Reversible Conversion of Monomeric Human Prion Protein Between Native and Fibrilogenic Conformations

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Prion propagation involves the conversion of cellular prion protein (PrP^C) into a disease-specific isomer, PrP^{Sc}, shifting from a predominantly α-helical to β-sheet structure. Here, conditions were established in which recombinant human PrP could switch between the native α conformation, characteristic of PrP^C, and a compact, highly soluble, monomeric form rich in β structure. The soluble β form (β-PrP) exhibited partial resistance to proteinase K digestion, characteristic of PrP^{Sc}, and was a direct precursor of fibrillar structures closely similar to those isolated from diseased brains. The conversion of PrP^C to β-PrP in suitable cellular compartments, and its subsequent stabilization by intermolecular association, provide a molecular mechanism for prion propagation.

Prion diseases are associated with the accumulation of a conformational isomer (PrP^{Sc}) of host-derived prion protein (PrP^C) with an increase in its β-sheet secondary structure content (*1*) and the acquisition of partial resistance to digestion with proteinase K (PK). According to the protein-only hypothesis, PrP^{Sc} is the principal or sole component of transmissible prions (*2*). Although the structure of PrP^C has been determined (*3*), the insolubility of PrP^{Sc}, which is isolated from tissue in a highly aggregated state, has precluded high-resolution structural analysis.

Human $PrP^{91–231}$ can be expressed in large amounts in *Escherichia coli* and purified as a highly soluble, monomeric protein with a single intact disulfide bridge (*4*). Analysis by circular dichroism (CD) spectropolarimetry revealed a structure rich in α-helical content (~50%) with little β sheet (~20%) (Fig. 1A). One-dimensional (1D) ^1H nuclear magnetic resonance (NMR) spectra (Fig. 1C) and two-dimensional ^1H-^{15}N correlation NMR spectra (*5*) of this material showed it to be conformationally similar to the mouse and hamster prion proteins (*3, 6*) and to another human $PrP^{90–231}$ construct (*7*).

Reduction of the disulfide bond in human $PrP^{91–231}$ and lowering the pH to 4.0, in a dilute acetate buffer without additives, generated a highly soluble protein that can be concentrated to at least 12 mg/ml. When subjected to gel filtration, it eluted as a monomeric species (Fig. 2). The CD signal in the amide region of the spectrum (Fig. 1A) suggests that this highly soluble, reduced species adopted a different conformation from the native cellular form of PrP (α-PrP). The native state is characterized by a strong α-helical signal, whereas the reduced form is qualitatively dominated by β sheet. This type of secondary structural transition occurs in proteins that go from a soluble monomeric state to an aggregated fibrous (amyloid) form in which β-structure is stabilized by intermolecular interactions (*8*). However, to our knowledge, it is unprecedented for a protein to undergo such an extensive β-sheet conversion while remaining in a monomeric state at

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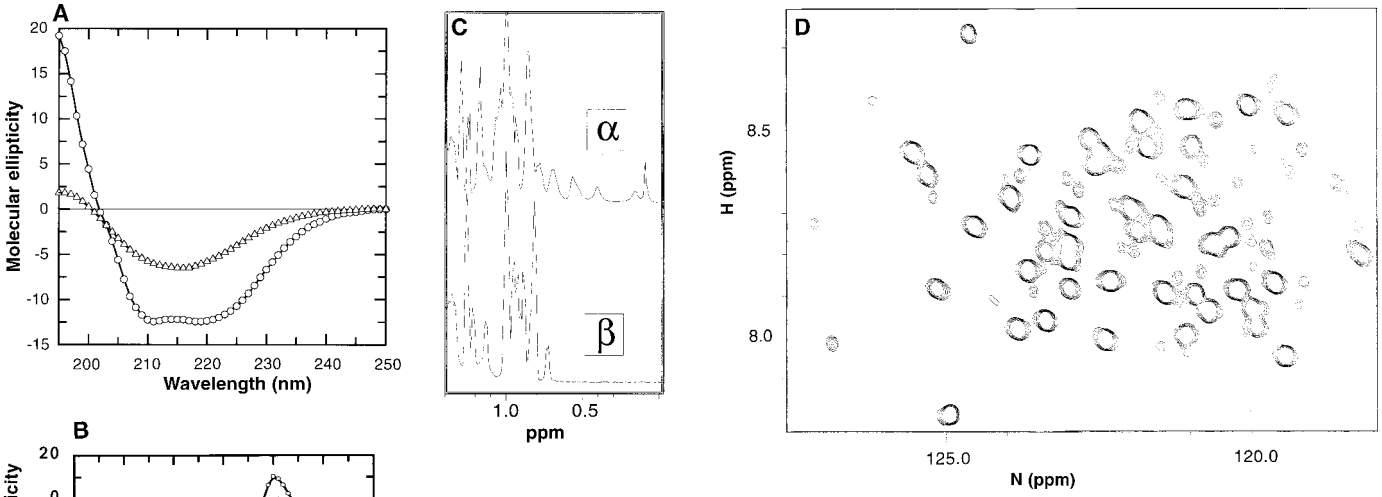


Fig. 1. Secondary and tertiary structure of the two human PrP isoforms (*26*). (**A**) Far-UV CD spectra (*27*). Oxidized human PrP (pH 8.0) (○) displays a typically α-helical spectrum. Reduced human PrP (pH 4.0) (△) displays a β-sheet spectrum with little or no helix present. (**B**) Near-UV CD spectra for oxidized human PrP (pH 8.0) (○), reduced human PrP (pH 4.0) (△), and denatured human PrP (□). The oxidized protein displays extensive tertiary organization, whereas denatured PrP lacks any distinct tertiary interactions. Reduced human PrP displays an intermediate level of tertiary organization. (**C**) ^1H NMR spectra of the upfield regions of the α and β forms of human $PrP^{91–231}$ (*28*). This includes the resonances at higher field than 0.7 ppm characteristic of strong tertiary interactions between methyl groups and aromatic rings in the α form. (**D**) Expanded region of a ^1H-^{15}N HSQC spectrum of the β form of human $PrP^{91–231}$ showing its chemical shift dispersion, which is much reduced relative to the α form (*7*).

high protein concentrations and in the absence of denaturants. This is in contrast to the folding intermediates of mouse $PrP^{121–231}$ (*9*) and human $PrP^{90–231}$ (*10, 11*). We also observe the equilibrium folding intermediate of α-PrP described in (*10*), but in contrast to β-PrP, this is poorly soluble and has an apparent molecular weight of 40 kD (Fig. 2) indicative of tertiary disorder and expanded molecular volume. In marked contrast, the folded β-PrP we observe is populated, and therefore stable, in the absence of denaturant with an apparent molecular volume indistinguishable from that of α-PrP (Fig. 2).

Using amide CD alone, we cannot be certain whether β-PrP is sufficiently condensed to have immobilized side chains characteristic of the native state of orthodox, globular proteins. However, the aromatic CD spectra contained signals from aromatic side chains in asymmetric environments. Compared to the native, oxidized molecule, the β form retained a signal from aromatic residues, but with diminished intensity (Fig. 1B). Thus, although the β conformation contained less extensive, weaker tertiary interactions than those present in α-PrP, some remained. This finding is consistent with the gel filtration behavior of reduced human $β\text{-}PrP^{91–231}$, which revealed the same level of compactness as the α-PrP conformation (Fig. 2).

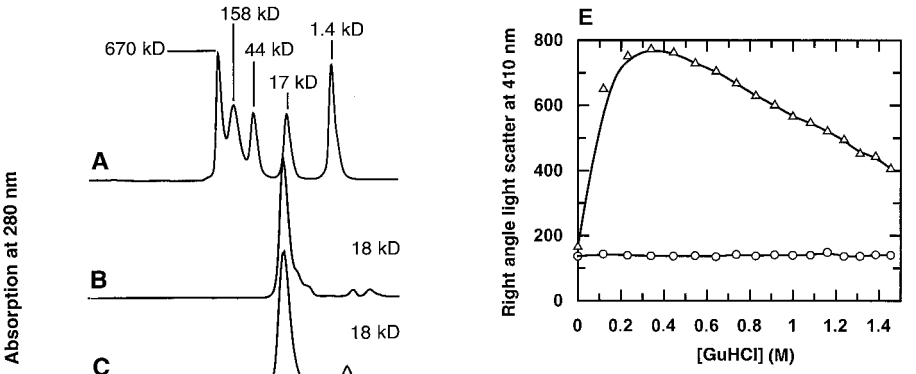


Fig. 2. Determination of the apparent molecular weight of PrP by size-exclusion chromatography (*29*). (**A**) Elution profile of standards used to construct a calibration curve of molecular weight (MW) versus elution time. (**B**) Oxidized human PrP (pH 8.0) elutes with an apparent MW of 18 kD. (**C**) Reduced human PrP (pH 4.0) also elutes as an 18-kD monomer. (**D**) Oxidized human PrP (pH 4.0) partially denatured with 1 M GuHCl, which results in aggregation and precipitation. Clarified supernatant contains a denatured form of PrP with an increased molecular volume (apparent MW, 40 kD). (**E**) β-PrP is more prone than α-PrP to form high-MW aggregates (*30*). Right-angle light scattering of α-PrP (○) shows that no high-MW aggregates are formed upon addition of GuHCl. In contrast, β-PrP readily forms high-MW aggregates upon addition of low concentrations of GuHCl (△). Identical results are obtained with other common salts (for instance, NaCl and KCl).

The availability of the β form of PrP as a monomeric species at 0.75 mM provided the opportunity to examine its physical properties using NMR. The 1D ^1H NMR spectrum of native human $PrP^{91–231}$ exhibited wide chemical shift dispersion characteristic of a fully folded globular protein, whereas the 1D ^1H and ^1H-^{15}N heteronuclear single-quantum coherence (HSQC) spectra of the β form of PrP exhibited considerably less chemical shift dispersion (Fig. 1, C and D). This lack of dispersion is characteristic of the loss of fixed side-chain interactions, which, in conjunction with the aromatic CD results, suggests some similarities with molten globule states (*12–14*). In addition, proton and nitrogen linewidths of the β form (Fig. 1D) were comparable to those observed in the folded and unfolded regions of the α-PrP conformation. This finding confirms the gel filtration results and indicates that the β form was monomeric at the extremely high concentrations required for NMR. The mobile unstructured regions of β-PrP were assigned from the sharpness and height of the peaks. Residues 91 to 126 and residues 229 and 230 were mobile in β-PrP; moreover, this is the same region that is unstructured in α-PrP (*3*). Thus, most of the rearrangement from α helix to β sheet has probably occurred within the structured region of α-PrP.

Within the structured region of β-PrP we could identify 57 peaks out of an expected 97 in the HSQC spectrum (Fig. 1D). None of these correlated with peak positions in the α-PrP spectrum, indicating no residual contamination from the oxidized form. The intensity ratio of weak and strong peaks within the HSQC spectrum from the unstructured and structured regions of β-PrP was identical to the ratio seen with α-PrP, indicating that a high degree of heterogeneity was unlikely.

The switch from α to β conformation was reversible. When the reduced β form was exposed to a higher pH (8.0), the native α conformation was restored. However, the rates of conversion in either direction were extremely slow, requiring days for completion (*15*). This high kinetic barrier, however, could be circumvented by fully denaturing and refolding at the appropriate pH to generate either isoform.

The solubility of the two isoforms was not equivalent. The α form of PrP can be titrated with the denaturant guanidine hydrochloride (GuHCl) to determine equilibrium parameters for the folding pathway (*4*). However, the

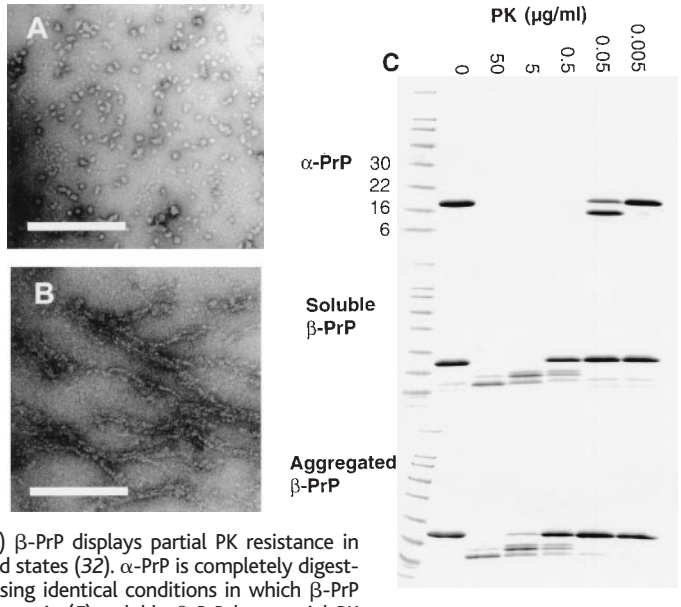


Fig. 3. β-PrP assembles into fibrils (*31*). Two forms of protein aggregates are seen with negative stain electron microscopy. (**A**) The most common form is small (diameter ~10 nm), irregularly shaped, and seen in all samples. (**B**) The other form is fibrillar and its proportion increases with incubation. These fibers intertwine with increased time of incubation. Scale bars, 200 nm. For biosafety reasons, electron microscopy was performed on mouse $PrP^{91–231}$ treated in an identical manner to human protein. (**C**) β-PrP displays partial PK resistance in monomeric and aggregated states (*32*). α-PrP is completely digested by PK at 0.5 μg/ml. Using identical conditions in which β-PrP remains soluble and monomeric (*5*), soluble β-PrP has partial PK resistance, with the majority undigested at 0.5 μg/ml. Aggregated β-PrP exhibits increased PK resistance, with some surviving intact at 5 μg/ml.

β form of PrP was also highly soluble in aqueous buffers, whereas titration with GuHCl led to intermolecular associations and a visible precipitate (Fig. 2E). Physiological concentrations of other salts (150 mM NaCl or 150 mM KCl) also caused aggregation, indicating that this was simply an effect of ionic strength. Salt-precipitated material was initially composed of irregular spherical particles (Fig. 3A) that associated over several hours to form fibrils (Fig. 3B) with a diameter of up to 20 nm and lengths up to 500 nm. The morphology and dimensions of these fibrils are within the range described for scrapie-associated fibrils extracted from brains affected by prion disease (*16*).

As with native PrP^C, α-PrP was extremely sensitive to digestion with PK (Fig. 3C). However, β-PrP showed marked protease resistance. This PK resistance was a function of the structural reorganization of the monomeric β form, with only a moderate further increase associated with aggregation (Fig. 3C). The different patterns of proteolytic cleavage fragments seen upon PK digestion of α-PrP and β-PrP provide further evidence of a major conformational rearrangement in β-PrP. In contrast, the partially structured β-sheet conformation of reduced hamster $PrP^{90–231}$ is fully sensitive to PK digestion (*17*).

Unusually for a protein with a predominantly helical fold, most of the residues in $PrP^{91–231}$ have a preference for β conformation. This suggests that PrP is balanced between radically different folds with a high energy barrier between them—one dictated by local secondary structural propensity (the β conformation) and one requiring the precise docking of side chains (the native α conformation). Such a balance would be influenced by mutations causing inherited prion diseases (*18*). Individuals homozygous for valine at polymorphic residue 129 of human PrP (where either methionine or valine can be encoded) are more susceptible to iatrogenic Creutzfeldt-Jakob disease (*19*), and valine has a much higher β propensity than methionine. Our results support this hypothesis, because the molecule is capable of slow conversion between α and β conformations. Furthermore, the β form can be locked by intermolecular association, thus supplying a plausible mechanism of propagation of a rare conformational state. The precise subcellular localization of PrP^{Sc} propagation remains controversial, although considerable evidence implicates late endosome–like organelles or lysosomes (*20–23*). This α-PrP to β-PrP conversion, caused by reduction and mild acidification, may be relevant to the conditions that PrP^C would encounter within the cell after its internalization during recycling (*24*). Reduction and acidification within the endosomal pathway is required for activation of diphtheria toxin (*25*). Such a mechanism could underlie prion propagation and hence could account for the transmitted, sporadic, and inherited etiologies of prion disease. Initiation of a pathogenic self-propagating conversion reaction, with accumulation of aggregated β-PrP, may be induced by exposure to a "seed" of aggregated β-PrP after prion inoculation, or as a rare stochastic conformational change, or as an inevitable consequence of expression of a pathogenic PrP^C mutant that is predisposed to form β-PrP.

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11. In the absence of denaturant, there is a calculable population of molecules in these intermediate states; the low proportion defines these states as highly unstable in such solvent conditions. The intermediate can be considered stable where it is the predominantly populated species, which occurs at ~2.3 M urea at pH 4.0 for mouse $PrP^{121–231}$.
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26. Recombinant human PrP in the oxidized α form was purified as described (*4*) and dialyzed into 10 mM Na acetate and 10 mM tris-acetate (pH 8.0). For conversion of this material to the β form, the protein was reduced and denatured in 100 mM dithiothreitol (DTT) in 6 M GuHCl, 10 mM Na acetate, and 10 mM tris-acetate (pH 8.0) for 16 hours. The protein was refolded by dialysis against 10 mM Na acetate, 10 mM tris-acetate, and 1 mM DTT (pH 4.0), and precipitated material was removed by centrifugation at 150,000*g* for 8 hours.
27. For CD measurements, protein was incubated in 10 mM Na acetate and 10 mM tris-acetate at either pH 8.0 (α-PrP) or pH 4.0 (β-PrP) and molecular ellipticity (shown in degrees mol^{-1} cm^{-1} × 10^3) was recorded between 190 and 250 nm. Near-ultraviolet (UV) CD spectra were recorded between 250 and 310 nm. All data were recorded at 25°C.
28. NMR spectra shown were acquired at 293 K on a Bruker DRX-500 spectrometer and processed using Felix (Molecular Simulations Inc.). For α-PrP, the proportions were 1 mM human $PrP^{91–231}$ in 20 mM sodium acetate–d_3, 2 mM sodium azide, and 10% D_2O (v/v), pH 5.55. For β-PrP, the proportions were 0.75 and 0.12 mM human $PrP^{91–231}$ in 20 mM sodium acetate–d_3, 2 mM sodium azide, and 10% D_2O (v/v), pH 4.
29. A Bio-Sil 125-5 size exclusion column (Bio-Rad) was calibrated with molecular weight standards. The α-PrP was loaded in a volume of 100 μl (200 μg) and eluted with 30 ml of 10 mM Na acetate, 10 mM tris-acetate, and 50 mM NaCl (pH 8.0). β-PrP was loaded in a volume of 100 μl (200 μg) and eluted with 30 ml of Na acetate, 10 mM tris-acetate, and 50 mM NaCl (pH 4.0).
30. Either oxidized human PrP (pH 8.0) was diluted to 1 mg/ml in 2 ml of 10 mM Na acetate and 10 mM tris-acetate (pH 8.0), or reduced human PrP (pH 4.0) was diluted to 1 mg/ml in 2 ml of the same buffer at pH 4.0. The presence of aggregated material was monitored by right-angle light scattering at 410 nm. Aliquots (30 μl) of 6 M GuHCl were added and the solution was allowed to equilibrate before each reading was taken.
31. The β-PrP protein at a concentration of 0.27 mg/ml in 10 mM Na acetate and 10 mM tris-acetate (pH 4.0) was treated with 1/9 volumes of a 5 M stock of GuHCl to give final protein and denaturant concentrations of 0.25 mg/ml and 0.5 M, respectively. A 3-μl portion of this protein solution was pipetted onto carbon-coated EM grids and stained with uranyl acetate. Electron micrographs were recorded at ~1 μm underfocus at 40,000× magnification.
32. α-PrP, and β-PrP as a monomer and aggregate, were digested with varying concentrations of PK (British Drug Houses) at 37°C for 1 hour and diluted to 1 mg/ml in 10 mM Na acetate and 10 mM tris-acetate (pH 8.0). Digestion was terminated by addition of Pefabloc (Boehringer). Samples were heated to 100°C for 5 min in SDS loading buffer before electrophoresis. Gels were Coomassie-stained.

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