Communication

pH-dependent Stability and Conformation of the Recombinant Human Prion Protein PrP(90–231)*

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A recombinant protein corresponding to the human prion protein domain encompassing residues 90–231 (huPrP(90–231)) was expressed in Escherichia coli in a soluble form and purified to homogeneity. Spectroscopic data indicate that the conformational properties and the folding pathway of huPrP(90–231) are strongly pH-dependent. Acidic pH induces a dramatic increase in the exposure of hydrophobic patches on the surface of the protein. At pH between 7 and 5, the unfolding of hPrP(90–231) in guanidine hydrochloride occurs as a two-state transition. This contrasts with the unfolding curves at lower pH values, which indicate a three-state transition, with the presence of a stable protein folding intermediate. While the secondary structure of the native huPrP(90–231) is largely α-helical, the stable intermediate is rich in β-sheet structure. These findings have important implications for understanding the initial events on the pathway toward the conversion of the normal into the pathological forms of prion protein.

Prion diseases comprise a group of transmissible neurodegenerative disorders. The best known animal forms of the disease are scrapie and bovine spongiform encephalopathy; the human versions include kuru, Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker disease, and fatal familial insomnia (1–4). All of these disorders are characterized by cerebral accumulation of an abnormal protein, designated PrPsc, which has a strong tendency to aggregate into insoluble fibrils. According to the “protein only hypothesis” (5, 6), PrPres constitutes the sole component of the infectious pathogen responsible for the transmission of prion disease.

PrPsc is derived from a host-encoded glycoprotein, the prion protein (PrP). The transition between the cellular form of PrP, designated PrPc, and PrPsc occurs by a post-translational mechanism and appears to take place without any detectable covalent modifications to the protein molecule (7). One of the main characteristics distinguishing PrPc from PrPsc is the resistance of the latter to proteolytic digestion (8, 9). Furthermore, recent spectroscopic studies indicate that the two isoforms have profoundly different conformation; while the secondary structure of PrPc consists largely of α-helices (10), PrPsc appears to be rich in β-sheet structure (10–13). In line with these observations, the current view is that prion diseases may be classified as disorders resulting from abnormal protein folding and that the key event in the pathogenic process is the transition between the “benign” conformation of PrPc and the “pathological” conformation of PrPsc. It is believed that the propagation of the disease can be described according to nucleation-dependent polymerization and/or template-assisted models (6, 14–17). However, the molecular mechanism and potential intermediate forms of PrP underlying the conformational transition between the normal and pathological isoforms of the protein remain unknown.

Recent studies have provided an insight into the three-dimensional structure of the recombinant mouse prion protein fragment 121–231 (18, 19). However, bacterial expression of larger PrP fragments proved to be more difficult, resulting in an aggregated protein that could only be solubilized using refolding procedures (20, 21). In the present study, we have developed an expression system that produces soluble protein corresponding to human prion protein domain encompassing residues 90–231 (huPrP(90–231)). Spectroscopic data show that the stability and conformation of the protein are strongly pH-dependent and suggest that conditions of acidic pH may be conducive to the transition between the conformational states characteristic of PrPc and PrPsc.

MATERIALS AND METHODS

Plasmid Construction—The DNA sequence corresponding to the huPrP(90–231) (Met129 variant) was amplified by polymerase chain reaction from a clone containing the coding region (22) using the primers 5′-CAAGCTTGAGGAGGTTGTTCCTCT-3′ and 5′-GAATTCCTGACGGATCTCGATCTCTC-3′ and 5′-CATGTTGGTGGACCCGCAGGAGG-3′. The amplified DNA was digested with EcoRI and BamHI (sites indicated in bold in the primer sequences) and cloned into the PET22b(+) vector (Novagen). The cloned protein contained the vector sequence MDGINSDFPD to the N terminus of the huPrP(90–231). The final construct was confirmed by sequencing the coding region using a Sequenase 2.0 kit (Amersham Corp.) and transformed into an Escherichia coli expression strain, B834(DE3) (Novagen). DNA manipulations were performed as described by Sambrook et al. (23).

Protein Expression and Purification—A midlog phase liquid culture of B834(DE3) cells in 2 × YT medium containing 0.1 mg/ml ampicillin was diluted 9-fold with a fresh medium (to a total volume of 2 liters) and grown at 37 °C until the optical density at 600 nm reached a value of approximately 2. At that time, protein expression was induced by the addition of 0.25 mM isopropyl-β-D-thiogalactoside, and the cultures were grown for an additional hour. Cells from four liters of medium were harvested by centrifugation and sonicated in 200 ml of buffer A (50 mM potassium phosphate, 5 mM EDTA, pH 7.8). After removal of cellular debris, the nucleic acids and some acidic proteins were removed by batch-mode chromatography (in buffer A), using DEAE-Sephadex A-50 (Pharmacia Biotech Inc.) followed by QA resin (Sigma) (in each step, about 2 g of preequilibrated resin was used to treat material recovered from a 4-liter cell culture). The protein was dialyzed overnight at 4 °C against 10 mM phosphate buffer, pH 6.0, and purified by cation-exchange chromatography on a prepacked 5-mL FPLC SP HiTrap column (Pharmacia). About 100 ml of crude material was loaded on the column, and the protein was eluted using a linear gradient of NaCl (0–0.2 M) and a flow rate of 5 ml/min. Individual fractions were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting; those containing huPrP(90–231) (between approximately 5 and 50 ml of gradient volume) were pooled, dialyzed against 50 mM potassium phos-
phate buffer, pH 7.0, and concentrated to a volume of approximately 6 ml. After this step, the huPrP(90–231) preparation was 80–85% pure. Final purification of the protein was accomplished by size-exclusion chromatography on a 1.6 × 60-cm HiLoad Superdex 75 column (Pharmacia). Two ml of a concentrated material was applied on the column (preequilibrated in 50 mM potassium phosphate, pH 7), and the protein was eluted at a flow rate of 1 ml/min. The protein was finally dialyzed against 10 mM potassium phosphate, pH 7.0, concentrated to 1 mg/ml and stored in small aliquots at −70 °C.

Equilibrium Unfolding in GdnHCl and Circular Dichroism Spectroscopy—To study the unfolding of huPrP(90–231) in GdnHCl, native protein was diluted (to a final concentration of approximately 0.1 mg/ml) in a 50 mM buffer (sodium phosphate at pH 7.2 or sodium acetate at pH 5 and below) containing different concentrations of GdnHCl. Samples were incubated for 24 h at room temperature, and the ellipticity at 222 nm was measured in a 1-mm cell by averaging the signal over 2 min. The denaturation curves were analyzed according to the two- or three-state protein unfolding models (24, 25).

Purities were incubated for 24 h at room temperature, and the ellipticity at 222 nm was measured in a 1-mm cell by averaging the signal over 2 min. The denaturation curves were analyzed according to the two- or three-state protein unfolding models (24, 25). Full far-UV CD spectra of huPrP(90–231) in the presence of 1 M GdnHCl were obtained in a 0.2-mm cell at a protein concentration of 0.8 mg/ml. All CD measurements were carried out at room temperature on a Jasco 600 spectropolarimeter. Protein concentration was determined by using a molar extinction coefficient at 280 nm of 20,800 M⁻¹ cm⁻¹, as calculated based on the extinction coefficients of aromatic residues (26).

Bis-ANS Binding—Protein from a stock solution was diluted to a concentration of 0.05 mg/ml in 50 mM Tris, pH 8.8 and 8.0, or phosphate-citric acid, pH 7–2.6, buffers containing 8 M urea. The CD spectrum characteristic of proteins rich in a-helical structure (27) was observed at pH below 5. Representative unfolding curves at low pH (4.0 and 3.6) show two distinctive transition regions and clearly indicate the presence of a relatively stable folding intermediate state (Fig. 3). The unfolding curve at pH 3.6 could be successfully approximated by a three-state transition model (25), yielding for the first transition ΔG° and C_m of 13 kJ/M and 0.5 M, respectively. The thermodynamic parameters for the second transition are ΔG° of 21 kJ/M and C_m of 2.4 M.

To gain insight into the conformational properties of huPrP(90–231) under conditions corresponding to the presence of a stable folding intermediate, we measured the far-UV CD spectra of the protein at various pH values in the presence of 1 M GdnHCl. In the pH range between 7.2 and 5, the spectra in 1 M denaturant are essentially indistinguishable from those in the absence of GdnHCl and indicate a largely a-helical structure of the protein. These spectra drastically differ from those obtained in the presence of 1 M GdnHCl at pH 4 and 3.6, i.e., under conditions leading to the formation of a stable folding intermediate of huPrP(90–231). In the latter spectra (Fig. 4), the double minimum at 222 and 208 nm is replaced by a single minimum at approximately 215 nm, a feature characteristic of proteins rich in b-sheet structure (27). It should be noted that at a protein concentration equivalent to that used in CD measurements, the elution pattern of the folding intermediate on a size exclusion column (Superose 6) is indistinguishable from that of native huPrP(90–231), indicating, in either case, a monomeric state of the protein (data not shown for brevity).

Binding of Bis-ANS—The fluorescence of bis-ANS is strongly dependent on the polarity of the environment: it is very weak in water and increases greatly upon binding to hydrophobic sites of proteins. This compound has been widely used to probe conformational changes in proteins as well as to assess the exposure of hydrophobic patches on the protein surface (28, 29).
As shown in Fig. 5, there is very little fluorescence of bis-ANS in the presence of huPrP(90–231) in the pH range between 9 and 6. However, further decrease in pH results in a dramatic (about 30-fold) enhancement of the fluorescence intensity, which reaches maximum at around pH 4.4. Since the fluorescence of bis-ANS alone is pH-independent, the acid-induced fluorescence enhancement in the presence of huPrP(90–231) thus reflects binding of the probe to the protein as a result of an increased exposure of hydrophobic patches on the surface of the protein molecule. The apparent pK of this conformational transition in huPrP(90–231) is about 5.1.

**DISCUSSION**

The key molecular event in the pathogenesis of prion diseases appears to be a conformational change in PrP, resulting in the conversion of a large portion of the protein molecule from an α-helical conformation to a β-sheet-rich, protease-resistant structure (1–4, 8–13). Recent structural studies of the C-terminal domain (residues 121–231) of mouse PrP have revealed that this part of the protein forms an autonomous folding unit, consisting of three α-helices and two short β-strands (18, 19).

However, very little information is currently available regarding the molecular basis of the conformational transition(s) that predispose conversion of PrPSEN to PrPRES. In addition, little is known about the role of the N-terminal portion in the folding of different PrP isoforms. To address these issues, we developed a bacterial expression system that allowed us to obtain a recombinant protein corresponding to human PrP domain 90–231. This region of PrP appears to be of special functional importance, since it encompasses the entire sequence of protease-resistant protein found in prion diseased brain, contains all known point mutations associated with human prion disorders, and is sufficient for the propagation of the disease (1–4).

Comparison of the CD data for huPrP(90–231) with those recently published for mouse PrP fragment 121–231 (18) indicates that the percentage of α-helical structure in the longer protein is reduced (mean residue ellipticity at 222 nm, θ222, of −15,600 and −12,000 degree cm² dmol⁻¹ for mouse PrP(121–231) and huPrP(90–231), respectively). However, the θ222 values for both proteins became remarkably similar when the data for huPrP(90–231) were normalized assuming that the N-terminal fragment 90–120 does not contribute to the ellipticity at 222 nm. This observation, together with apparently similar thermodynamic stability of mouse PrP(121–231) and huPrP(90–231) (ΔG₀ for GdnHCl unfolding at pH 7 of 21.8 and 20 kJ/M, respectively), strongly supports the recent prediction that the C-terminal domain comprising residues 121–231 constitutes the only polypeptide segment within PrP with a global fold and ordered structure (30) and argues against the model postulating a fourth α-helix between residues 109 and 122 (31).

The lack of ordered structure in the N-terminal portion of huPrP(90–231) is also indicated by the fluorescence properties of the sole tryptophan residue at position 99. While generally consistent with structural data available for the shorter polypeptide corresponding to mouse PrP(121–231), the properties of huPrP(90–231) appear to differ in some respects (e.g. GdnHCl denaturation curves at pH 5) from those reported for the recombinant protein corresponding to Syrian hamster PrP(90–231) (21). It is conceivable that these discrepancies reflect species-specific differences in the primary structure of PrP. However, it should also be noted that the polypeptide corresponding to hamster PrP was expressed as an insoluble (presumably misfolded) protein aggregate; its purification in-

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volved chromatography in an organic solvent, followed by a refolding step from a concentrated solution of GdnHCl (20, 21). This treatment may result in a structure that does not represent the native conformation of PrP. Our expression system produces a secreted protein that is soluble, hence not requiring any refolding treatment.

The availability of a soluble recombinant protein corresponding to a major portion of human prion protein allows studies on the pathway of PrP folding and the nature of protein folding intermediates that are likely to precede formation of PrPres from PrPC 

from PrP  

in GdnHCl is at least as high as the free energy of its unfolding in GdnHCl occurs as a simple two-state transition typical for many small globular proteins. In contrast, the unfolding curves in the pH range 5–7, the denaturation of huPrP(90–231) in GdnHCl involves a massive increase in the exposure of hydrophobic surfaces, without affecting the overall secondary structure of huPrP(90–231). Furthermore, acidic pH induces a profound change in the unfolding pathway of the protein. Thus, in the pH range 5–7, the denaturation of huPrP(90–231) in GdnHCl occurs as a simple two-state transition typical for many small globular proteins. In contrast, the unfolding curves at lower pH values indicate a three-state transition, with the presence of protein folding intermediate. This intermediate appears to have a remarkably high thermodynamic stability; the free energy of its unfolding in GdnHCl is at least as high as that of the native protein at pH 7.2. Most importantly, while the secondary structure of the native huPrP(90–231) is largely α-helical, spectroscopic properties of the intermediate state are characteristic of a protein rich in β-sheet structure.

It should be emphasized that the folding intermediate described above is soluble and thus unlikely to represent the infectious species (although infectivity has not been tested in the present study). However, the finding that the recombinant huPrP(90–231) can exist in two stable monomeric forms that, depending on environmental conditions, have markedly different secondary structures has important implications for understanding the pathway involved in the PrPC  

PrP  

conversion. In this context, we postulate that the properties of the intermediate state (surface-exposed hydrophobic patches, β-sheet-rich structure) render this conformer as a good candidate for the prototype of a soluble PrP folding intermediate that may immediately precede the formation of an insoluble PrPres aggregate. In the present in vitro experiments, the transition to an intermediate state was promoted by acidic conditions. This is of potential significance, since the conversion of PrPC to PrPres appears to take place, at least in part, along the endocytic pathway which contains acidic compartments (34, 35). However, one can envisage that under in vivo conditions the formation of a PrP folding intermediate similar to that found in this study may also be facilitated by other factors (e.g. interaction with auxiliary proteins (36)). Furthermore, the properties of the protein may be affected by the mutations specific to hereditary forms of prion disease (4). The ability to produce large quantities of soluble PrP in a bacterial expression system provides a basis for studying the effect of these pathogenic mutations on both the folding pathway and conformational stability of PrP.

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