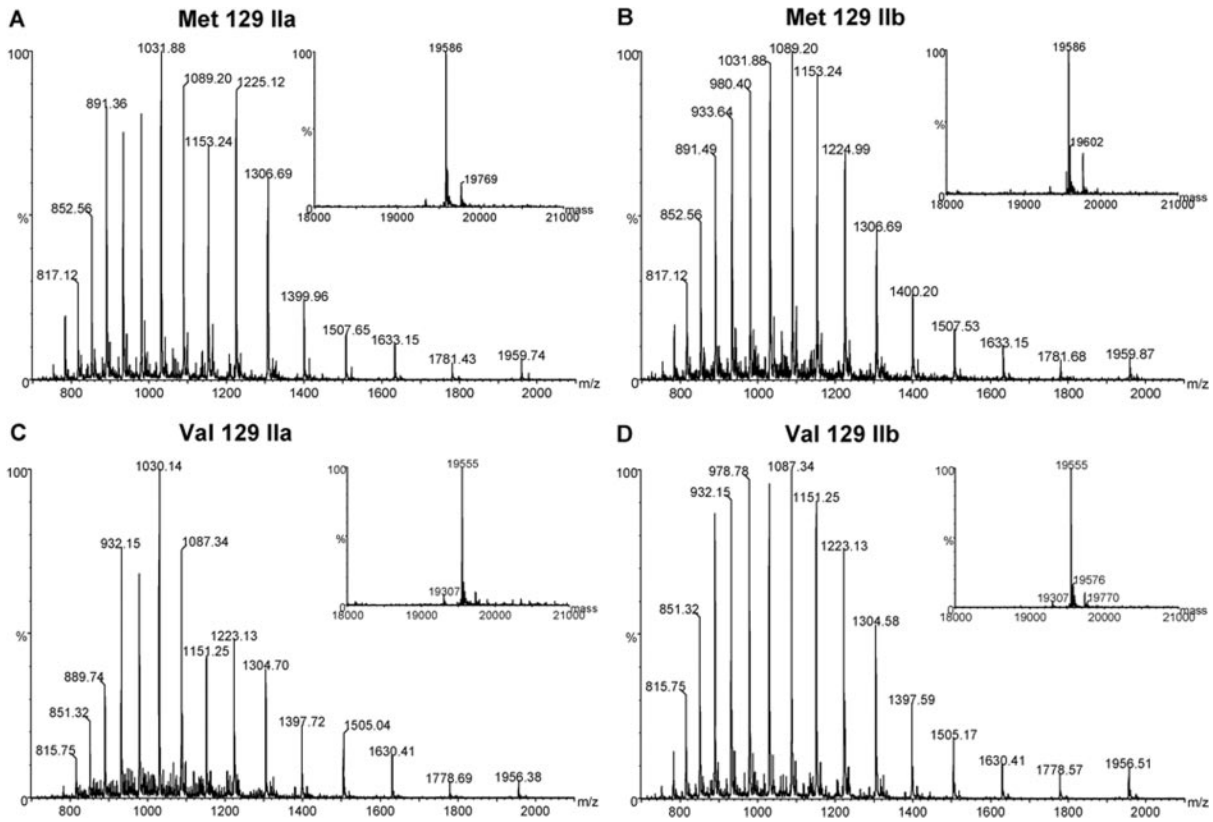

FIG. 4. **Nanospray mass spectrometry analysis of peaks IIa and IIb after 12 days of oligomerization.** Peaks IIa and IIb that were resolved by reversed-phase HPLC during the oligomerization of Met^{129} and Val^{129} were manually collected and immediately subjected to mass spectrometry analysis. *A* and *B* are electrospray mass spectra and deconvoluted (see *insets*) mass spectra of peaks IIa and IIb, respectively, of the Met^{129} oligomer. *C* and *D* are electrospray mass spectra and deconvoluted (see *insets*) mass spectra of peaks IIa and IIb, respectively, of Val^{129} oligomer.

Two important observations can be clearly made from reversed-phase chromatograms. First, the proportions of the two forms of PrP-Met^{129} and PrP-Val^{129} were different; the proportion of protein eluting in peak IIa was higher for PrP-Met^{129} after 12 days of incubation (Fig. 3, *A versus B*), while for PrP-Val^{129} there was more protein that eluted in peak IIb. Second, the proportions of the two peaks changed over time at different rates for Met^{129} and Val^{129} variants. In particular, the proportion of protein eluting in peak IIb decreased at a higher rate in the oligomer of PrP-Met^{129} (Fig. 3*A*) than in the oligomer of PrP-Val^{129} (Fig. 3*B*). Peak IIb of the oligomer from both allelomorphs seemed to have a retention time similar to that of the corresponding α-helical monomers. Because RP-HPLC uses differences in the hydrophobic properties to achieve separation between bio-molecules, it can be concluded that the protein population present in the highly retained peak IIb has more surface-exposed hydrophobic residues than the protein population present in peak IIa. Furthermore, the protein population present in peak IIb appears to undergo conversion to become recruited into the population that eluted in peak IIa. Over time, this conversion seemed to occur much quicker with PrP-Met^{129} than with PrP-Val^{129} (Fig. 3, *A versus B*).

Because of the long storage time of both allelomorphs in SEC elution buffer at room temperature, we have used mass spectrometry to assess the integrity of the samples (see supplementary Fig. 3). After 2 and 12 days of oligomerization, both allelomorphs had molecular masses that matched those predicted from the amino acid sequences. However, after 30 days of oligomerization, the deconvoluted mass spectra of both variants showed an additional species with a molecular mass that corresponded to the cleavage of the N-terminal two residues from the proteins (see supplementary Fig. 3). The two variants showed the same level of cleavage that increased over time to yield, 300 days later, proteins that were completely lacking two residues from the N terminus (see supplementary Fig. 3). The cleavage occurred in the peptide bond linking an aspartyl residue to a prolyl residue (Fig. 5*F*). The lability of Asp-Pro bonds under acidic conditions has been reported previously (32) and provides a simple explanation for our observation. The cleavage occurred at the same rate for both Met^{129} and Val^{129} variants and at a region of the N terminus that was PK-sensitive (see below) and, therefore, was unlikely to impact the differences that were observed during oligomerization of the two allelomorphs.

The Oligomers of Met^{129} and Val^{129} Show Similar Proteinase K Resistance—Limited PK digestion has been used widely to distinguish the disease isoform, PrP^{Sc}, from PrP^{C} as well as to probe differences between PrP^{Sc} strains (33, 34). We therefore used limited PK digestion in combination with on-line capillary HPLC mass spectrometry to probe differences between Met^{129} and Val^{129} variants during oligomerization. After treatment for up to 60 min with a 1:24 ratio of PK to protein, capillary HPLC mass spectrometry demonstrated that digestion of the oligomers from both allelomorphs yielded a range of short peptides that eluted before 20 min (*insets* in Fig. 5, *A* and *B*) that were liberated from the N- and C-terminal His tag regions of the protein. The central and C-terminal domains remained intact and eluted after 20 min as two peaks from the reversed-phase column (*insets* in Fig. 5, *A* and *B*). These polypeptides separated into two peaks that started either from residues 117 or 135, respectively, as a result of differential cleavage within the hydrophobic region (Fig. 5*F*). Comparison of deconvoluted mass spectra of polypeptides starting at 117 between PrP-Met^{129} (Fig. 5*A*) and PrP-Val^{129} (Fig. 5*B*) oligomers showed

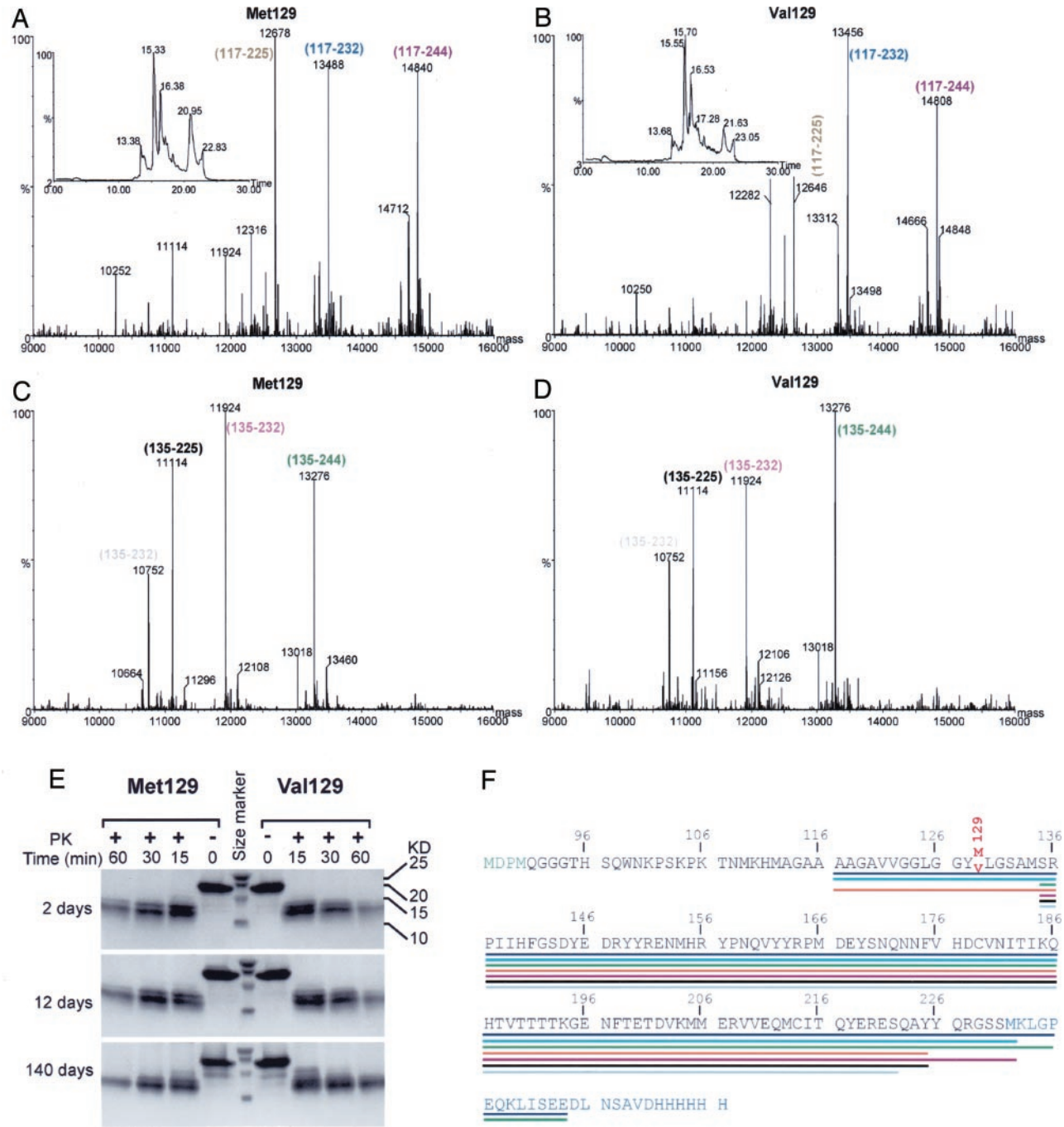

FIG. 5. **Nanospray mass spectrometry and SDS-PAGE analysis of the proteinase K digests from oligomeric recHuPrP$^{90-231}$.** *A*, deconvoluted mass spectrum of polypeptides eluting at 23.35 min from C-18 reversed-phase capillary HPLC (see *inset*) of Met^{129} oligomers. These correspond to residues 117–225 (12678 Da), 117–232 (13488 Da), and 117–244 (14840 Da). *B*, deconvoluted mass spectrum of polypeptides eluting at 22.90 min (see *inset*) of Val^{129} oligomers. These correspond to residues 117–225 (12 646 Da), 117–232 (13,456 Da), and 117–244 (14,808 Da). *C*, deconvoluted mass spectrum of polypeptides eluting at 21.33 min (see *inset* in *A*) of Met^{129} oligomers. These correspond to residues 135–222 (10752 Da), 135–225 (11,114 Da), 135–232 (11,924 Da), and 135–244 (13,276). *D*, deconvoluted mass spectrum of polypeptides eluted at 21.55 min (see *inset* in *B*) of Val^{129} oligomers. These correspond to residues 135–222 (10,752 Da), 135–225 (11,114 Da), 135–232 (11,924 Da), and 135–244 (13,276 Da). *E*, Coomassie-stained SDS-PAGE of limited proteinase K digests from Met^{129} and Val^{129} after 2, 12, and 140 days of oligomerization. The digestion was carried out at 37 °C at the indicated incubation times with a ratio 1:24 PK to protein. *F*, the PK-resistant fragments that were identified by mass spectrometry are overlaid on the amino acid sequence of human PrP Met/Val^{129}. The residues that were introduced into the recombinant HuPrP for cloning and purification purposes are indicated in *green* at the N terminus and in *blue* at the C terminus.

three major PK-resistant cores with matching cleavage sites for each variant. The differences in masses (32 Da) between these PK-resistant fragments were due only to the presence of methionine or valine at position 129. Similarly, the comparison of deconvoluted mass spectra of polypeptides that started at 135 between PrP-Met^{129} (Fig. 5*C*) and PrP-Val^{129} (Fig. 5*D*) oligomers revealed four major PK-resistant polypeptides with identical molecular masses for Met^{129} and Val^{129}. Most, if not all, of the C-terminal His tag remained PK-sensitive (Fig. 5*F*) during oligomerization, indicating that it was unlikely to interfere with the oligomerization process. SDS-PAGE separation of the PK digests at various times of oligomerization (Fig. 5*E*) provided additional evidence that oligomers from PrP-Met^{129} and PrP-Val^{129} have similar PK-resistant properties.

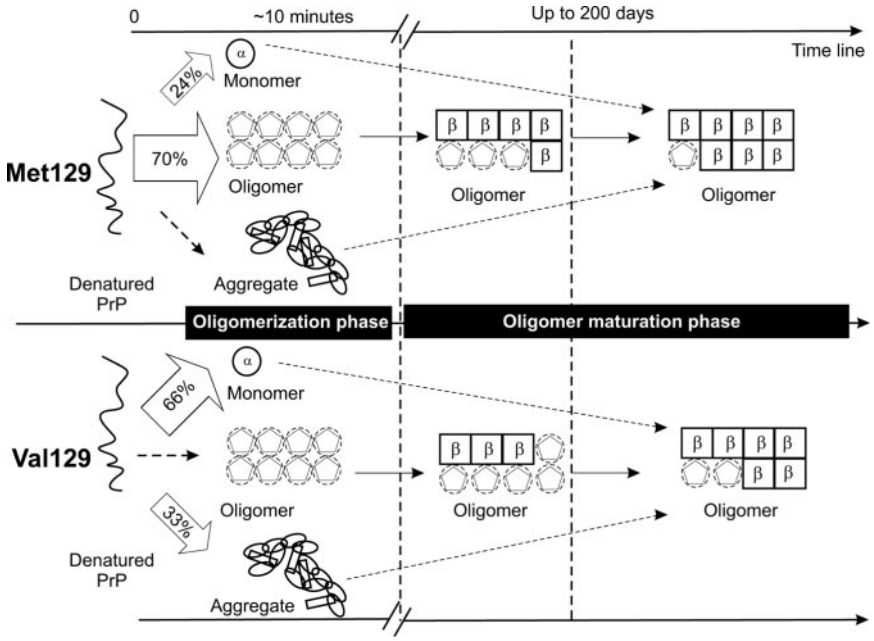

FIG. 6. **Schematic model illustrating *in vitro* formation and maturation of oligomer isoforms of recHuPrP$^{90-231}$ Met^{129} and Val^{129}.** Under appropriate conditions (low pH and high protein concentration) the acquisition of non-native form can happen from either denatured, aggregated, or native forms and involves two steps: a rapid oligomerization phase followed by a slow maturation phase characterized by a structural rearrangement of the oligomers. The presence of a methionine at codon 129 affects both the kinetics of oligomerization and those of the maturation phase.

DISCUSSION

In this work we show that the polymorphism at codon 129 affects the kinetics of oligomerization that leads to non-native isoforms of human prion protein. Starting from an unfolded state and when forced rapidly to adopt the oligomeric form under low pH and at high protein concentration, the Met^{129} variant clearly exhibited a preference for the oligomeric state, whereas the Val^{129} variant favored the α-helical-rich, monomeric state. The apparent molecular mass of the oligomers from both PrP variants was estimated to be around 400 kDa, close to that reported previously (26). We have ruled out the possibility that the denatured starting protein contained any oligomeric forms of PrP by SEC analysis under denaturing conditions using 6 M guanidine hydrochloride, 50 mM Tris-HCl, pH 7.2, as elution buffer. Indeed the chromatograms showed only a monomeric population of both PrP variants (see supplementary Fig. 4). How then does residue 129 influence this folding behavior? The first clue can be derived from the solution structure of human PrP determined by NMR (35). Residue 129 lies within the first β-strand (residues 128–131) in human PrP. Riek *et al.* (36) proposed that this short β-sheet might be a "nucleation site" for a conformational transition from PrP^{C} to PrP^{Sc} that could include the loops connecting the β-sheet to the first helix. Prion peptide models have also indicated the possible involvement of the region containing the first β-strand in the conformational switch of PrP (37, 38). To our knowledge there is no published structure of the Val^{129} variant of human PrP; however, molecular dynamic simulations of Syrian hamster PrP$^{90-231}$ at neutral pH showed that when a valine residue was present at position 129 instead of a methionine residue, the proportion of protein with a three-stranded, antiparallel β-sheet structure increased from 0 to 23% (39). The three β-strands spanned residues 115–116, 119–122, and 129–130 without affecting the thermodynamic stability of PrP, as was demonstrated experimentally (23). Molecular dynamic simulations of Syrian hamster PrP$^{109-219}$ at low pH revealed that regions around β-strand 1 and β-strand 2 were potential sites for conversion to β-structures (25). In particular, the first β-strand served as a structural nucleus for the formation of new, and propagation of existing, β-like structures (25). The interactions between Met^{129} and Val^{122} and between Tyr^{128} and Tyr^{162} illustrated the role of tertiary interactions between side chains nucleating secondary structure formation, as has been observed for helix formation in barnase (40).

Our experimental data show that, under conditions that favor rapid formation of oligomers, SEC can be used to partition protein folded into aggregated, monomeric and oligomeric states so that differences in folding of Met^{129} and Val^{129} variants become clear. It has been shown that folding of recombinant, murine prion protein into α-helix-rich conformation is extremely rapid (41) and is under kinetic control (28). We show in this work that the propensity of recombinant human prion protein to fold into monomeric α-helical-rich population is increased when residue 129 was occupied by a valine. However, when the same position is occupied by methionine a tendency for oligomerization can be observed, with comparatively little monomeric α-helical-rich population. These differences in folding behavior can be explained by a fundamental event in protein folding, which is driven by a small number of specific residues involved in the formation of a well defined, specific nucleus that then allows the rest of the structure to coalesce efficiently around it (42, 43). The high β-sheet propensity of valine residues as compared with methionine residues (44, 45) makes PrP in which valine is present at residue 129 more favorable to adopt the native local β-sheet that would temporarily stabilize the Val^{129} allelomorph in the monomeric α-helical PrP.

Differences between the oligomers formed from Met^{129} and Val^{129} could be seen when we explored the hydrophobicity of the oligomers as they evolved over time. These differences were consistent with early events that were observed during the rapid misfolding process. That is, the proportion of oligomer subunits with hydrophobic properties resembling those of the α-monomer was higher in PrP-Val^{129} than in the PrP-Met^{129}. Over the course of several weeks of incubation, the oligomerization process in both variants occurred at the expense of the α-helical-like population that became a substrate for oligomer recruitment (28, 46). The presence of the α-helical-like species in the oligomer reflected the heterogeneous nature of this non-native form of PrP. This finding was consistent with the previously published supra-molecular electrospray ionization mass spectrometry results (26). On the basis of these observations we propose a model (Fig. 6) that depicts the formation of non-native β-oligomers *in vitro*. Under appropriate conditions (low pH and high protein concentration) the acquisition of this non-native form can happen from either denatured, aggre-

gated, or native forms and involves two steps: a rapid oligomerization phase followed by a slow maturation phase characterized by a structural rearrangement of the oligomers. The presence of a methionine at codon 129 affects both the kinetics of oligomerization and those of the maturation phase.

A hallmark of the disease isoform, PrP^{Sc}, is its partial resistance to proteinase K digestion compared with normal cellular PrP. This property has also been used to probe differences between PrP^{Sc} conformations. We found that once oligomers from both allelomorphs are formed they show similar proteinase K resistance that does not change throughout the oligomer maturation process. This indicates that if one relies solely on PK digestion to investigate PrP conformations, one could miss the detection of dynamic conformational changes influenced by the polymorphism at codon 129 in human PrP during disease pathogenesis. A similar PK cleavage site (starting at residue 117) in the protease-resistant core of the oligomers from both human PrP variants has been reported for recombinant mouse $PrP^{23–231}$ (47). It is important to emphasize that this PK cleavage site is not the same as the termini around codon 90 found for authentic PrP^{Sc} (48).

It has been reported that two mutations in α-synuclein that are linked to early-onset Parkinson's disease can accelerate the oligomerization but not fibrilization suggesting that formation of nonfibrillar oligomer is likely to be critical in disease pathogenesis (49). There is no evidence so far that these β-oligomers exist *in vivo* or can directly cause prion diseases either alone or with the help of other factors. The high affinity and newly reported ligands that we have isolated in our laboratory (50, 51) would help investigating the presence *in vivo* of the β-oligomers. The observed differences in the misfolding behavior between Met^{129} and Val^{129} variants could provide a kinetic explanation to the observed high susceptibility of individuals that are methionine homozygote to sporadic as well as to variant CJD. The attempt to model a heterozygote situation by mixing Met^{129}/Val^{129} (1:1) and allowing the rapid refolding to occur has yielded some promising results in that the amount of oligomers formed was reduced by ~30% as compared with the homozoygote-like situation in the Met^{129} alone, indicating certain degree of inhibition of the oligomerization in the heterozygote.

Phylogenetically, methionine is the ancestral amino acid at codon 129 (52), and Val^{129} is a mutation found in humans. Worldwide *PRNP* haplotype diversity and coding allele frequencies suggest that strong balancing selection at this locus occurred during the evolution of modern human and made heterozygosity at *PRNP* a significant selective advantage (53). Our data could provide a mechanism based on prion protein folding properties as an additional selective pressure against, particularly, Met^{129} homozygosity.

REFERENCES

1. Prusiner, S. B. (1996) *Trends Biochem. Sci.* **21,** 482–487
2. Weissmann, C. (1996) *FEBS Lett.* **389,** 3–11
3. Will, R. G., Ironside, J. W., Zeidler, M., Cousens, S. N., Estibeiro, K., Alperovitch, A., Poser, S., Pocchiari, M., Hofman, A., and Smith, P. G. (1996) *Lancet* **347,** 921–925
4. Bruce, M. E., Will, R. G., Ironside, J. W., McConnell, I., Drummond, D., Suttie, A., McCardle, L., Chree, A., Hope, J., Birkett, C., Cousens, S., Fraser, H., and Bostock, C. J. (1997) *Nature* **389,** 498–501
5. Hill, A. F., Desbruslais, M., Joiner, S., Sidle, K. C., Gowland, I., Collinge, J., Doey, L. J., and Lantos, P. (1997) *Nature* **389,** 448–450 and 526
6. Prusiner, S. B. (1991) *Science* **252,** 1515–1522
7. Prusiner, S. B. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13363–13383
8. Collinge, J., Palmer, M. S., and Dryden, A. J. (1991) *Lancet* **337,** 1441–1442
9. Windl, O., Dempster, M., Estibeiro, J. P., Lathe, R., de Silva, R., Esmonde, T., Will, R., Springbett, A., Campbell, T. A., Sidle, K. C., Palmer, M. S., and Collinge, J. (1996) *Hum. Genet.* **98,** 259–264
10. Palmer, M. S., Dryden, A. J., Hughes, J. T., and Collinge, J. (1991) *Nature* **352,** 340–342
11. Brown, P., Cervenakova, L., Goldfarb, L. G., McCombie, W. R., Rubenstein, R., Will, R. G., Pocchiari, M., Martinez-Lage, J. F., Scalici, C., and Masullo, C. (1994) *Neurology* **44,** 291–293
12. Baker, H. E., Poulter, M., Crow, T. J., Frith, C. D., Lofthouse, R., and Ridley, R. M. (1991) *Lancet* **337,** 1286
13. Cervenakova, L., Goldfarb, L. G., Garruto, R., Lee, H. S., Gajdusek, D. C., and Brown, P. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13239–13241
14. Parchi, P., Giese, A., Capellari, S., Brown, P., Schulz-Schaeffer, W., Windl, O., Zerr, I., Budka, H., Kopp, N., Piccardo, P., Poser, S., Rojiani, A., Streichemberger, N., Julien, J., Vital, C., Ghetti, B., Gambetti, P., and Kretzschmar, H. (1999) *Ann. Neurol.* **46,** 224–233
15. Hill, A. F., Joiner, S., Wadsworth, J. D., Sidle, K. C., Bell, J. E., Budka, H., Ironside, J. W., and Collinge, J. (2003) *Brain* **126,** 1333–1346
16. Collinge, J., Sidle, K. C., Meads, J., Ironside, J., and Hill, A. F. (1996) *Nature* **383,** 685–690
17. Hauw, J. J., Sazdovitch, V., Laplanche, J. L., Peoc'h, K., Kopp, N., Kemeny, J., Privat, N., Delasnerie-Laupretre, N., Brandel, J. P., Deslys, J. P., Dormont, D., and Alperovitch, A. (2000) *Neurology* **54,** 1641–1646
18. Goldfarb, L. G., Petersen, R. B., Tabaton, M., Brown, P., LeBlanc, A. C., Montagna, P., Cortelli, P., Julien, J., Vital, C., Pendelbury, W. W., Haltia, M., Wills, P. R., Hauw, J. J., McKeever, P. E., Monari, L., Schrank, B., Swergold, G. D., Autilio-Gambetti, L., Gajdusek, D. C., Lugaresi, E., and Gambetti, P. (1992) *Science* **258,** 806–808
19. Goldfarb, L. G., Haltia, M., Brown, P., Nieto, A., Kovanen, J., McCombie, W. R., Trapp, S., and Gajdusek, D. C. (1991) *Lancet* **337,** 425
20. Dlouhy, S. R., Hsiao, K., Farlow, M. R., Foroud, T., Conneally, P. M., Johnson, P., Prusiner, S. B., Hodes, M. E., and Ghetti, B. (1992) *Nat. Genet.* **1,** 64–67
21. Dermaut, B., Croes, E. A., Rademakers, R., Van den Broeck, M., Cruts, M., Hofman, A., van Duijn, C. M., and Van Broeckhoven, C. (2003) *Ann. Neurol.* **53,** 409–412
22. Croes, E. A., Dermaut, B., Houwing-Duistermaat, J. J., Van den Broeck, M., Cruts, M., Breteler, M. M., Hofman, A., van Broeckhoven, C., and van Duijn, C. M. (2003) *Ann. Neurol.* **54,** 275–276
23. Liemann, S., and Glockshuber, R. (1999) *Biochemistry* **38,** 3258–3267
24. Riek, R., Wider, G., Billeter, M., Hornemann, S., Glockshuber, R., and Wuthrich, K. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 11667–11672
25. Alonso, D. O., DeArmond, S. J., Cohen, F. E., and Daggett, V. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 2985–2989
26. Baskakov, I. V., Legname, G., Baldwin, M. A., Prusiner, S. B., and Cohen, F. E. (2002) *J. Biol. Chem.* **277,** 21140–21148
27. Jackson, G. S., Hill, A. F., Joseph, C., Hosszu, L., Power, A., Waltho, J. P., Clarke, A. R., and Collinge, J. (1999) *Biochim. Biophys. Acta* **1431,** 1–13
28. Baskakov, I. V., Legname, G., Prusiner, S. B., and Cohen, F. E. (2001) *J. Biol. Chem.* **276,** 19687–19690
29. Gill, A. C., Ritchie, M. A., Hunt, L. G., Steane, S. E., Davies, K. G., Bocking, S. P., Rhie, A. G., Bennett, A. D., and Hope, J. (2000) *EMBO J.* **19,** 5324–5331
30. Lu, B. Y., Beck, P. J., and Chang, J. Y. (2001) *Eur. J. Biochem.* **268,** 3767–3773
31. Lu, B. Y., and Chang, J. Y. (2001) *Biochemistry* **40,** 13390–13396
32. Landon, M. (1977) *Methods Enzymol.* **47,** 145–149
33. Safar, J., Wille, H., Itri, V., Groth, D., Serban, H., Torchia, M., Cohen, F. E., and Prusiner, S. B. (1998) *Nat. Med.* **4,** 1157–1165
34. Bessen, R. A., and Marsh, R. F. (1994) *J. Virol.* **68,** 7859–7868
35. Zahn, R., Liu, A., Luhrs, T., Riek, R., von Schroetter, C., Lopez Garcia, F., Billeter, M., Calzolai, L., Wider, G., and Wuthrich, K. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 145–150
36. Riek, R., Hornemann, S., Wider, G., Billeter, M., Glockshuber, R., and Wuthrich, K. (1996) *Nature* **382,** 180–182
37. Nguyen, J., Baldwin, M. A., Cohen, F. E., and Prusiner, S. B. (1995) *Biochemistry* **34,** 4186–4192
38. Tahiri-Alaoui, A., Bouchard, M., Zurdo, J., and James, W. (2003) *Protein Sci.* **12,** 600–608
39. Santini, S., Claude, J. B., Audic, S., and Derreumaux, P. (2003) *Proteins* **51,** 258–265
40. Wong, K. B., Clarke, J., Bond, C. J., Neira, J. L., Freund, S. M., Fersht, A. R., and Daggett, V. (2000) *J. Mol. Biol.* **296,** 1257–1282
41. Wildegger, G., Liemann, S., and Glockshuber, R. (1999) *Nat. Struct. Biol.* **6,** 550–553
42. Matouschek, A., Kellis, J. T., Jr., Serrano, L., and Fersht, A. R. (1989) *Nature* **340,** 122–126
43. Vendruscolo, M., Paci, E., Dobson, C. M., and Karplus, M. (2001) *Nature* **409,** 641–645
44. Kim, C. A., and Berg, J. M. (1993) *Nature* **362,** 267–270
45. Fasman, G. D. (1989) *Trends Biochem. Sci.* **14,** 295–299
46. Baskakov, I. V., Aagaard, C., Mehlhorn, I., Wille, H., Groth, D., Baldwin, M. A., Prusiner, S. B., and Cohen, F. E. (2000) *Biochemistry* **39,** 2792–2804
47. Qin, K., Yang, D. S., Yang, Y., Chishti, M. A., Meng, L. J., Kretzschmar, H. A., Yip, C. M., Fraser, P. E., and Westaway, D. (2000) *J. Biol. Chem.* **275,** 19121–19131
48. Oesch, B., Westaway, D., Walchli, M., McKinley, M. P., Kent, S. B., Aebersold, R., Barry, R. A., Tempst, P., Teplow, D. B., Hood, L. E., Prusiner, S. B., and Weissmann, C. (1985) *Cell* **40,** 735–746
49. Conway, K. A., Lee, S. J., Rochet, J. C., Ding, T. T., Williamson, R. E., and Lansbury, P. T., Jr. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 571–576
50. Sayer, N. M., Cubin, M., Rhie, A., Bullock, M., Tahiri-Alaoui, A., and James, W. (2004) *J. Biol. Chem.* **279,** 13102–13109
51. Rhie, A., Kirby, L., Sayer, N., Wellesley, R., Disterer, P., Sylvester, I., Gill, A., Hope, J., James, W., and Tahiri-Alaoui, A. (2003) *J. Biol. Chem.* **278,** 39697–39705
52. Schatzl, H. M., Da Costa, M., Taylor, L., Cohen, F. E., and Prusiner, S. B. (1995) *J. Mol. Biol.* **245,** 362–374
53. Mead, S., Stumpf, M. P., Whitfield, J., Beck, J. A., Poulter, M., Campbell, T., Uphill, J. B., Goldstein, D., Alpers, M., Fisher, E. M., and Collinge, J. (2003) *Science* **300,** 640–643