Isolation and characterization of a polymerized prion protein

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A polymerized form of recombinant mouse prion protein (mPrP) domain 23–231 [mPrP-(23–231)], designated mPrP-z, was generated at acidic pH (pH 2–5) in the presence of selected concentrations of denaturant (2 M guanidinium chloride or 5 M urea). This isoform of mPrP is stable in acidic solution after removal of denaturant. It can be isolated and purified using reversed-phase HPLC or size-exclusion HPLC. mPrP-z bears structural properties that partially resemble those of scrapie prion. Unlike the native mPrP-(23–231) (mPrP-N), mPrP-z exhibits a high content

of β -sheet structure, as shown by CD spectroscopy, and exists as an oligomer with an approximate molecular mass of 340 000 Da, as measured by light scattering. However, similarly to mPrP-N, mPrP-z contains the intact disulphide bond and is sensitive to digestion by proteinase K.

Key words: mouse, prion protein isoforms, prion protein polymerization, prion protein unfolding.

INTRODUCTION

Prion disease afflicts both animals (bovine spongiform encephalopathy or ‘mad cow’ disease, scrapie of sheep, etc.) and humans (Creutzfeldt–Jacob disease, etc.) [1–8]. The chemical event that underlies the cause of prion disease is the conversion (conformational change) of a host-derived cellular prion protein (PrP^C) to the infectious scrapie prion protein (PrP^{Sc}) [2,9,10]. There are no detectable chemical differences between PrP^C and PrP^{Sc}. Both have the same molecular mass and amino acid sequences [11]. However, they differ by their different conformation and relevant physicochemical properties. In contrast with PrP^C, which is soluble, susceptible to enzyme digestion and rich in α -helical structure, PrP^{Sc} has a high content of β -sheet structure, partial resistance to proteolytic digestion and is insoluble in non-denaturant solvents [2,5,9,12]. It is also known that propagation of infectious prion requires the interaction between PrP^C [9,10,13–17] and PrP^{Sc}, and that PrP^{Sc} exists as diverse strain-related conformational isoforms that are segregated by energy barriers [9,18–20]. Despite lingering controversy [21], collective and mounting evidence strongly indicates that PrP^{Sc} alone constitutes the infectious prion pathogen. However, the exact molecular mechanism of the conformational change of prion protein remains unclear, and conditions that lead to the conversion of PrP^C into PrP^{Sc} have yet to be identified. Numerous efforts were undertaken by different laboratories to search for conditions *in vitro* that would transform PrP^C into PrP^{Sc}-like proteins [12,22–31]. Most of these studies exploited the folding and unfolding behaviours of recombinant prion proteins in order to identify transient intermediates that display structural properties of PrP^{Sc}.

Isoforms of PrP^{Sc}-like proteins were indeed found as folding intermediates of prion proteins. A reduced form of human prion protein was shown to exhibit β -sheet structure and an increased tendency to form aggregates and to resist proteolysis, all structural hallmarks of PrP^{Sc} [12]. During the process of oxidative folding of reduced mouse prion protein (mPrP), three novel isoforms of PrP^{Sc}-like protein were detected and isolated when folding was accomplished in the presence of urea and under mild acidic conditions [29,30]. A cysteine-free variant of prion protein

that lacked disulphide bonds has a similar tendency to form the β -sheet structure [31]. Renaturation of SDS-denatured SHaPrP-(90–231) (the hamster prion protein) also allows identification of two stable intermediates: a dimeric α -helical state and a tetrameric β -sheet state [26].

Alternatively, β -sheet structures of prion protein were also observed as unfolding intermediates of the native prion protein without disrupting the intact disulphide bond. Unfolding by elevated temperatures converted cellular hamster PrP-(90–231) irreversibly to a stable β -sheet form [22]. An equilibrium unfolding intermediate rich in β -sheet structure was identified by various laboratories. Such an unfolding intermediate was observed under mild acidic conditions in the presence of a low concentration of either urea or guanidinium chloride (GdmCl) [23–25,27]. Conflicting data exist as to the state of oligomerization of this unfolding intermediate, and whether salt is required in the case of its urea-induced formation [27]. In addition, the chemical nature of this β -sheet-rich isoform of prion protein has yet to be characterized.

We report in the present study the production and isolation of a soluble aggregate of mPrP generated in acidic solution in the presence of a mild concentration of denaturant. This polymerized form of recombinant mPrP domain 23–231 [mPrP-(23–231)], designated mPrP-z, was purified for the first time, and its properties were investigated here. Although mPrP-z bears predominantly a β -sheet structure, it does not exhibit an increased resistance to digestion with proteinase K, which is a common property of PrP^{Sc}-like proteins [2,9,12].

EXPERIMENTAL

Materials

mPrP-(23–231) was expressed and purified using the method developed by Hornemann et al. [32], and was purified further by reversed-phase (RP)-HPLC for the purpose of unfolding experiments. The plasmid pRBI-PDI-T7 for expression of mPrP-(23–231) was kindly supplied by Professor Rudi Glockshuber (Institute for Molecular Biology and Biophysics, ETH-Hoenggerberg, Zürich, Switzerland). Dithiothreitol and α -chymotrypsin

Abbreviations used: BCA, bicinchoninic acid; GdmCl, guanidinium chloride; MALDI-TOF, matrix-assisted laser-desorption/ionization–time-of-flight; mPrP, mouse prion protein; mPrP-(23–231), mPrP domain 23–231; mPrP-z, polymerized form of recombinant mPrP-(23–231); mPrP-N, native mPrP-(23–231); PrP^C, cellular prion protein; PrP^{Sc}, scrapie prion protein; RP, reversed-phase; SEC, size-exclusion chromatography.

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were purchased from Sigma. Bicinchoninic acid (BCA) assay reagent kit was obtained from Pierce. Proteinase K was from Boehringer Mannheim GmbH (Mannheim, Germany). NAP-5 columns were from Pharmacia. All other chemicals have a grade purity of > 99 %.

Polymerization of mPrP at acidic pH in the presence of denaturant

Freeze-dried native mPrP-(23–231) (mPrP-N; 1 mg/ml) was dissolved in buffers of selected pH (2.0–6.0) and different concentrations of urea (2–6 M) and GdmCl (0.5–4 M). The reactions were performed at 23 °C. The intermediates were trapped in a time-dependent manner by removing aliquots of the sample and mixing with 2 vols. of 4 % (v/v) trifluoroacetic acid. Acid-trapped samples were analysed by HPLC with the following buffers/conditions. For RP-HPLC: solvent A was water containing 0.088 % (v/v) trifluoroacetic acid; solvent B was 90 % (v/v) acetonitrile in water containing 0.084 % trifluoroacetic acid; the gradient employed was 28–48 % solvent B over a 25 min period; the column was a ZORBAX 300 SB-C18 (dimensions 4.6 mm × 25 cm); and the flow rate was 0.5 ml/min. For size-exclusion chromatography (SEC): the column was TSK-GEL G3000 SWXL (dimensions 30 cm × 7.80 mm) equilibrated and eluted with either sodium acetate buffer (20 mM, pH 4.0) containing 0.2 M NaCl, or an aqueous solution containing 0.2 % trifluoroacetic acid (pH ≈ 2.0), or a 40 % (v/v) aqueous acetonitrile solution containing 0.1 % trifluoroacetic acid (pH ≈ 2.0).

Depolymerization of mPrP-z

mPrP-z (freeze-dried) was incubated in either 20 mM sodium acetate buffer, pH 4.0, or 0.1 M Tris/HCl, pH 8.0, containing different concentrations of urea (2–8 M) and GdmCl (2–6 M). The reactions were performed at 23 °C. The kinetics of the depolymerization were monitored by removing aliquots of the sample at different time points and mixing with 2 vols. of 4 % (v/v) trifluoroacetic acid. Acid-trapped samples were then analysed by SEC, using the conditions described above.

CD spectroscopic measurements

Far-UV CD spectra were recorded on a Jasco J-715 spectropolarimeter. The protein concentration was 0.2 mg/ml in 20 mM sodium acetate buffer, pH 4.0. The spectra were recorded in a 0.1 cm cuvette in the far-UV region (190–250 nm). mPrP-z was prepared at pH 4.0 in the presence of 2 M GdmCl. The denaturant was removed by passing the sample through a NAP-5 column eluted with 20 mM sodium acetate buffer, pH 4.0. The protein concentration was verified by BCA assay. Protein secondary structure was estimated using the Softsec[®] Program (Softwood Company, Brookfield, CT, U.S.A.) provided by the Jasco.

Determination of the molecular mass of the proteins by light scattering and MS

The average molecular mass of protein was measured by a right-angle light-scattering method using a TDA triple detector array (Viscotek, Houston, TX, U.S.A.). Lysozyme (Viscotek; molecular mass 14307 Da) and BSA were used as calibration standards. The triple-detector array (light scattering, viscometry and refraction index) was connected to an Agilent 1100 HPLC isocratic pump system and an auto-sampler. The system was equilibrated with specified buffer at a flow rate of 1.0 ml/min. Samples were injected through the auto-sampler directly into the detector array. The molecular mass of prion protein was also

measured by matrix-assisted laser-desorption/ionization–time-of-flight (MALDI-TOF) MS with a time-of-flight detector Voyager-DE[®] STR, from PerSeptive Biosystems. Horse myoglobin (Sigma; molecular mass 16952 Da) and carbonic anhydrase (also from Sigma; molecular mass 29022 Da) were used as calibration standards.

Proteolysis of mPrP

mPrP-N and mPrP-z (1 mg/ml) were digested by proteinase K and α -chymotrypsin in 100 mM *N*-ethylmorpholine buffer (pH 8.1). The mass ratio of enzyme to protein ranges from 0.01–0.000001 (10–0.001 μ g/ml). Digestion was performed at 37 °C for 1 h, and was stopped by adding 2 vols. of 4 % (v/v) trifluoroacetic acid. Digested samples were freeze-dried and analysed by SDS/PAGE (15 % gels).

Determination of the state of the disulphide bond

Both mPrP-N and mPrP-z were allowed to react with vinylpyridine with or without prior reduction. Reduction of the proteins was performed at 23 °C for 90 min in 0.1 M Tris/HCl, pH 8.0, containing 100 mM dithiothreitol and 8 M urea. Modification with vinylpyridine was accomplished in the same buffer using an excess molar concentration of the reagent (0.2 M) over dithiothreitol. Reaction with vinylpyridine was allowed to proceed at 23 °C for 45 min. Vinylpyridine-modified samples were purified by RP-HPLC, freeze-dried and then analysed by MALDI-TOF MS.

RESULTS

Preparation and isolation of mPrP-z from mPrP-N

In the presence of a mild concentration of denaturant and acidic pH, mPrP-N undergoes a structural transformation, leading to the process of polymerization. This reaction can be measured by the yield of polymerized product, and followed by both RP-HPLC and SEC. mPrP-z is stable under acidic conditions and can be isolated and purified for further structural analysis. Figure 1 shows chromatograms of the time-course conversion of

Figure 1 Polymerization of the mPrP-N at pH 4.0 in the presence of 2 M GdmCl

The reactions were performed at 23 °C and intermediates were trapped in a time-dependent manner by mixing aliquots of the sample with 2 vols. of 4 % trifluoroacetic acid in water. Acid-trapped samples were analysed by RP-HPLC and SEC (see the Experimental section for further details). 'N' indicates mPrP-N; 'Z' denotes mPrP-z. mPrP-N and mPrP-z were eluted at 17.8 min and 18.1 min respectively on RP-HPLC, and at 11.3 min and 11.2 min respectively on SE-HPLC.

Figure 2 Effect of varying pH (A and B) and the influence of different concentrations of denaturant (C and D) on the kinetics of polymerization of mPrP-N

(A) and (B): reactions were carried out at 23 °C in the presence of optimized concentrations of GdmCl (2 M) or urea (5 M). Protein concentration was 1.0 mg/ml. The rate of conversion of mPrP-N into mPrP-z was analysed by SEC using the conditions described in the Experimental section. The pH values shown are: 2 (Δ); 3 ($\blacktriangle$); 4 ($\bullet$); 5 ($\blacksquare$); and 6 ($\square$). (C and D), the reactions were carried out at 23 °C at optimized pH (pH 4 for GdmCl and pH 2 for urea) in the presence of different concentrations of denaturant. The protein concentration was 1.0 mg/ml. The rate of conversion of mPrP-N into mPrP-z was analysed by SEC using the conditions described in the Experimental section. The concentrations of denaturant are indicated on the right of each curve.

Table 1 Molecular mass of mPrP forms

The data in the second column from the right were measured by right-angle light scattering (RALS) in an acidic solution containing 60% water, 40% acetonitrile and 0.1% trifluoroacetic acid. The data in the rightmost column were measured by RALS in 20 mM acetate at the pH of 4.0. VP, vinylpyridine.

Species	Expected size (Da)	Measured by MALDI-TOF MS modified with VP without reduction	Measured by MALDI-TOF MS modified with VP after reduction	Measured by RALS at pH 2 (kDa)	Measured by RALS at pH 4 (kDa)
mPrP-N	23107.4	23107.4	23319.5	31.8 $\pm$ 1.6	23.5 $\pm$ 0.2
mPrP-z	—	23109.8	23322.5	353.8 $\pm$ 8.7	314.9 $\pm$ 3.7

mPrP-N into mPrP-z at pH 4.0 in the presence of 2 M GdmCl. Baseline separation of mPrP-N and mPrP-z can be achieved by SEC. Kinetic analysis of the formation of mPrP-z was thus primarily on the basis of data analysed by SEC.

Polymerization of mPrP-N to form mPrP-z appears to occur under a wide range of conditions that include both an acidic pH and the presence of denaturant. Our first objective was to establish the optimized conditions for the production of mPrP-z. In order to achieve this aim, we performed a systematic study whereby we examined the collective effects of varying the pH (pH 2–6) and different concentrations of urea (2–6 M) or GdmCl (0.5–4 M). The results, summarized in Figure 2, lead us to conclude that the most efficient conditions for the production of mPrP-z are the combination of either pH 4.0 and 2 M GdmCl, or pH 2.0 and 5 M urea.

The effect of pH is most significant in the case of urea-induced polymerization. Low pH (2.0) is required for the near-quantitative recovery of mPrP-z (Figure 2B). At 5 M urea, an increase of pH from 2.0 to 3.0 reduces the yield of mPrP-z by 70%.

Figure 3 Dependence of the rate of polymerization upon the concentration of mPrP-N (A), and formation of mPrP-z under three different conditions in the absence or presence of 150 mM NaCl (B)

(A) The reactions were performed at 23 °C at pH 4.0 in the presence of 2 M GdmCl. The rate of conversion of mPrP-N into mPrP-z was analysed by SEC using the conditions described in the Experimental section. The concentrations of prion protein are indicated on the line of each curve. (B) Formation of mPrP-z under three different conditions in the absence or presence of NaCl (150 mM). These conditions are: 2 M GdmCl, pH 4.0 (Δ); 2 M GdmCl, pH 4.0, plus NaCl ($\blacktriangle$); 5 M urea, pH 2.0 ($\circ$); 5 M urea, pH 2.0, plus NaCl ($\bullet$); 3.5 M urea, pH 4.0 ($\square$); and 3.5 M urea, pH 4.0, plus NaCl ($\blacksquare$). Protein concentration was 1.0 mg/ml. Reactions were performed at 23 °C, and samples were trapped and analysed as described in the Experimental section.

Figure 4 Characterization of mPrP-z and mPrP-N by SEC

Top panel: samples were eluted with 40% acetonitrile in water containing 0.1% trifluoroacetic acid, pH 2. Bottom panel: samples were eluted with 20 mM sodium acetate, pH 4.0, containing 200 mM NaCl. The column was TSK-GEL G3000 SWXL (30 cm $\times$ 7.8 mm). Flow rate was 1.0 ml/min. Bovine BSA (66,000) and ribonuclease A (RNaseA; 14,000) were used as standards.

concentration of urea is less crucial. A high yield of mPrP-z was similarly obtained at pH 2.0 in the presence of 4 M and 6 M urea (Figure 2D). We also found that, at pH 4.0, the optimized concentration of urea is 3.5 M, consistent with the previous observation [23,27]. However, the yield of mPrP-z is low in comparison with that performed at pH 2.0 and 5 M urea.

In the case of GdmCl-induced polymerization, the concentration of denaturant and the range of pH are approximately equally as critical. For example, at 2 M GdmCl, approx. 90% of mPrP-z was recovered within 4 h when the reaction was performed at pH 4.0. As the pH was increased from 4.0 to 6.0, the yield of mPrP-z decreased to < 10% (Figure 2A). At the optimal pH (4.0), adjustment of the concentration of GdmCl by one molar unit from 2 M to either 1 or 3 M decreases the yield of mPrP-z by almost 40% (Figure 2C).

Production of mPrP-z is also dependent upon protein concentration. The higher the protein concentration, the greater the yield of mPrP-z (Figure 3A). Under otherwise-optimal conditions (pH 4.0 and 2 M GdmCl), an increase in the protein concentration from 0.1 to 2.0 mg/ml improved the recovery of mPrP-z from 40% to 92% after 24 h of reaction. This observation is not surprising, given that a high protein concentration facilitates intermolecular interactions and promotes the polymerization of denatured mPrP-N. The influence of salt on the formation of mPrP-z was also investigated. The inclusion of NaCl (150 mM) produced no discernible effect on the recovery of mPrP-z when the reaction was performed at either pH 4.0 and 2 M GdmCl or pH 2.0 and 5 M urea. However, it increased the yield of mPrP-z 4-fold when the reaction was performed at 3.5 M urea and pH 4.0 (Figure 3B). These results are striking, but consistent with those observed by Surewicz and co-workers [27].

Figure 5 Far-UV CD spectra of mPrP-z and mPrP-N

The trace labelled 'N' represents the spectrum of mPrP-N. The trace labelled 'Z (Purified)' represents that of mPrP-z. In between are traces showing the incubation of mPrP-N at 2 M GdmCl at pH 4.0 after time periods of 1 min, 6 min and 90 min. Denaturant was removed by passing the solution through a NAP-5 column equilibrated with 20 mM sodium acetate. Spectra were obtained in 20 mM sodium acetate buffer, pH 4.0, at a protein concentration of 0.2 mg/ml.

Evidence that mPrP-z exists in an oxidized state and a polymerized form

The molecular mass of mPrP-z was characterized by both SEC and right-angle light scattering using a triple detector array model TDA 310 (Viscotek; Table 1). In acidic solutions of pH 2.0 and 4.0, mPrP-z was eluted close to the void volume of the TSK-GEL G3000 column, and displayed a molecular mass greater than that of BSA (66000; Figure 4). Analysis by light scattering at both pH 2.0 and 4.0 revealed the molecular mass of mPrP-z to be in the range of 315000–355000 Da (corresponding to greater-than-decameric size) (Table 1).

The disulphide bond of the prion protein remains intact in mPrP-z. This conclusion was based on the analysis of molecular mass of mPrP-z modified with vinylpyridine (a cysteine-specific reagent), both before and after reduction with dithiothreitol. The data are summarized in Table 1. It is important to mention that, during the analysis by MALDI-TOF MS, polymerized mPrP-z apparently dissociates to form the monomeric form of prion protein. Without prior reduction, vinylpyridine-modified mPrP-z exhibits a molecular mass of 23109, identical with that of mPrP-N. After reduction followed by vinylpyridine modification, the molecular mass of mPrP-z increases by 210 Da from 23109 to 23319, accounted for by two molecules of conjugated vinylpyridine (molecular mass 105 Da). These data indicate that the two cysteines of mPrP-z are incorporated into a disulphide bond, and are not accessible for vinylpyridine modification.

mPrP-z displays a high content of β -sheet structure, but lacks resistance to proteolytic digestion

The far-UV CD spectra of mPrP-N and purified mPrP-z were measured in the sodium acetate buffer (20 mM, pH 4.0; Figure 5). mPrP-N exhibits a double minimum at 208 and 222 nm, characteristic of the spectra for α -helical rich proteins, and

Figure 6 Proteolytic digestion of mPrP-N and its isoforms

mPrP-(23–231) isomers were digested by proteinase K and α -chymotrypsin in *N*-ethylmorpholine acetate buffer (50 mM, pH 8.1). The protein concentration was 1 mg/ml. The mass ratio of enzyme to substrate was 0.00001 and 0.0001 for proteinase K and α -chymotrypsin respectively. Proteolysis was allowed to proceed at 37 °C for 1 h, and then stopped by addition of 2 vols. of 4% aqueous trifluoroacetic acid, freeze-dried before analysis by SDS/PAGE (15% gels). N, Z and R represent mPrP-N, mPrP-z and mPrP-R respectively, where mPrP-R is the reduced form of mPrP-(23–231).

consistent with results published previously [32–34]. On the other hand, the CD spectra of mPrP-z differs significantly from that of mPrP-N. It displays a single minimum at approx. 215 nm, a unique CD signal for proteins rich in β -sheet structure, similar to both previous observations [23,24] and those found in the case of one reduced form of human prion protein [12] and four isoforms of reduced mPrP [30]. Using the Softsec[™] program provided by Jasco, mPrP-z was estimated to contain 72% β -sheet structure, 25% random coil and α -helix structure. A time-course CD measurement (Figure 5) also indicates that the formation of mPrP-z is accompanied by an increase in β -sheet structure. The CD data are consistent with those obtained from SEC analysis (Figure 1). After 90 min of sample incubation, approx. 80% of mPrP-z was generated.

However, mPrP-z is indistinguishable from mPrP-N in terms of susceptibility towards proteolysis. We compared the susceptibility to limited proteolysis using both α -chymotrypsin and proteinase K. In both cases, mPrP-N and mPrP-z were readily digested by the enzymes (Figure 6). When digestions were carried out for 1 h at 37 °C with 0.001% (w/w) of proteinase K, approx. 55% of mPrP-N and 65% of mPrP-z were proteolysed. The sensitivity of mPrP-z towards proteolysis contrasts sharply with that of four different isoforms of reduced mPrP (mPrP-a, mPrP-b, mPrP-c and mPrP-R) [29,30]. Under the same conditions, all four reduced forms of prion isomers were almost completely resistant to proteolysis. Results obtained from the digestion of mPrP-R are also presented in Figure 6 (lanes labelled with 'R').

mPrP-z could be depolymerized into the monomeric form at basic pH in the presence of denaturant

mPrP-z was isolated by SEC and eluted with an acidic solution (pH 2.0) that consists of 60% water, 40% acetonitrile and 0.1% trifluoroacetic acid. Isolated mPrP-z is completely stable in the freeze-dried form when stored at –20 °C for up to 60 days. This was verified by analysis of freeze-dried samples after reconstitution in the same acidic solution (results not shown). mPrP-z also remains stable when incubated at pH 4 (50 mM sodium acetate buffer) in the absence of denaturant for at least 48 h without sample precipitation or depolymerization. As the pH of the

Figure 7 Depolymerization of mPrP-z to form mPrP-N under conditions of basic pH in the presence of denaturant

Purified mPrP-z was freeze-dried. The reactions were initiated by dissolving samples in Tris/HCl (0.1 M, pH 8.0) containing various concentrations of GdmCl and urea. The reactions were performed at 23 °C. The intermediates were trapped in a time-dependent manner by mixing aliquots of the sample with 2 vols. of 4% aqueous trifluoroacetic acid, and analysed by SEC using the conditions described in the Experimental section. Top panel: SEC of the time-dependent depolymerization of mPrP-z in the presence of 6 M GdmCl and 8 M urea. Bottom panel: effects of the concentration of GdmCl and urea on the kinetics of depolymerization of mPrP-z.

100 mM Tris/HCl buffer was adjusted to 8.0, mPrP-z rapidly formed insoluble aggregates and precipitated out of the solution.

However, mPrP-z may be depolymerized and converted back into mPrP-N in the alkaline buffer containing high concentrations of denaturant. Studies were conducted at pH 8.0 in the presence of various concentrations of GdmCl and urea. The results (Figure 7) clearly show that the depolymerization of mPrP-z is promoted by the high concentration of denaturant. At pH 8.0, complete depolymerization may be achieved within 24 h in the presence of 8 M urea.

DISCUSSION

Identification of *in vitro* conditions that induce the conversion of PrP^C into PrP^{Sc} is of fundamental importance in understanding the underlying chemistry of prion diseases [2,9,10]. Numerous isoforms of PrP^{Sc}-like proteins have been observed during the progression of the folding and unfolding pathways of prion proteins by different laboratories [22–31]. Among them, an unfolding intermediate exhibiting β -sheet structure was found to exist at pH 3.5 in the presence of 3.5 M urea [23,27]. A similar unfolding intermediate of human prion protein exists at pH 3.6 in the presence of 1–2 M GdmCl [24,25]. However, most of them have not been isolated from the chemical environments that induce their formation. Using mPrP-z, we have demonstrated and confirmed in this study that this unique structural change in the prion protein induced by the combination of acidic pH and mild denaturant concentration is a process that leads to the non-

covalent polymerization of prion protein. Most importantly, we report here the isolation of this soluble aggregate of mPrP (mPrP-z). mPrP-z is stable in acidic solution, and can be removed from the denaturant and isolated by either RP-HPLC or size-exclusion HPLC. The purified product of mPrP-z is also stable at -20°C in freeze-dried form for up to 2 months without any detectable degradation. The mechanism of acid stabilization of mPrP-z is not immediately obvious.

Formation of mPrP-z requires the simultaneous presence of denaturant and acidic pH. Experiments conducted in our laboratory have shown that neither denaturant nor acid solution alone is able to effectively generate mPrP-z. Results from a systematic analysis also revealed two optimal sets of conditions for the production of mPrP-z (Figure 2). These conditions most probably induce the conformational change of mPrP-N to a partially unfolded monomeric intermediate, which then serves as a precursor to form the β -sheet rich oligomer (mPrP-z). Whether acquisition of β -sheet structure occurs before or after polymerization still remains to be determined. The resolution of this question will entail the isolation and characterization of the transient monomeric intermediate. In any case, such a transient intermediate must be partially unfolded, short-lived and prone to form aggregates, and is likely to adopt a unique structure. Another contentious issue is the effect of NaCl on the urea- and acidic-pH-induced transformation of prion protein to the β -sheet rich structure. Surewicz and co-workers [27] reported that NaCl (150 mM) is required for the formation of a β -sheet equilibrium intermediate during the urea (pH 4.0)-induced unfolding of human prion protein. Using the model of mPrP, our data demonstrate that NaCl is not an essential element, although its presence may greatly enhance the formation of mPrP-z under selected conditions (Figure 3B). When unfolding of mPrP-N was carried out at a concentration of urea of 3.5 M and pH 4.0, the inclusion of NaCl (150 mM) improved the yield of formation of mPrP-z by 400%.

mPrP-z shares some structural properties with PrP^{Sc} [9,10]. It exists as an oligomer (approximately a decamer), exhibits a high content of β -sheet structure, but fails to demonstrate resistance to proteolysis. The properties of mPrP-z thus differ from those shown by an oligomer of human prion protein, huPrP-(90–231), produced at pH 4.0 in the presence of 0.5–1.0 M GdmCl [24,25]. As compared with normal huPrP-(90–231), this reported oligomer showed a marked increase in resistance to proteinase K digestion at pH 4.0. mPrP-z and mPrP-N, on the other hand, are about equally susceptible to the protease digestion either at pH 8.0 (Figure 6) or at pH 4.0 (results not shown). It should be mentioned here that mPrP-z may be structurally different (e.g. degree of polymerization) from the oligomer of human prion protein [24] that was generated at pH 4.0 in the presence of 0.5–1 M GdmCl. In addition, proteolysis of the oligomerized human prion protein was conducted in the presence of denaturant [25].

The lack of resistance to proteolysis implies that mPrP-z is unlikely to be a pathogenic form of prion protein. However, it remains possible that mPrP-z may represent an unfolding intermediate that leads to the transformation of PrP^C→PrP^{Sc}. Accumulation of PrP^{Sc} has been shown to occur at the endosomal lumen of scrapie-infected cells [35–38], which contain acidic compartments possessing a pH that is compatible with the conditions used for generating mPrP-z described in the present study. If mPrP-z is indeed an intermediate or a precursor for the formation of toxic prion plaque, then it may also represent a valuable marker for the diagnosis of pre-clinical infection of prion diseases. At present, most diagnoses were confirmed by the post-mortem testing of brain tissue using antibodies raised

against PrP^{Sc}. However, antibodies used in currently approved test systems are not highly specific for PrP^{Sc}. Most of them bind about equally well to both PrP^{Sc} (protease-resistant form of PrP) and PrP^C (protease-sensitive form of PrP). Therefore, samples must be pre-treated with protease to remove any PrP^C before the antibody assay of PrP^{Sc} is performed (for reviews, see [39,40]). Most importantly, post-mortem diagnosis has only limited value in the containment of prion diseases. The most pressing issue in combating the prion disease is the development of a diagnostic tool that can reliably identify animal and human subjects that are incubating the disease, but which show no symptoms. Development of a practical method for detecting pre-clinical prion infection will at the least require production of antibodies that recognize selectively and exclusively the molecules of PrP^{Sc}, its aggregates or intermediates, or precursors and surrogate markers that are specifically associated with the formation of PrP^{Sc}. Isolation of stable mPrP-z has provided a putative candidate for such a marker.

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