# Isolation of Isoforms of Mouse Prion Protein with $PrP^{SC}$-like Structural Properties†

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ABSTRACT: Three novel conformational isomers of mouse prion protein mPrP(23−231) were prepared by incubating the reduced mPrP(23−231) in the presence of urea at mild acidic conditions. They are stable isomers that can be separated and isolated by reversed phase HPLC. These isomers, designated mPrP-**a**, mPrP-**b**, and mPrP-**c**, all exist in reduced state and monomeric form. They all exhibit a high content of $\beta$-sheet structure upon oligomerization at near-neutral pH. They are also partially resistant to proteolysis by proteinase K and chymotrypsin. These structural properties are hallmarks of pathogenic prion protein ($PrP^{SC}$).

Prion infections may cause neuro-degenerative diseases afflicting both humans and animals (*1*−*6*). It has been shown that prion diseases develop by a "protein only" mechanism (*1*, *7*, *8*). The underlying principle of prion disease is the conversion of a host-derived cellular prion protein ($PrP^{C}$)[1] to the infectious scrapie prion protein ($PrP^{SC}$) (*2*, *8*). There is no detectable chemical difference between $PrP^{C}$ and $PrP^{SC}$. Both $PrP^{C}$ and $PrP^{SC}$ have the same molecular weight and amino acid sequence (*9*). They differ by their conformation, and this conformational difference is associated with a considerable dissimilarity of their physicochemical properties. Unlike $PrP^{C}$, which is soluble, susceptible to enzyme digestion, and rich in α-helical structure, $PrP^{SC}$ is highly insoluble, is partially resistant to proteolytic digestion, and has a high $\beta$-sheet content (*8*, *10*). Aside from the distinctive structural properties of $PrP^{C}$ and $PrP^{SC}$, the issue of prion strains (*8*, *11*, *12*), defined by different incubation times to produce disease and varied deposition patterns of $PrP^{SC}$, implies and predicts that prion protein molecules must have the capability of forming multiple and stable conformational isomers with $PrP^{SC}$ properties.

To date, the majority of structural data was obtained from the study of $PrP^{C}$. The 3-D structures of various lengths of mouse $PrP^{C}$ were determined by NMR experiments (*13*−*15*). The well-resolved core domain of the prion protein is formed of three helices and a short two-stranded $\beta$-sheet. On the other hand, structural analysis of $PrP^{SC}$ has been hampered by its intractable solubility. $PrP^{SC}$ can be solubilized and unfolded in solutions containing guanidine thiocyanate and refolded under diluted solutions to a $PrP^{C}$-like state. However, conditions that would allow recovery of prion infectivity have not been identified (*8*, *16*). As a result, numerous efforts have also been pursued to search for conditions that would generate prion protein isomers with increased content of $\beta$-sheet structure and characteristics of $PrP^{SC}$. $PrP^{C}$ contains one disulfide bond, is structurally plastic, and may exist in various conformations depending on the conditions (*8*, *17*). The $\beta$-sheet structure can be attained with or without disruption of the disulfide bond. At elevated temperatures, $PrP^{C}$ converts irreversibly to a stable $\beta$-sheet form (*17*). A scrapie-like prion protein rich in $\beta$-sheet was found to populate as unfolding intermediate at pH 4.0 in the presence of 3.5 M of urea (*18*). A similarly stable unfolding intermediate was observed at pH 3.6−4.0 in 1-2 M GdnCl (*17*, *19*, *20*). Reduction of the disulfide bond in the presence of dithiothreitol (100 mM) and 6 M GdnCl also converts $PrP^{C}$ to a structure that exhibits a high content of $\beta$-sheet, is prone to aggregation, and is resistant to proteinase K (*10*). Synthetic fragments of wild-type PrP were shown to adopt $\beta$-sheet-rich conformations, to form amyloid, but failed to cause disease in rodents (*21*). Cell-free induction of protease-resistant prion protein in the presence of preexisting $PrP^{SC}$ has also been demonstrated (*22*, *23*). Despite mounting efforts, conditions that would permit fractionation and isolation of stable prion protein isomers remain to be identified.

The native form of recombinant mouse prion protein is generally defined by the formation of its single disulfide bond ($Cys^{179}$−$Cys^{214}$) and characterized by a typical α-helical far-UV CD spectra, with minima at 208 and 222 nm (*2*, *3*, *25*). The native mouse prion protein mPrP(23−231) (mPrP-**N**) was prepared by oxidation of reduced inclusion bodies at basic pH in the presence of 8 M urea (*25*). This protocol is remarkable, but at the same time appears to be intriguing and preposterous because a high concentration of urea is known to impede rather than promote protein folding and the formation of native structure. To understand this unusual property of prion protein, we have recently performed a

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[1] Abbreviations: GdnCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; mPrP(23−231), murine prion protein domain 23−231; $PrP^{C}$, cellular prion protein; $PrP^{SC}$, scrapie prion protein; TFA, trifluoroacetic acid; IPTG, isopropyl $\beta$-D-thiogalactopyranoside; DTT, 1,4-dithio-DL-threitol; PMSF, phenylmethanesulfonyl fluoride; HPLC, high-performance liquid chromatography; MALDI-TOD MS, matrix-assisted laser desorption/ionization mass spectrometry with a time of flight detector; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CD, circular dichrosim; BSA, bovine serum albumin; RNase A, ribonuclease A.

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systematic study to examine the effects of pH, redox agents, and denaturants on the behavior of oxidative folding of reduced mPrP(23−231) (mPrP-**R**), which was prepared by treating mPrP-**N** with 6 M GdnCl and 100 mM dithiothreitol (*24*). Our results confirm that the presence of denaturant (urea or GdnCl) is indeed required for the efficient oxidative folding of mPrP-**R**. More specifically, urea and GdnCl are apparently needed for the efficient oxidation of the single disulfide bond, an essential step for the formation of mPrP-**N**. Following removal of the denaturant by dialysis, mPrP-**N** with characteristic α-helical CD spectra was then generated. Our data also reveal many unique properties of mouse prion protein. Numerous factors that are known to facilitate the oxidative folding of disulfide proteins, for instance, alkaline pH and the inclusion of redox agents, have no observed effect on the folding of mPrP-**R** (*24*). These findings lead us to conclude that mPrP-**R** must adopt a rather rigid structure, unlike the conventional form of reduced proteins, which are typically disordered. In addition, mPrP-**R** also exhibits *β*-sheet structure and is prone to form aggregates. These properties, which are structural hallmarks of $PrP^{SC}$ (*2*, *3*), were similarly observed in the case of the reduced form and Cys-free mutant of human prion protein (*8*, *26*).

Most importantly, during the course of our study (*24*), we have observed that under selected conditions, mPrP-**R** displays a tendency to form three additional species that are distinct from mPrP-**N** and amenable to chromatographic separation. These three novel species, designated mPrP-**a**, mPrP-**b**, and mPrP-**c**, accumulated when folding of mPrP-**R** was allowed to proceed in the presence of selected concentrations of urea under a chosen range of pH. They display sufficient stability to be fractionated by reversed phase HPLC using the solvent system of water/acetonitrile/trifluoroacetic acid (TFA). However, the properties of these three species of prion protein have yet to be characterized. Here we extend these studies (*24*) to identify optimal conditions of their preparation and also to purify sufficient quantities of these novel species of prion protein for further characterization of their structures and properties.

## EXPERIMENTAL PROCEDURES

*Materials.* The plasmid pRBI-PDI-T7 for expression of mPrP(23−231) was kindly supplied by Dr. Rudi Glockshuber (ETH, Switzerland). Tryptone and yeast extract were from Fisher Scientific. Isopropyl *β*-D-thiogalactopyranoside (IPTG), 1,4-dithio-DL-threitol (DTT), phenylmethanesulfonyl fluoride (PMSF), and α-chymotrypsin were purchased from Sigma. Proteinase K was from Boehringer Mannheim GmbH (Germany). Amicon ultrafiltration unit and YM10 membranes were from Millipore. Sephadex Fast-Flow and NAP-5 columns were from Pharmacia. All other chemicals were of analysis grade. Protein samples for the refolding study were further purified by reversed phase HPLC.

*Expression and Purification of Recombinant mPrP(23−231).* This was achieved using the method developed by Hornemann et al. (*25*). Protein concentrations were measured by using the Bradford method (from Bio-Rad). The purified protein was verified by Edman sequencing, matrix-assisted laser desorption/ionization mass spectrometry with a time-of-flight detector (MALDI-TOF MS), SDS−PAGE, and circular dichroism (CD).

*Oxidative Folding of mPrP(23−231).* The native protein was first reduced and denatured by incubation in Tris-HCl buffer (0.1 M, pH 8.0) containing 100 mM DTT and 6 M GdnCl at 23 °C for 90 min. The reducing reagent and denaturant were removed by passing the solution through a NAP-5 column (Pharmacia, G-25) equilibrated in 20 mM sodium acetate buffer (pH 4.0). Samples were then made ready for folding experiments by concentration of the protein to 2 mg/mL using Ultrafree centrifugal filters (Millipore, Biomax, 5K). To initiate folding, freshly prepared reduced/denatured protein was diluted to 0.2 mg/mL in buffers of selected pH and concentrations of urea. Folding reactions were performed at 23 °C and trapped in a time course manner by mixing aliquots of the sample with an equal volume of 4% TFA in water. Acid-trapped samples were analyzed by HPLC using the conditions described in the caption of Figure 1.

*Identification of Conformational Isomers of mPrP(23−231).* Prion protein isomers purified by HPLC were freeze-dried and modified with 4-vinylpyridine in the Tris-HCl buffer (pH 8.0) containing 8 M urea. The reaction was allowed for 30 min at 23 °C. Modified samples were desalted by a NAP-5 column using 0.5% TFA or by HPLC using the same conditions as stated in the caption of Figure 1. Samples were analyzed by MALDI-TOF MS.

*Stop/Go Folding of Conformational Isomers of mPrP(23−231).* Purified prion protein isomers were freeze-dried and solubilized (2 mg/mL) in acetate buffer (20 mM, pH 4.0). To start the folding, the samples were diluted with Tris-HCl buffer (0.1 M, pH 8.0) containing 4.4 M urea to a final protein concentration of 0.2 mg/mL. Folding reactions were performed at 23 °C and trapped in a time course manner by mixing aliquots of the sample with an equal volume of 4% TFA in water.

*Measurement of CD Spectrometry.* Far-UV CD was recorded on a Jasco J-715 spectropolarimeter. Protein concentration was 0.2 mg/mL in 20 mM sodium acetate buffer (pH 5.0). The spectra were recorded in a 0.1 cm cuvette in the far-UV region (190-250 nm).

*Molecular Weight Determination by Light Scattering and Mass Spectrometry.* The measurement was performed using a right-angle light scattering instrument (TDA model 301, Viscotek, Houston, TX). The polymerization state of prion protein species was assessed on the basis of the measured molar masses. The triple detector array (light scattering, viscometry, and refraction index) was connected to a Hewlett-Packard 1100 HPLC pump system, an autosampler, and a UV detector. The system was equilibrated with specified buffer at a flow rate of 1.0 mL/min. Samples were injected through the autosampler directly into the detector array. Protein concentration was verified by the on-line UV detector. The average *dn*/d*c* value of 0.175 was selected for all samples. Calculation was completed using the software provided by the manufacturer. The molecular weights of bovine serum albumin (BSA; 66000) and ribonuclease A (13000) were used as standards for calibration. Molecular mass of prion protein isomers were also measured using a MALDI-TOF mass spectrometer from Perceptive Biosystems (Voyger-DE STR).

*Proteolysis of mPrP(23−231).* mPrP(23−231) was digested by α-chymotrypsin and proteinase K in *N*-ethylmorpholin acetate (50 mM, pH 8.1). The ratio of substrate to

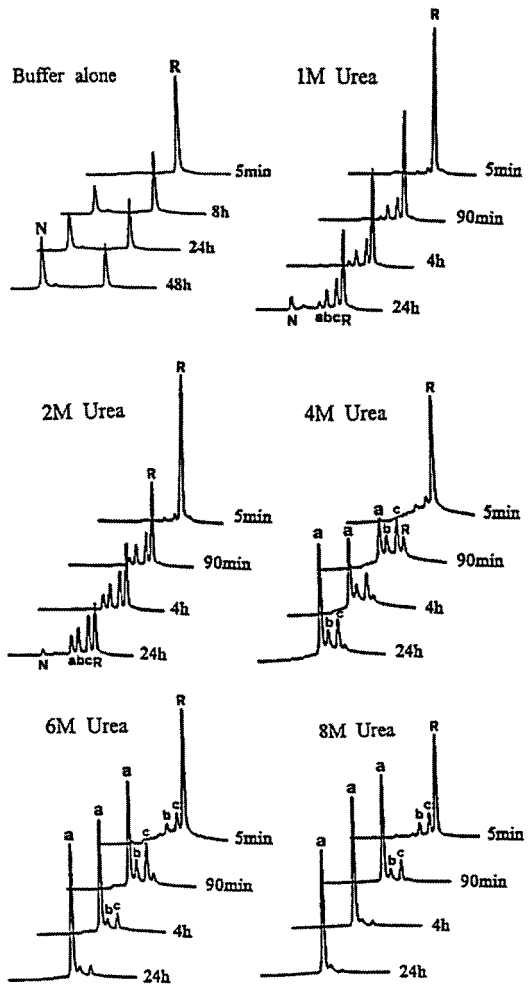


FIGURE 1: Folding of reduced mPrP(23−231) at pH 4.0 in the presence of different concentrations of urea. Folding reactions were performed at 23 °C and trapped in a time course manner by mixing aliquots of the sample with an equal volume of 4% TFA in water. Acid-trapped samples were analyzed by HPLC using the following conditions. Solvent A was water containing 0.1% TFA. Solvent B was 90% acetonitrile in water (by volume) containing 0.1% TFA. The gradient was 28−50% solvent B in 25 min. The column was a Zorbax 300 SB-C18 4.6 mm × 25 cm. The flow rate was 0.5 mL/min. The temperature of the column was 23 °C. **N** indicates the native mPrP(23−231), and **R** indicates the reduced mPrP(23−231). They were eluted at 17.8 and 22 min, respectively. **a**−**c** indicate the three novel isomers of reduced mPrP(23−231).

enzyme was from 100:1 to 1000000:1 (10−0.001 $\mu$g/mL). Proteolysis was achieved at 37 °C for 1 h, stopped by the addition of 2 volume of 4% TFA, and then freeze-dried. Treated samples were analyzed by SDS−PAGE.

## RESULTS AND DISCUSSION

*Identification and Isolation of Novel Species of Mouse Prion Protein.* The studies were conducted by examining the cumulative effects of six different pH values (3, 4, 5, 6, 7, and 8) and six different concentrations of urea (0, 1, 2, 4, 6, and 8 M). The optimal pH for the preparation of these three isomers was found to be 4.0, and the result is illustrated in Figure 1. In the buffer alone, reduced mPrP(23−231) (mPrP-**R**) folds directly to form native (oxidized) species (mPrP-**N**), albeit with a slow rate constant. Approximately 56% of the native mPrP(23−231) were recovered after 48 h. When folding was performed at pH 4.0 in the presence

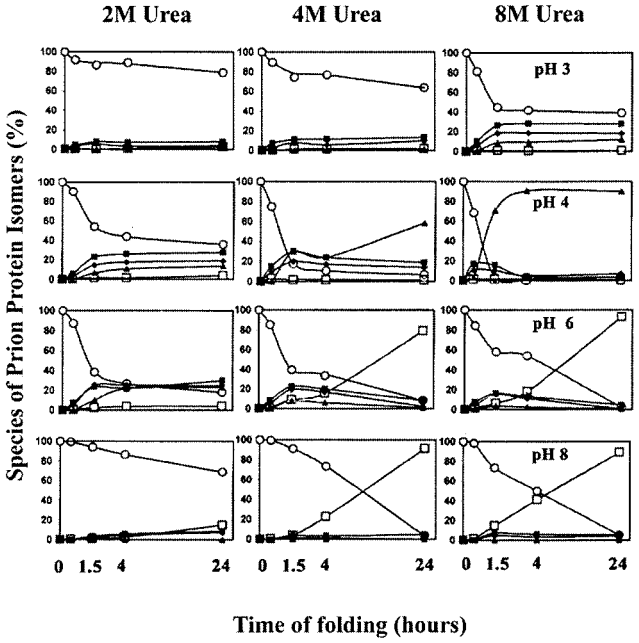


FIGURE 2: Combined effects of varying pH and urea concentration on the folding of mPrP(23−231). Folding was carried out at 23 °C. Protein concentration was 0.2 mg/mL. Folding intermediates were trapped in a time course manner by mixing aliquots of the sample with an equal volume of 4% aqueous TFA and analyzed by HPLC using the conditions described in the caption of Figure 1. Recoveries of five different species of prion protein isomers were based on HPLC peak area integration: (□) mPrP-**N**; (○) mPrP-**R**; (▲) mPrP-**a**; (♦) mPrP-**b**; (■) mPrP-**c**.

of urea, mPrP-**R** was unable to form the native structure. Instead, it converted to three distinct species (mPrP-**a**, mPrP-**b**, and mPrP-**c**) (Figure 1). Their relative recovery depends on the time of folding and the concentration of urea. Prolonged folding in the presence of high concentrations of urea (6−8 M) generates one predominant species (mPrP-**a**) with a purity of >94%. The results found at pH 5 are similar to those obtained at pH 4.0.

As the pH increases from 5.0 to 6.0, the folding behavior of the reduced mPrP-**R** exhibits a dramatic change. Under these conditions, mPrP-**a**, mPrP-**b**, and mPrP-**c** appear as transient intermediates, and mPrP-**R** is able to fold to form mPrP-**N** quantitatively during extended incubation at high concentrations (4−8 M) of urea (Figure 2). Similar results were obtained at pH 7.0. At pH 8, only negligible amounts of mPrP-**a**, mPrP-**b**, and mPrP-**c** were observed and the folding proceeds directly from mPrP-**R** to mPrP-**N** (Figure 2).

Surprisingly, mPrP-**a**, mPrP-**b**, and mPrP-**c** were not detected in the presence of GdnCl. When mPrP-**R** was allowed to refold in buffer at pH 4.0 containing GdnCl (2 or 4 M), >95% of the protein remained as starting material (mPrP-**R**) after 24 h of incubation. mPrP-**R** was also stable at pH 2.0 regardless of the concentration of urea.

*mPrP-**a**, mPrP-**b**, and mPrP-**c** Exist in Reduced State and Monomeric Form.* mPrP-**a**, mPrP-**b**, and mPrP-**c** were separated by reversed phase HPLC and isolated in the HPLC solution that consists of ∼60% water, ∼40% acetonitrile, and ∼0.1% TFA. They are completely stable in lyophilized form when stored at −20 °C for up to 60 days. This was verified by HPLC analysis of lyophilized samples after

Table 1: Molecular Weight of Prion Protein Isomers Measured by Mass Spectrometry and Light Scattering

| | | measured by MALDI | | measured by LS | | |
|---|---|---|---|---|---|---|
| species | expected | before modification[a] | after modification[b] | in HPLC solution[c] (kDa) | at pH 2.0[d] (kDa) | at pH 5.0[e] (kDa) |
| mPrP-**N** | 23107.4 | 23105.9 | 23106.7 | 23.3 ± 0.5 | 28.0 ± 0.4 | 22.3 ± 0.9 |
| mPrP-**a** | 23109.4 | 23107.8 | 23319.5 | 23.2 ± 0.2 | 28.3 ± 2.1 | 288 ± 13.6 |
| mPrP-**b** | 23109.4 | 23107.2 | 23319.7 | 27.5 ± 0.2 | 25.8 ± 0.6 | 290 ± 4.2 |
| mPrP-**c** | 23109.4 | 23108.1 | 23320.1 | 27.6 ± 0.3 | 24.7 ± 0.5 | 220 ± 13.6 |
| mPrP-**R** | 23109.4 | 23107.9 | 23320.7 | 28.5 ± 0.3 | 28.2 ± 0.7 | 211 ± 3.2 |

[a] Determined by MALDI before modification with vinylpyridine. [b] Determined by MALDI after modification with vinylpyridine. [c] Measured by right angle light scattering in the HPLC solution consisting of 60% water, 40% acetonitrile, and 0.1% TFA. [d] Measured by right angle light scattering in the aqueous acidic solution containing 0.2% TFA (pH 2.0). [e] Measured by right angle light scattering in the sodium acetate buffer (20 mM, pH 5.0).

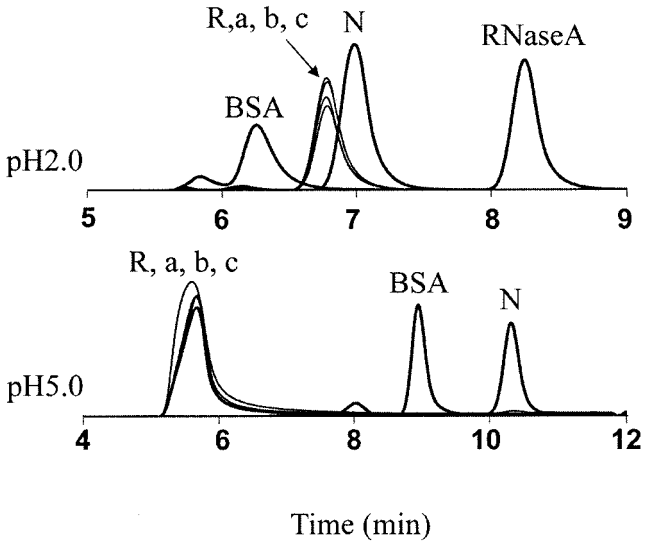


FIGURE 3: Size exclusion chromatography profiles of the native (N) and four reduced isoforms (R, a, b, and c) of mPrP(23−231): (top) samples were eluted with aqueous solution containing 0.2% TFA (pH 2.0); (bottom) samples eluted with sodium acetate buffer (20 mM, pH 5.0) containing NaCl (200 mM). The column was a TSK Gel G3000 SWXL (30 cm × 7.8 mm). The flow rate was 1 mL/min. The column temperature was 23 °C. BSA (66000) and RNase A (14000) were used as standards.

reconstitution in the same HPLC solution used for their isolation (data not shown).

All three species, mPrP-**a**, mPrP-**b**, and mPrP-**c**, contain two free cysteines and exist in reduced state. This evaluation was based on the molecular weight of species modified with vinylpyridine (a cysteine-specific reagent) and measured by MALDI-TOF MS. The data are summarized in Table 1. Modification with vinylpyridine increases the molecular weights of mPrP-**R**, mPrP-**a**, mPrP-**b**, and mPrP-**c** by 210 Da from 23110 to 23320, accounted for by 2 mol of conjugated vinylpyridine (MW = 105). The molecular mass of the native species remains unchanged after modification with vinylpyridine (Table 1).

Because mPrP-**R**, in both cases of human (*8*, *26*) and murine species (*24*), is known to form aggregate, it raises the possibility that mPrP-**a**, mPrP-**b**, and mPrP-**c** may simply represent oligomeric species of mPrP-**R**. To clarify this question, the molecular masses of the three novel species together with mPrP-**R** and mPrP-**N** were analyzed by size exclusion chromatography (Figure 3) as well as right angle light scattering using a Triple Detector Array model TDA 310 (Viscotek) (Table 1). Measurements by these two different techniques were conducted in three different solutions: (1) in the acidic HPLC solution (water/acetonitrile, 60:40 by volume, containing 0.1% TFA) used for isolation of mPrP-**a**, mPrP-**b**, and mPrP-**c**; (2) in the acidic aqueous solution containing 0.2% TFA (pH 2.0); and (3) in the acetate buffer of pH 5.0 (20 mM). In the first two acidic solutions, all species display molecular weights within the limit of 23000−28000, indicating that the vast majority of prion protein species exist in monomeric state (Table 1 and Figure 3). At pH 5.0, mPrP-**N** remains as a monomer. However, mPrP-**a**, mPrP-**b**, mPrP-**c**, and mPrP-**R** rapidly form aggregates, with measured molecular masses ranging from 210000 to 280000 Da (corresponding to decamer) (Table 1; Figure 3).

The only plausible interpretation of these results is that mPrP-**a**, mPrP-**b**, and mPrP-**c** differ from mPrP-**R** by their distinctive conformation rather than the state of oligomerization or the consequence of covalent modification. They also demonstrate that the reduced form of prion protein is capable of adopting diverse conformations that are segregated by energy barrier and stable in the acidic solution.

*Aggregates of mPrP-a, mPrP-b, and mPrP-c Exhibit β-Sheet Structure and Resistance to Proteolysis.* The far-UV CD spectra of mPrP-**a**, mPrP-**b**, mPrP-**c**, mPrP-**R**, and mPrP-**N** were measured both in the acidic solution (0.2% TFA, pH 2.0) and in the sodium acetate buffer (20 mM, pH 5.0). Under both conditions, mPrP-**N** stays as monomer (Table 1) and exhibits a double minimum at 208 and 222 nm, characteristics for α-helical-rich proteins (*27*) and consistent with those observed earlier (*13*, *14*, *25*) (Figure 4). On the other hand, CD spectra of mPrP-**a**, mPrP-**b**, mPrP-**c**, and mPrP-**R** differ significantly from that of mPrP-**N** and are determined by the state of their oligomerization. In monomeric state (pH 2.0), all four reduced isoforms exhibit coil structure with only a single strong dichroic band at ∼200 nm. In oligomeric form at pH 5.0, all reduced isoforms display a single minimum at ∼216 nm, a unique CD signal for proteins rich in β-sheet structure. These results (Figures 3 and 4) clearly show that oligomerization of mPrP-**a**, mPrP-**b**, mPrP-**c**, and mPrP-**R** is accompanied by a conformational change from random coil to predominant β-sheet structure. Whether acquisition of β-sheet structure occurs before or after polymerization remains to be demonstrated. Clarification of this issue will entail isolation and characterization of the transient monomeric intermediate rich in β-sheet structure. Such a transient intermediate, if it exists at all, must be very short-lived and highly prone to form aggregate.

MPrP-**a**, mPrP-**b**, mPrP-**c**, and mPrP-**R** are also distinguished from the mPrP-**N** by their marked resistance against proteolysis. We compared their susceptibility to limited

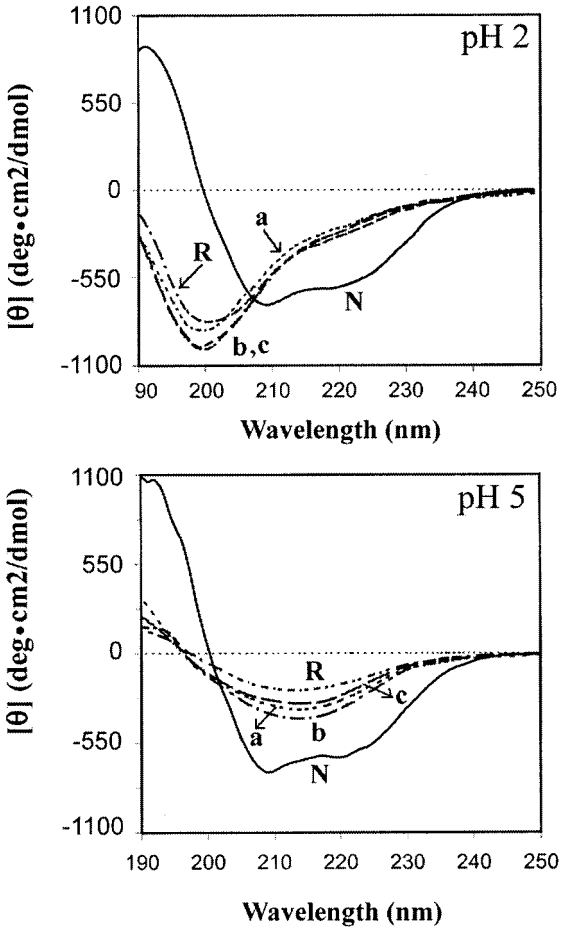


FIGURE 4: Far-UV CD spectra of the conformational isomers of mPrP(23−231): (top) spectra recorded in the aqueous solution containing 0.2% TFA (pH 2.0); (bottom) spectra obtained in the sodium acetate buffer (20 mM, pH 5.0). Solid line represents the spectrum of mPrP-**N**. Dashed and broken lines are spectra of mPrP-**R**, mPrP-**a**, mPrP-**b**, and mPrP-**c**. Protein concentration was 0.2 mg/mL.

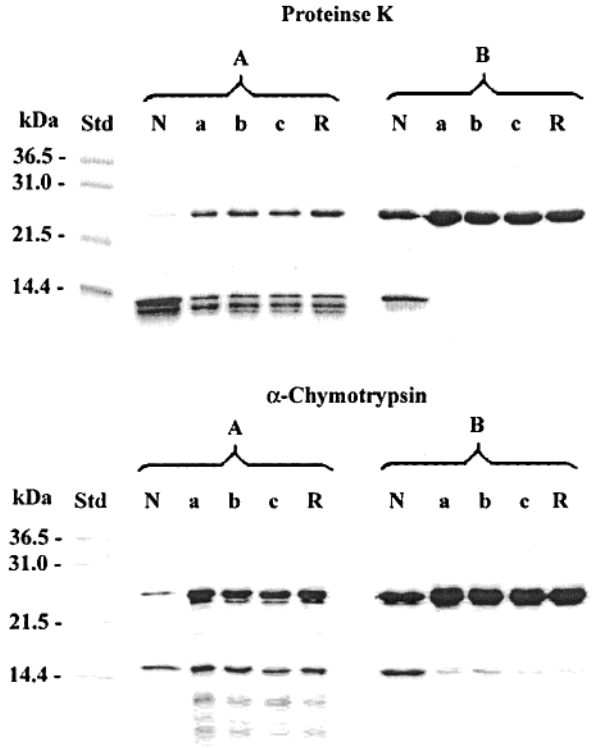


FIGURE 5: Proteolytic digestion of native and isoforms of mPrP-(23−231). mPrP(23−231) isomers were digested by proteinase K and α-chymotrypsin in *N*-ethylmorpholine acetate buffer (50 mM, pH 8.1). Protein concentration was 1 mg/mL. The weight ratios of enzyme to substrate were 1/1000 (A) and 1/10000 (B). Proteolysis was achieved at 37 °C for 1 h and stopped by the addition of 2 volumes of 4% aqueous TFA, freeze-dried, and then analyzed by SDS−PAGE (15%).

proteolysis using both α-chymotrypsin and proteinase K. In both cases, the four reduced isomers were shown to be more resistant than the mPrP-**N** (Figure 5). When digestions of prion proteins (1 mg/mL) were carried out for 1 h at 37 °C with 0.1 and 0.01 $\mu$g/mL of proteinase K and chymotrypsin, the relative susceptibility between mPrP-**N** and the reduced form of prion protein isomers was evident (Figure 5). For instance, in the presence of 0.01 $\mu$g/mL of proteinase K, all reduced forms of isomers remained practically intact, whereas ~50% of the native mPrP(23−231) became fragmented under the same conditions. At protease concentration >0.1 $\mu$g/mL, almost all species of prion protein isomers are completely digested. Resistance against proteolysis is likely to be a consequence of oligomerization of reduced prion protein under the selected experimental conditions. Alternatively, these results may also imply that the four reduced isomers are more stable then the native prion protein.

*Stop/Go Folding of Conformational Isomers of Mouse Prion Protein.* Although mPrP-**a**, mPrP-**b**, and mPrP-**c** are stable as monomer in the acidic HPLC solution, they are able to fold to form the native (oxidized) mPrP(23−231) (mPrP-**N**) after reconstitution in alkaline buffer containing urea. Their folding pathways, carried out in the Tris-HCl buffer (0.1 M, pH 8.0) containing 4 M urea, are shown Figure 6. These three isomers were incapable of converting directly to mPrP-**N**. Instead, all three must fold back to mPrP-**R**, which then converts to the native structure. For mPrP-**a** to reach the native structure, it used pathways via either mPrP-**b** or mPrP-**c** as intermediates; (1) mPrP-**a** ⇒ mPrP-**b** ⇒ mPrP-**R** ⇒ mPrP-**N**; or (2) mPrP-**a** ⇒ mPrP-**C** ⇒ mPrP-**R** ⇒ mPrP-**N**. In contrast to mPrP-**a**, both mPrP-**b** and mPrP-**c** directly fold back first to mPrP-**R** and then to mPrP-**N**. The results also demonstrate that under identical conditions, mPrP-**b** more efficiently converts back to mPrP-**R** and subsequently to the native structure than either mPrP-**a** or mPrP-**c**. It should be mentioned that mPrP-**a**, mPrP-**b**, and mPrP-**c** are not oligomers of mPrP-**R**. Analysis by size exclusion chromatography (TSK Gel 3000SW) and light scattering has demonstrated that mPrP-**a**, mPrP-**b**, and mPrP-**c** differ from mPrP-**R** by their distinctive conformation. However, these conversion reactions are most likely accompanied by reversible oligomerization of prion protein isomers. This is illustrated by the HPLC chromatograms with the reduced recoveries of prion protein isomers and the rising baseline hump during the pathway of conversion (e.g., compare the 5, 15, and 45 min samples of mPrP-**b** conversion to the mPrP-**N** in Figure 6). A systematic analysis of the oligomerization of mPrP-**a**, mPrP-**b**, and mPrP-**c** will be required to understand this side reaction.

The most striking finding of this analysis is that these three isomers together with mPrP-**R** are partitioned by kinetic rather than thermodynamic control. Isomers mPrP-**b** and mPrP-**c** are apparently segregated by an energy barrier that is far greater than that between mPrP-**b** and mPrP-**R** or

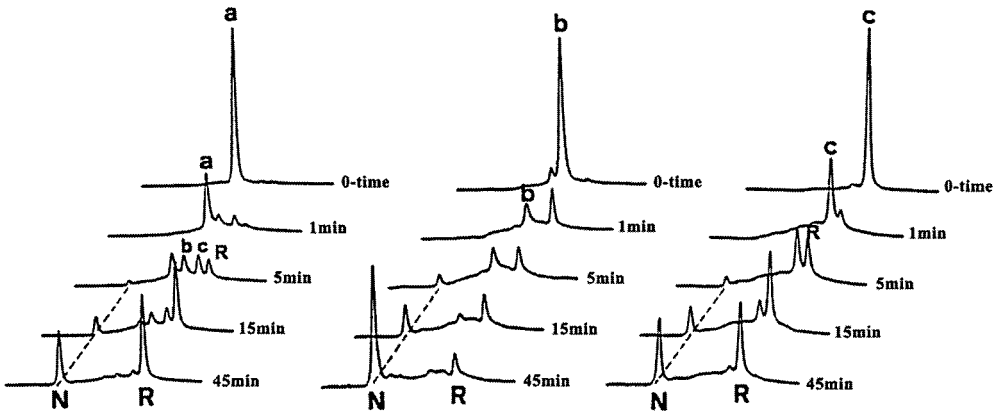


FIGURE 6: Stop/go folding of prion protein isomers mPrP-**a**, mPrP-**b**, and mPrP-**c**. Purified prion protein isomers were freeze-dried and solubilized (2 mg/mL) in acetate buffer (20 mM, pH 4.0). To initiate the folding, the samples were diluted with Tris-HCl buffer (0.1 M, pH 8.0) containing 4.4 M urea to a final protein concentration of 0.2 mg/mL. Folding reactions were performed at 23 °C. Methods for the trapping of the folding intermediates and sample analysis by HPLC are similar to those described in the caption of Figure 1.

between mPrP-**c** and mPrP-**R**. Kinetic partition of protein conformations is not a prevailing phenomenon in the field of protein folding. Nonetheless, it has been demonstrated in numerous cases (*28*, *29*) and has been predicted as one of the unique properties of prion protein molecules (*2*, *8*).

*Reduced Form of Prion Protein Isomers Displays $PrP^{SC}$-like Structural Properties.* The prion disease is caused by the conversion of cellular prion protein ($PrP^{C}$) to the infectious $PrP^{SC}$. According to the "protein-only" model (*2*, *8*), $PrP^{SC}$ differs from $PrP^{C}$ by conformation, acts as template for $PrP^{C} \Rightarrow PrP^{SC}$ transformation and propagation, and exists as diverse strains-associated isomers. Elucidating the underlying mechanism of prion disease will thus demand identification and isolation of stable conformational isomers of prion protein that exhibit $PrP^{SC}$-like structural properties (*8*, *30*).

We have demonstrated in this study that such stable prion protein isomers do exist. Three novel conformational isoforms of mouse prion protein were produced by incubating mPrP-**R** in the mild acidic solution containing urea. MPrP-**a**, mPrP-**b**, and mPrP-**c** are distinguished from $PrP^{C}$ (mPrP-**N**) by a higher content of $\beta$-sheet structure (Figure 3) and a higher tendency to form aggregates (Table 1) and to resist proteolytic digestion (Figure 4). These structural properties are characteristics of infectious $PrP^{SC}$ (*2*). These data clearly demonstrate that mPrP-**a**, mPrP-**b**, and mPrP-**c**, together with the reduced mPrP-**R**, all possess $PrP^{SC}$-like structures. It is important to keep in mind that although the above-mentioned species possess $PrP^{SC}$-like properties, they do not necessarily represent $PrP^{SC}$. The infectious activity of these novel isomers of mouse prion protein, if any, has yet to be verified. Furthermore, $PrP^{SC}$ has been shown to exist in oxidized form and contains a disulfide bond (*30*, *31*). Observations in different laboratories also argue against the involvement of disulfide bond reduction in the pathogenesis of prion disease (*32*, *33*).

Finally, our finding may have raised more questions than it answered. What is the molecular mechanism that stabilizes mPrP-**a**, mPrP-**b**, and mPrP-**c** under acidic conditions? What is their long-term stability under different pH conditions? Do mPrP-**a**, mPrP-**b**, and mPrP-**c** assume defined structures that can be elucidated by NMR or X-ray crystallography? Are mPrP-**a**, mPrP-**b**, and mPrP-**c** isoforms of $PrP^{SC}$, intermediates along the pathway of $PrP^{C} \Rightarrow PrP^{SC}$ transformation, or merely structural artifacts totally irrelevant to $PrP^{SC}$? We need to stress that application of acid trapping was intended to quench the disulfide formation during the oxidative folding of mPrP (*24*). Detection of mPrP-**a**, mPrP-**b**, and mPrP-**c** by acid trapping was serendipitous, not by design. Despite these lingering questions, our experimental data are completely reproducible. These data indicate that the reduced form of mouse prion protein is able to exist as at least four diverse conformational isoforms that are segregated by energy barrier, stable under acidic conditions, and amenable to HPLC separation. The ability to isolate prion protein isomers with $PrP^{SC}$-like structures provides evidence to confirm a unique feature of the dogma of prion disease, that is, the prion protein molecule is capable of adopting stable conformational isomers segregated by kinetic barriers (*8*). Most importantly, it will facilitate the search for potential candidates of pathogenic variants of prion protein.

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## REFERENCES

1. Prusiner, S. B. (1982) *Science 216*, 136−144.
2. Prusiner, S. B. (1999) *Proc. Natl. Acad. Sci. U.S.A. 95*, 13363−13383.
3. Weissmann, C., and Aguzzi, A. (1997) *Curr. Opin. Neurobiol. 7*, 695−700.
4. Hill, A. F., Derbruslais, M., Joiner, S., Sidle, K. C., Gowland, I., Collinge, J., Doey, L. J., and Lantos, P. (1997) *Nature 389*, 448−450.
5. Will, R., Cousens, S., Farrington, C., Smith, P., Knight, R., and Ironside, J. (1999) *Lancet 353*, 9157−9158.
6. Gajdusek, D. C. (1977) *Science 197*, 943−960.
7. Griffith, J. S. (1967) *Nature 215*, 1043−1044.
8. Cohen, F. E., and Prusiner, S. B. (1998) *Annu. Rev. Biochem. 67*, 793−819.
9. Stahl, N., Baldwin, M. A., Teplow, D. B., Hood, L., Gibson, B. W., Burlingame, A. L., and Prusiner, S. B. (1993) *Biochemistry 32*, 1991−2002.
10. Jackson, G. S., Hosszu, L. L. P., Power, A., Hill, A. F., Kenney, J., Saibil, H., Craven, C. J., Waltho, J. P., Clarke, A. R., and Collinge, J. (1999) *Science 283*, 1935−1937.
11. Bessen, R. A., Kocisko, D. A., Raymond, G. J., Nandan, S., Lansbury, P. T., and Caughey, B. (1995) *Nature 375*, 698−700.

12. 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.
13. Riek, R., Hornemann, S., Wider, G., Billeter, M., Glockshuber, R., and Wuthrich, K. (1996) *Nature 382*, 180−182.
14. Donne, D. G., Viles, J. H., Groth, D., Mehlhorn, I., James, T. L., Cohen, F. E., Prusiner, S. B., Wright, P. E., and Dyson, H. J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 3452−3457.
15. Daggett, V. (1998) *Curr. Opin. Biotechnol. 9*, 359−365.
16. Prusiner, S. B., Groth, D., Serban, A., Stahl, N., and Gabizon, R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2793−2797.
17. Zhang, H., Stockel, J., Mehlhorn, I., Groth, D., Baldwin, M. A., Prusiner, S. B., James, T. L., and Cohen, F. E. (1997) *Biochemistry 36*, 3543−3553.
18. Hornemann, S., and Glockshuber, R. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 6010-6014.
19. Swietnicki, W., Petersen, R., Gambetti, P., and Surewicz, W. K. (1997) *J. Biol. Chem. 272*, 27517−27520.
20. Swietnicki, W., Morillas, M., Chen, S. G., Gambetti, P., and Surewicz, W. K. (2000) *Biochemistry 39*, 424−431.
21. Gasset, M., Baldwin, M. A., Lloyd, D. H., Gabriel, J. M., Holtzman, D. M., Cohen, F., Fletterick, R., and Prusiner, S. B. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10940−10944.
22. Caughey, B., Raymond, G. J., Priola, S. A., Kocisko, D. A., Race, R. E., Bessen, R. A., Lansbury, P. T., Jr., and Chesebro, B. (1999) *Mol. Biotechnol. 13*, 45−55.
23. Kocisko, D. A., Come, J. H., Priola, S. A., Chesebro, B., Raymond, G. J., Lansbury, P. T., and Caughey, B. (1994) *Nature 370*, 471−474.
24. Lu, B.-Y., Beck, P. J., and Chang, J.-Y. (2001) *Eur. J. Biochem. 268*, 3767−3773.
25. Hornemann, S., Korth, C., Oesch, B., Riek, R., Wider, G., Wuthrich, K., and Glockshuber, R. (1997) *FEBS Lett. 413*, 277−281.
26. Maiti, N. R., and Surewicz, W. K. (2001) *J. Biol. Chem. 276*, 2427−2431.
27. Woody, R. W. (1995) *Methods Enzymol. 246*, 34−71.
28. Sinclair, J. F., Ziegler, M. M., and Baldwin, T. O. (1994) *Nat. Struct. Biol. 5*, 320−326.
29. Matthews, C. R. (1993) *Annu. Rev. Biochem. 62*, 653−683.
30. Muramoto, T., Scott, M., Cohen, F., and Prusiner, S. B. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 15457−15462.
31. Turk, E., Teplow, D. B., Hood, L. E., and Prusiner, S. B. (1988) *Eur. J. Biochem. 176*, 21−30.
32. Horiuchi, M., and Caughey, B. (1999) *Structure 7*, R231−R240.
33. Hermann, L. M., and Caughey, B. (1998) *Neuroreport 9*, 2457−2461.

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