Partially Unfolded Forms of the Prion Protein Populated under Misfolding-promoting Conditions

CHARACTERIZATION BY HYDROGEN EXCHANGE MASS SPECTROMETRY AND NMR

Roumita Moulick, Ranabir Das, and Jayant B. Udgaonkar

From the National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bengaluru 560065, India

Background: Folding intermediates of proteins are known to initiate misfolding.

Results: Two partially unfolded forms (PUFs) of the prion protein have been characterized structurally and energetically.

Conclusion: One of the PUFs is structurally similar to an initial intermediate in prion misfolding.

Significance: Identification of aggregation-prone intermediates on the prion protein’s folding pathway is the key to understanding its amyloidogenic propensity.

The susceptibility of the cellular prion protein (PrP\text{C}) to convert to an alternative misfolded conformation (PrP\text{Sc}), which is the key event in the pathogenesis of prion diseases, is indicative of a conformationally flexible native (N) state. In the present study, hydrogen-deuterium exchange (HDX) in conjunction with mass spectrometry and nuclear magnetic resonance spectroscopy were used for the structural and energetic characterization of the N state of the full-length mouse prion protein, moPrP(23–231), under conditions that favor misfolding. The kinetics of HDX of 34 backbone amide hydrogens in the N state were determined at pH 4. In contrast to the results of previous studies on the human and Syrian hamster prion proteins at a higher pH, various segments of moPrP were found to undergo different extents of subglobal unfolding events at pH 4, a pH at which the protein is known to be primed to misfold to a \( \beta \)-rich conformation. No residual structure around the disulfide bond was observed for the unfolded state at pH 4. The N state of the prion protein was observed to be at equilibrium with at least two partially unfolded forms (PUFs). These PUFs, which are accessed by stochastic fluctuations of the N state, have altered surface area exposure relative to the N state. One of these PUFs resembles a conformation previously implicated to be an initial intermediate in the conversion of monomeric protein into misfolded oligomer at pH 4.

The conformational conversion of native mammalian cellular prion protein (PrP\text{C}) into misfolded oligomeric or fibrillar forms (PrP\text{Sc}) is a key event in the pathogenesis of transmissible spongiform encephalopathies (1–3). The ability of PrP to convert into misfolded conformations appears to be a consequence of its conformational flexibility (4). Indeed, at pH 4 where the prion protein is prone to misfolding, the native (N) state of PrP has an unusually high specific heat capacity, which is indicative of a very malleable structure (5). It appears therefore that under such conditions, the N state is in dynamic equilibrium with conformations of high energy (N* states) characterized by altered topology and exposure of hydrophobic regions. N* states have been suggested to be the monomeric precursors to amyloid forms during the aggregation of globular proteins under native conditions (6, 7). Hence, a structural and energetic characterization of these high energy states is extremely important for understanding the susceptibility of PrP\text{C} to convert into alternative misfolded forms that may differ in their morphology, toxicity, and other amyloidogenic properties (8–10).

The native state hydrogen-deuterium exchange (HDX) method enables the characterization of high energy intermediates, often termed partially unfolded forms (PUFs), present in equilibrium with the N state without the need of having to perturb the amino acid sequence of the protein. PUFs have been shown to be populated on (11–15) or off (16–18) the folding pathways of many proteins. However, no PUFs could be detected in earlier native state HDX-NMR studies carried out at pH 5.5 on the human PrP and Syrian hamster PrP (19, 20). Nevertheless, folding intermediates could be detected in kinetic studies of the folding of human PrP (21, 22) as well as of pathogenic variants of ovine PrP (23) whose population correlated well with the amyloidogenicity of the protein. However, similar kinetic studies on moPrP at pH 7 could not detect any intermediate forms (24). If the N state of PrP does indeed exist in dynamic equilibrium with PUFs, these PUFs are more likely to be populated to detectable extents under conditions where the N state is destabilized, such as at a lower pH (5, 25).

The propensity of the prion protein to oligomerize or fibrillize is well correlated with acidic pH (26–31); indeed, misfolded-
ing and oligomerization of PrP in the cell may originate in the endocytic pathway in late endosomes or in lysosomes, which have a low internal pH (26, 27). PrP misfolds and oligomerizes at low pH in the presence of 150 mM NaCl (29, 32). The misfolding transition has an apparent pK_a of 4.7, and at pH 4, nearly all (> 95%) of the protein molecules are misfolded and oligomerized (32). Hence, if misfolding does originate from a PUF, then such a form can be expected to be nearly maximally populated at pH 4 where the protein is also destabilized, and the PUF should be detectable by native state HDX carried out at this pH.

In the present study, native state HDX mass spectrometry (HDX-MS) as well as HDX-NMR studies were carried out on the full-length mouse prion protein, moPrP(23–231), at pH 4. At this pH, the protein is known to be primed to misfold, which it does to near completion upon the addition of salt (28, 29, 32, 33). Fortuitously, from the viewpoint of the applicability of the HDX methodology, pH 4 is close to the pH at which the intrinsic rate of HDX is minimal, which allows more amide hydrogens to be probed. Consequently, exchange rates for 34 backbone amide hydrogens were obtained in this study in comparison with the ~23 (19) and ~28 (20) seen in previous HDX-NMR studies that were carried out at a higher pH.

Here, it is shown that the backbone amides of all the residues in the N-terminal unstructured domain as well as 57 of 105 backbone amides of residues in the structured C-terminal domain of moPrP exchange out very fast with those from the C-terminal domain exchanged likely through local structural fluctuations of the backbone. Residues in the two β-strands (β1 and β2), three α helices (α1, α2, and α3), and the loops connecting α1 and β2 are shown to exchange through denaturant-dependent, subglobal unfolding events. The data suggest that the N state is in equilibrium with at least two PUFs. The two PUFs differ in their solvent-exposed surface areas, which are greater than that of the N state, and in their stabilities. In contrast to previous HDX-NMR studies carried out at a higher pH, there is no evidence for residual structure in the unfolded state at pH 4. Importantly, one of the two PUFs appears to be structurally similar to a conformation that has been implicated both in computational (34) and experimental studies (35) to be a crucial initial intermediate in misfolding.

**Experimental Procedures**

**Protein Expression and Purification**

The full-length recombinant mouse prion protein, moPrP (23–231), encoded in the pET-17b(+) plasmid was expressed in *Escherichia coli* BL21(DE3) CodonPlus (Stratagene) cells and purified as described previously (29). The protein was lyophilized and stored at −20 °C. The concentration of the protein was determined by absorbance measurements at 280 nm using an extinction coefficient of 62,160 M⁻¹ cm⁻¹ (36). The recombinant moPrP(23–231) lacks the first 22-residue signal sequence that is cleaved off in the formation of mature protein in vivo.

**Chemicals and Buffers**

All the experiments utilized buffers containing 20 mM sodium acetate and variable concentrations of urea (obtained from USB Corp.) in the range 0–2 M. Urea was deuterated by dissolving it in D₂O, flash freezing the solution, and lyophilizing it. This cycle was repeated three times to ensure complete deuteration of the urea. The deuteration or exchange buffer consisted of 20 mM sodium acetate dissolved in D₂O adjusted to pD 4 (pD = pD_pred + 0.4) using DCI. All solutions were filtered using 0.22-µm Millipore syringe filters before use. The concentrations of urea stock solutions were determined prior to use by refractive index measurements using an Abbe refractometer. All chemicals used were obtained from Sigma (unless mentioned otherwise).

**Peptide Map of moPrP**

Lyophilized protein was dissolved in Milli-Q water at pH 2.5 to a final concentration of 3.5 µM. This sample was injected into the HDX module (Waters) where the sample was digested by pepsin (at a 50 µl/min flow rate), and the peptic fragments were separated on a C₁₈ reverse phase chromatography column using a gradient of 3–40% acetonitrile before being fed into a Waters Synapt G2 HD mass spectrometer. All columns were kept at 4 °C, and the mobile phases were chilled on ice. The fragments were identified using Protein Global Lynx software. The fragments obtained corresponded to all structured parts of the protein including the loops connecting the secondary structures. However, no peptide could be obtained for a stretch of 14 residues (168–181) corresponding to the N-terminal segment of α2.

**Hydrogen Exchange**

**Deuteration of moPrP**—Lyophilized protein was dissolved in deuteration buffer (20 mM sodium acetate dissolved in D₂O at pD 4) to a final concentration of ~20 µM. This stock solution was heated to 65 °C for 10 min to unfold and deuterate the protein, immediately kept on ice for 15 min for refolding, and then kept at room temperature (37). The stock solution was concentrated using a 10-kDa Centricron filter unit from Millipore to 180 µM. The mass of the deuterated protein was checked using a Synapt G2 HD mass spectrometer, and the protein was found to be completely deuterated.

**HDX-MS of moPrP**—Lyophilized protein was dissolved in 20 mM sodium acetate, pH 4 to a final concentration of 20 µM. To initiate exchange, 25 µl of protonated protein were mixed with 475 µl of deuteration buffer in the presence of 0–2 mM urea for varying lengths of time, at 25 °C. The reaction was quenched by mixing with ice-cold 500 mM glycine, pH 2.4, and the solution was desalted into ice-cold water, pH 2.5 using a Sephadex G-25 HiTrap desalting column in conjunction with an ÄKTA Basic HPLC. The desalted samples were injected into the HDX module (Waters) coupled to a nanoACQUITY UPLC. A gradient of 3–40% acetonitrile (0.1% HCOOH) at a flow rate of 40 µl/min was used for elution of the protein from an analytical C₁₈ reverse phase chromatography column in 10 min. The extent of exchange was determined by measuring the increase in the mass of the protein in the Synapt G2 HD mass spectrometer.

For experiments in which HDX was followed by pepsin digestion, the protein was dissolved to a final concentration of 180 µM in 20 mM sodium acetate, pH 4. For online pepsin digestion, a flow rate of 50 µl/min of water (0.05% HCOOH) was
used. The peptides eluted were collected using a peptide trap column, washed to remove salt, and eluted as above. All columns were kept at 4 °C in the cold chamber of the HDX module. The peptides separated on the column were detected using the Synapt G2 HD mass spectrometer. The mass spectrometer parameters were set as follows: source temperature, 35 °C; desolvation temperature, 100 °C; capillary voltage, 2.8 kV. To determine the number of exchanged deuteriums that are lost due to back-exchange during sample processing after quenching of the exchange reaction, 25 μl of completely deuterated protein were diluted in 475 μl of deuterium buffer, the reaction was quenched as above, and the sample was then processed in an identical way.

**Data Analysis**

Pepsin digestion and separation of the pepitic fragments of the protonated protein labeled in deuteration buffer for different times yielded pepitic fragments (corresponding to sequence segments in the intact protein) with differential retention of protons with time. The percentage of proton retention in each pepitic fragment was determined using Equation 1.

\[
\% \text{ H Retention} = \frac{(M_2 - M(t)) \times 100}{(M_0 - M_p)} \tag{Eq. 1}
\]

Here, \(M(t)\) is the centroid mass of the isotopic envelope of the peptide at time \(t\) of exchange, \(M_0\) is the centroid mass of the isotopic envelope of the protonated peptide obtained from protonated protein, and \(M_p\) is the centroid mass of the isotopic envelope of the peptide obtained from completely deuterated protein that had been processed as indicated above. The plot of percent hydrogen retention versus \(t\) was fit to an exponential decay equation to yield the observed exchange rates \(k_{obs}\) and amplitudes of the different kinetic phases of the exchange process. The HDX reaction under the given experimental conditions occurs in the EX2 limit, and hence, the observed exchange rates can be used to calculate the free energy of opening of structure in the sequence segment, to exchange, using Equation 2 (13).

\[
\Delta G_{op} = RT \ln \frac{k_{int}}{k_{obs}} \tag{Eq. 2}
\]

Here, \(k_{int}\) is the intrinsic exchange rate, which is the rate of exchange in an unstructured peptide of identical sequence (38). In using Equation 2, \(k_{int}\) for each sequence segment was determined as the average \(k_{int}\) of those residues in the segment that were identified separately by HDX-NMR of the intact protein to have the slow exchanging amide hydrogens. \(R\) and \(T\) are the universal gas constant and the temperature at which the exchange reaction is carried out, respectively. The difference in the centroid mass of the isotopic envelope of the peptide at time \(t\) of exchange and that of the completely protonated peptide was used to obtain the number of deuteriums incorporated with time (Table 1).

**Sequential Backbone Assignment of the NMR Spectrum of moPrP**

Two-dimensional 15N heteronuclear single quantum coherence, HNCO, HNCA, HNCA-CA, HNOCAB, and HNCA-CA resonance experiments were carried out on 300 μM moPrP in 20 mM sodium acetate at pH 4 (in the presence of 5% D2O) for the backbone assignment. Data were processed using NMRPipe, and the assignment was done using the NMR data visualization and assignment software Sparky. A list of the assigned residues is given in supplemental Table S1. The NMR spectrum of the protein collected at pH 4 in this study is indistinguishable from that collected at pH 4.5 (39).

**HDX-NMR**

A 1H-15N two-dimensional selective optimized flip angle short transient heteronuclear multiple quantum coherence spectrum (40) was collected as the reference unexchanged sample spectrum by dissolving lyophilized protein in 20 mM sodium acetate, pH 4, 95% H2O, 5% D2O buffer to a final concentration of 300 μM. To monitor exchange, lyophilized protein was dissolved in 20 mM sodium acetate (95% D2O), pH 4 to a final concentration of 300 μM (exchange sample). A series of 1H-15N two-dimensional selective optimized flip angle short transient heteronuclear multiple quantum coherence spectra of the exchange sample were collected up to 40 days following an initial dead time of 10 min. The two-dimensional spectra were collected on a Bruker 800-MHz spectrometer with 1024 × 256 \((t_1 \times t_2)\) time points totaling up to an acquisition time of 5 min and processed using NMRPipe and Sparky. The NMR spectrum of the unexchanged sample was corrected for intensity differences arising due to differences in matching, tuning, and shimming by multiplying with the ratio of the intensity of the Cys213 amide (an isolated peak that exchanges on a 1-month time scale) in the unexchanged spectrum to that of the Cys213 amide at 10 min of exchange. This was the intensity-corrected unexchanged spectrum. The signal intensity for each residue in all exchange spectra was normalized with the signal intensity of that residue in the intensity-corrected unexchanged spectrum. The resulting decrease in the normalized signal intensity with increasing time of exchange was converted into a percent hydrogen occupancy versus \(t\) plot and fit to an exponential decay equation to yield an observed exchange rate. This rate was used to calculate the free energy of opening of structure to exchange using Equation 2. The intrinsic rates of exchange for individual residues were obtained as described elsewhere (38).

**Far-UV Circular Dichroism Spectra of moPrP at 25 °C**

Far-UV CD spectra of moPrP at pH 4 in 20 mM sodium acetate, and at pH 7 in 20 mM sodium phosphate were acquired on a Jasco J-815 spectropolarimeter using a protein concentration of 10 μM in a 0.1-cm-path length cuvette with a bandwidth of 1 nm, a scan speed of 50 nm/min, and a digital integration time of 1 s.

**1-Anilino-8-naphthalene Sulphonate (ANS) Binding of moPrP**

2 μM moPrP was incubated at 25 °C with 20 μM ANS dye in 20 mM sodium acetate buffer at pH 4 and in 20 mM sodium phosphate buffer at pH 7. The fluorescence spectra were obtained by exciting the samples at 385 nm and collecting the emission spectra from 400 to 600 nm.
Misfolding and Oligomerization of moPrP

For studying the misfolding of moPrP at pH 4 and pH 5.5, 100 μM moPrP was incubated at 25 °C for different times up to 3 weeks and at 37 °C for different times up to 24 h in 150 mM NaCl at the desired pH. 20 mM sodium acetate and 20 mM 2-(N-morpholino)ethanesulfonic acid were used as buffers at pH 4 and pH 5.5, respectively. To study the extent of misfolding, each sample was diluted 10-fold in the same buffer, and the CD spectra were acquired immediately on a Jasco J-815 spectropolarimeter using a 0.1-cm-path length cuvette with a scan speed of 50 nm/min, and a digital integration time of 1 s. The percentage of misfolding of moPrP was calculated from the CD spectra by monitoring the decrease in CD signal at 216 nm, a scan speed of 50 nm/min, and a digital integration time of 1 s. The percentage of misfolding was initiated by the addition of 150 mM NaCl. b shows the kinetics of misfolding at pH 4 (circles) and at pH 5.5 (squares) at 37 °C. Misfolding was monitored by the measurement of the CD signal at 216 nm. The error bars represent standard deviations from three independent experiments. The solid lines are exponential fits through the data. An earlier study has shown that the time course of misfolding as monitored by CD is the same as the time course of oligomerization as measured by size exclusion chromatography (32, 35). MRE, mean residue ellipticity; deg, degrees.

Results

Upon lowering the pH to 4, moPrP is known to become primed for misfolding and oligomerization. Fig. 1 shows that when the misfolding of moPrP was initiated by the addition of 150 mM NaCl at two different temperatures moPrP misfolded considerably faster at pH 4 than at pH 5.5. Fig. 1a shows that after 3 weeks of incubation in the presence of 150 mM NaCl at 25 °C, moPrP had misfolded completely into a β-sheet rich structure at pH 4, whereas it had not undergone any significant change in secondary structure at pH 5.5. The misfolding reaction at pH 5.5 at 25 °C was too slow for its rate to be measured. However, at 37 °C, the misfolding reactions became substantially faster. Fig. 1b shows that at 37 °C in the presence of 150 mM NaCl, moPrP misfolded completely within 24 h at pH 4 and that the reaction was much faster than at pH 5.5. Hence, in this study, the cooperativity of the unfolding reaction of moPrP was studied by carrying out native state HDX at pH 4 at 25 °C, in the presence of different concentrations (0–2 M) of urea. The HDX reaction was monitored by mass spectrometry for different times ranging from 5 s to 40 days (Fig. 2a). The slow exchanging amide hydrogens within each sequence segment studied by HDX-MS were identified by HDX-NMR of the intact protein at pH 4 at 25 °C, in the absence of urea.

Native State HDX of the moPrP at pH 4 Occurs in the EX2 Limit—When the unfolding and refolding kinetics of the structured C-terminal domain of moPrP (W144F/F174W variant of moPrP(121–231)) was studied using a continuous flow mixer at 25 °C and pH 4, the protein was found to refold with a rate of 11,000 s⁻¹. This refolding rate is much faster than the intrinsic rate of change, which is 0.01 s⁻¹, under the given exchange conditions. Hence, HDX in moPrP must occur in the EX2 limit (41). This is also evident from the observation that the single peak of the 27th charge state in the mass spectrum of moPrP shifted gradually to a higher mass to charge ratio (m/z) with increasing times of exchange (Fig. 2a) as expected when HDX occurs in the EX2 limit.

To obtain structural information, moPrP that had undergone HDX for a variable time as indicated above, was proteolyzed by passing through an online pepsin column. Pepsin cleaved the protein into fragments, each fragment corresponding to a segment of the protein. The increase in the m/z value, which is a measure of the number of backbone amide hydrogens that have exchanged with deuterium, was converted into percent hydrogen retention using Equation 1. Fig. 2b shows the mass spectra of three different sequence segments of the protein that correspond to three structured parts of the protein, with increasing time of HDX in the absence of denaturant. A gradual shift in the centroid of the peak (shown as a dashed line) to higher values as a result of the increase in the number of deuteriums incorporated is observed with increasing time of exchange. The three sequence segments of the protein are seen to differ greatly in their rates of exchange of their backbone amide hydrogens.

Fig. 3 shows the decrease in percent hydrogen retention with increasing time of HDX for five different sequence segments of the protein and the dependences of their rates of HDX on urea concentration. The data for sequence segment 149–153, which corresponds to the C-terminal end of α1, and segment 205–212, which corresponds to the central region of α3, fit to a single exponential equation, and the corresponding HDX rates differ greatly in their rates of exchange of their backbone amide hydrogens.

4 R. Moulick, R. Goluguri, and J. B. Udgaonkar, unpublished data.
increased with the increase in urea concentrations. The data for sequence segment 155–162, corresponding to the loop between α1 and α2 including β2, fit to a two-exponential equation, which corresponds to two kinetic phases of exchange. The fast rate is independent of urea concentration and is similar in value to the $k_{int}$ of HDX (38), whereas the slow exchange rate is dependent on urea concentration. The fast rate of HDX for segment 155–162 therefore corresponds to the average rate of HDX for the unprotected amides in the segment, and the slow rate corresponds to the average rate of HDX for the protected amides in the segments. The data for segment 190–197, which corresponds to the C-terminal end of α2 and the loop between α2 and α3, fit to a single exponential equation and has an exchange rate that is independent of urea concentration. For the segment 204–224, which spans α3, the amide hydrogens exchange out in three kinetic phases, a fast phase independent of urea concentration and two slow phases of exchange that are dependent on urea concentration with distinct rates.

Table 1 lists the various structured parts of moPrP with the sequence segments obtained from proteolytic fragmentation of the protein. The number of exchangeable backbone amide hydrogens in any segment is given by the number of backbone amide hydrogens corrected for the number of proline residues in the peptic fragment corresponding to the sequence segment. The first and second residues from the N terminus of any peptic fragment are known to exchange their amide hydrogens rapidly and hence have not been counted (42–44). The number of backbone amide hydrogens that exchange is obtained from the amplitudes of multiexponential fits to the time dependence of the number of deuteriums incorporated (not shown) in the given segment. Due to a loss in backbone amides because of back-exchange, the number of backbone amide hydrogens observed to exchange is lower than expected. Since, the percent hydrogen retention versus time curves were found to fit to a multiexponential equation, the relative amplitudes and the observed exchange rate of each kinetic phase for each sequence segment are shown. Here, for a given segment, the $k_{int}$ value is
Partially Unfolded Forms of Prion in Misfolding Conditions

FIGURE 3. HDX-MS of backbone amide hydrogens of moPrP in deuterated solvent at 25 °C and pH 4. The percent hydrogen retention versus time data for five peptide fragments, 149–153, 155–162, 190–197, 204–224, and 205–212, in the presence of 0, 1, and 2 M urea data, respectively, which belong to different structural parts of the protein. Tyr127, Ile138, Met153, Val160, Asn180, Lys203, Gln211, and Gln216 are residues in the N-terminal, central, and C-terminal regions of α3, respectively. The identification of the individual amide hydrogens that exchange slowly by HDX-NMR enabled the identification of the subset of residues within each proteolytic fragment whose exchange was studied by HDX-MS. The slow exchanging residues identified by HDX-NMR experiments are listed in supplemental Table S1 and Fig. 6.

Evaluation of Stability at the Residue Level Obtained from HDX-NMR Study on moPrP in the Absence of Urea—The HDX-NMR study on moPrP at pH 4 and 25 °C enabled the determination of the rates of exchange for the backbone amides of 34 individual residues. The slow exchanging amides observed in the HDX-MS experiments for each sequence segment could be identified by the HDX-NMR experiment (Fig. 6 and Table 2), making use of the fact that the slow exchanging residues are unlikely to be the first two residues in the proteolytic fragment corresponding to the sequence segment (42–44). A detailed description of the identification of the slow exchanging residues observed in HDX-MS by HDX-NMR is given in the supplemental results. Table 2 also includes the slow exchanging residues observed by HDX-NMR that were not observed by HDX-MS. The rates of exchange of different residues obtained from HDX-NMR and their individual $k_{\text{int}}$ rates were used to calculate $\Delta G_{\text{prop}}$ for each residue as shown in Fig. 6. Fig. 6a shows the $\Delta G_{\text{prop}}$ values for the exchange of these 34 amides mapped onto the sequence, and Fig. 6b shows these residues mapped onto the structured, globular domain of moPrP (moPrP(121–231)).

The identities of the slow exchanging residues obtained from HDX-NMR were used to evaluate $\Delta G_{\text{prop}}$ of the different proteolytic fragments for a range of denaturant concentrations. Fig. 7
TABLE 1
Parameters obtained from native state HDX of moPrP at pH 4 at 25 °C monitored by mass spectrometry
The relative amplitudes and rates of the multiple kinetic phases of exchange for the peptic fragments corresponding to sequence segments of moPrP were obtained from native state HDX-MS. In HDX-MS experiments, the amide hydrogens belonging to the first two residues of the peptide fragment corresponding to any sequence segment exchange too fast for their exchange to be observed (42–44). In sequence segment 127–132, it appears from the HDX-NMR data that Met128 may not be exchanging fast.

<table>
<thead>
<tr>
<th>Secondary structure</th>
<th>Sequence segment</th>
<th>No. of exchangeable backbone hydrogens</th>
<th>No. of deuteriums incorporated</th>
<th>Amplitude k_{int}</th>
<th>Exchange rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>β1</td>
<td>127–132</td>
<td>4</td>
<td>Fast, 1</td>
<td>43.0 ± 1.3</td>
<td>4×10^{-3}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slow, 1</td>
<td>49.0 ± 1.2</td>
<td>1×10^{-1}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slow, 1</td>
<td>52.5 ± 1.8</td>
<td>9×10^{-4}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slow, 1</td>
<td>37.9 ± 1.5</td>
<td>1×10^{-2}</td>
</tr>
<tr>
<td>α1</td>
<td>133–148</td>
<td>5</td>
<td>Fast, 2</td>
<td>32.9 ± 1.6</td>
<td>5×10^{-3}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slow, 3</td>
<td>54.4 ± 1.4</td>
<td>1×10^{-2}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slow, 2</td>
<td>88.8 ± 0.7</td>
<td>1×10^{-2}</td>
</tr>
<tr>
<td>Loop between α1 and α2 including β2 strand</td>
<td>154–167</td>
<td>9</td>
<td>Fast, 2</td>
<td>25.2 ± 2.2</td>
<td>3×10^{-3}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slow, 5</td>
<td>66.8 ± 2.5</td>
<td>2×10^{-5}</td>
</tr>
<tr>
<td>α2 and loop between α2 and α3</td>
<td>182–196</td>
<td>13</td>
<td>Fast, 3</td>
<td>71.1 ± 2.6</td>
<td>5×10^{-3}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slow, 1</td>
<td>23.3 ± 2.4</td>
<td>2×10^{-5}</td>
</tr>
<tr>
<td>Loop between α2 and α3</td>
<td>190–197</td>
<td>6</td>
<td>Fast, 2</td>
<td>90.9 ± 1.6</td>
<td>8×10^{-3}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slow, 3</td>
<td>62.4 ± 5.5</td>
<td>9×10^{-3}</td>
</tr>
<tr>
<td>α3</td>
<td>197–204</td>
<td>6</td>
<td>Fast, 1</td>
<td>24.6 ± 1.4</td>
<td>3×10^{-3}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slow, 3</td>
<td>69.4 ± 1.2</td>
<td>1×10^{-3}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slow, 1</td>
<td>18.4 ± 1.8</td>
<td>1×10^{-2}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slow, 2</td>
<td>70.2 ± 1.7</td>
<td>7×10^{-3}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slow, 3</td>
<td>27.4 ± 1.9</td>
<td>8×10^{-3}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slow, 5</td>
<td>20.9 ± 2.3</td>
<td>3×10^{-4}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slow, 1</td>
<td>51.1 ± 2.8</td>
<td>5×10^{-3}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slow, 1</td>
<td>37.0 ± 2.7</td>
<td>1×10^{-4}</td>
</tr>
</tbody>
</table>

FIGURE 4. Native state HDX of moPrP at pH 4 and 25 °C monitored by NMR. a–d show the progressive decrease in 1H–15N cross-peak intensities of a few representative residues, Asn155, Tyr159, Arg155, Tyr159, Asp157, Asn150, Asp155, Arg156, and Gin111; at 0 s, 10 min, 1 h, and 4 h of HDX, respectively.

shows the denaturation dependences of the stabilities of different segments of the protein. The stability of a proteolytic fragment was determined using the slower rates of HDX obtained from the exponential fits to the plots of percent hydrogen retention versus time of exchange. Here, the k_{int} for any segment is the average of the intrinsic rates of exchange of all slow exchanging residues in the segment identified by HDX-NMR. For all segments, the values obtained for ΔG_{op} are lower than the free energy of global unfolding (5). The exception is sequence segment 205–212 for which ΔG_{op} values correspond to the global unfolding free energy. The dependence of ΔG_{op} on urea concentration obtained from HDX-MS studies for different segments appears to merge with the global free energy of unfolding (ΔG_{u}) at denaturant concentrations greater than 2 M. In all cases, the change in accessible surface area upon unfolding, which is represented by the slope (m_{op}) of the linear fit to the urea concentration dependence of ΔG_{op}, is lower than the slope (m_{u}) of the linear least square fit to the urea concentration dependence (ΔG_{u}) obtained from global unfolding studies.

Fig. 8 is a plot of the ΔG_{op} of each segment in the absence of denaturant versus m_{op}. The ΔG_{op} values were observed to range from 2.0 to 4.8 kcal mol^{-1}, and the m_{op} values ranged from 0.3 to 1.1 kcal mol^{-1} M^{-1}. The values of ΔG_{op} and m_{op} for segments 190–197 and 197–201 were negligible, indicating that this region of the protein undergoes very fast exchange, exposing negligible surface area while doing so.

Discussion
The molecular mechanism of conversion of PrP^C to PrP^Sc, which is the fundamental event in almost all prion diseases, is still unknown. Studies indicating the unusual conformational plasticity of PrP^C (4, 5, 45) have led to the hypothesis that partially unfolded intermediate conformations referred to as PrP^st states are populated due to stochastic fluctuations of the native state, which then act as monomeric precursors to oligomeric or fibrillar forms (46). A battery of structural probes with an extensive set of perturbants of the native structure has been used to investigate the folding energy landscape of different prion proteins in an exhaustive search for partially structured intermediates. Studies of chemical denaturant-induced folding monitored by global probes such as fluorescence and circular
Partially Unfolded Forms of Prion in Misfolding Conditions

FIGURE 5. HDX-NMR of backbone amide hydrogens of moPrP in deuterated solvent at 25 °C and pH 4. a–h show the change in percent hydrogen occupancy with increasing times of exchange for residues Tyr127, Ile138, Met153, Val160, Asn180, Lys204, Gln211, and Gln216. The solid line through each curve represents an exponential decay fit through the data.

dichroism have identified unfolding or refolding intermediates (21–23), but little structural information is available on them. NMR studies of the relaxation dynamics as well as of the effects of pressure-induced and thermally induced perturbations have revealed the presence of disordered conformations in equilibrium with the native state (47–51). Misfolded conformations can be selectively populated by refolding unfolded prion protein in solvents having high ionic strength in the presence of denaturant, and such intermediate conformations may be monomeric or oligomeric (28, 30, 33, 52, 53). However, previous native state HDX-NMR studies were unsuccessful in identifying such intermediate conformations in equilibrium with the native state under non-perturbing solvent conditions (19, 20). The current study identified partially unfolded intermediate conformations under solvent conditions where the protein is monomeric and native, but destabilized and aggregation-prone.

moPrP Has Native Structure at pH 4—Although the stability of moPrP decreases by 1.3 kcal mol−1 with a decrease in pH from 7 to 4 (5, 54), the native structure remains intact. The circular dichroism spectra of moPrP at pH 4 and pH 7 are very similar but not identical as shown in Fig. 9a. The differences most likely arise due to the pH-induced disruption of hydrophobic contacts in the C-terminal region of α2, as reported in earlier studies (31, 55). To investigate whether moPrP at pH 4 has molten globule-like properties, ANS binding studies were carried out at a protein:ANS concentration ratio of 1:10 at pH 4 and pH 7. Fig. 9, b and c, indicate that moPrP did not bind to ANS at either pH. All these studies indicate that moPrP at pH 4 is monomeric and native and lacks molten globule-like properties.

Denaturant-independent Exchange—moPrP has a total of 105 residues in the structured domain of the protein involved in the formation of the two antiparallel β-strands and three α-helices with interconnecting loops as shown in Fig. 6b. Of these, only 34 residues exhibit slow exchange in a denaturant-dependent manner as identified by HDX-NMR experiments (supplemental Table S1). However, all 34 slow exchanging backbone amide hydrogens studied by HDX-NMR could not be studied by HDX-MS because some of the amide hydrogens belonged to residues that happened to be the first two residues in the proteolytic fragments corresponding to the sequence segments and hence, exchanged very fast in the fragments (42–44). In addition to these 34 residues, overlapping residues Arg155 and Asp177, Thr182 and Thr215, and Val202 and Val208 were also observed to be slow exchangers. However, because of the overlap of their peaks in the NMR spectrum, the individual exchange rates were not determined. Of the 99 residues that were assigned, 57 residues showed denaturant-independent exchange. Although, the exact mechanism of denaturant-independent exchange is unclear, previous studies have proposed a “local structural fluctuation” (56) model, which posits a transient opening of the exchanging residues one at a time without significant surface area exposure. Alternatively, a statistical-mechanical approach proposes denaturant-induced modulation of populations of exchange-competent (open) and exchange-incompetent (closed) states to result in no net change in ΔGop with a change in denaturant concentration (57). Residues in the loops are expected to exchange fast as loops are flexible and lack any ordered structure. This was observed in moPrP where, with the exception of Ile138, all residues in the loops linking β1, α1, β2, α2, and α3 exchange out at very fast rates that could not be determined by HDX-MS or HDX-NMR. However, residues in the N-terminal region of α1, two of which are H-bonded (Glu45 to Asn132 and Asp146 to both Asn142 and Asp143) and residues in the N- and C-terminal regions of α2 and α3 were also seen to exchange out in a denaturant-independent manner. It also seems that the N-terminal region of α1 is more dynamic than the rest of the helix. The C-terminal region of α2 has been shown by studies of NMR relaxation dynamics to be destabilized due to the protonation of His186 at low pH, resulting in the disruption of hydrophobic forces and increased fluctuations (55). At pH 3.6, this region becomes disordered. Molecular dynamics simulations indicate that the residues in

\[ \text{equation} \]

\[ \text{equation} \]

\[ \text{equation} \]
this region are “frustrated” in the helix and would be better accommodated in either a strand or in coiled structures (58, 59). Stabilization of this region by mutagenesis prevents the misfolding and oligomerization of the protein (32). A conformationally dynamic region is expected to exchange out fast in an HDX study. Incidentally, this region was observed to exchange very fast in previous HDX studies done at higher pH as well (19, 20).

Evidence for Subglobal Unfolding Events—Previous HDX-NMR studies were done at higher pH where the intrinsic rates of exchange are fast, and hence, only 23–28 residues could be studied (19, 20). In contrast, at the lower pH of 4, the exchange rates of 34 amide hydrogens could be determined.

The β1 strand, whose exchange could not be studied previously (19, 20), was observed to exchange out in a denaturant-dependent manner with an $m_{op}$ value of 0.4 kcal mol$^{-1}$ M$^{-1}$.

The β1 strand is docked against the loop 120–126 and β2 strand and seems to undergo partial unfolding and consequently significant change in surface exposure during exchange. Figs. 6a and 7a show the $\Delta G_{op}$ values for residues Tyr127 and Met128 in β1 as obtained from HDX-NMR and HDX-MS studies, respectively.

Most residues in the loop connecting the β1 strand and the N-terminal region of α1 exchange out at a very fast rate. Only residue Ile138 was observed to exchange slowly by both HDX-MS and HDX-NMR. The amide hydrogen of Ile138 is proximal to the methyl group of Met137 and the aromatic ring of Tyr149 and may be sterically hindered from exchange. Hence, local unfolding of the region would be essential for exposing the residue to solvent for exchange as evident from the exchange process having a denaturant dependence of 0.4 kcal mol$^{-1}$ M$^{-1}$.
The N-terminal residues in \( \alpha_1 \) exchange out with very different exchange rates when compared with the C-terminal residues in \( \alpha_1 \). The N-terminal residues Asp\(^{143} \)-Asp\(^{146} \) exchange fast in a denaturant concentration-independent manner. Arg\(^{147} \) and Tyr\(^{148} \) exchange with an \( m_{op} \) value of 0.4 kcal mol\(^{-1} \)M\(^{-1} \). The dependence of exchange on denaturant concentration for these residues is significantly different from that of the C-terminal region of \( \alpha_1 \) (residues Tyr\(^{149} \)-Asn\(^{152} \)), which shows a higher stability at 0M urea (Fig. 7, b and c) and an \( m_{op} \) value of 0.7 kcal mol\(^{-1} \)M\(^{-1} \). Residues Met\(^{153} \)-Tyr\(^{156} \) in the loop connecting \( \alpha_1 \) and \( \alpha_2 \) have exchange rates and consequently \( \Delta G_{op} \) values similar to residues in the C-terminal region of \( \alpha_1 \). This region shows a dependence of exchange rates on denaturant concentration identical to that of the C-terminal end of \( \alpha_1 \), which is unusual for residues in loop regions. It has been reported in the case of human PrP that the C-terminal residues 153–156 of \( \alpha_1 \) undergo a pH-induced elongation to form a 3\(_{10}\) helix at neutral pH (60) that becomes disordered at lower pH. In moPrP, residues Met\(^{153} \)-Tyr\(^{156} \) were observed to be protected at pH 4, resulting in slow HDX of these residues. It is likely that the elongated 3\(_{10}\) helix in the C-terminal end of \( \alpha_1 \) persists at pH 4.

Residues Gln\(^{159} \), Val\(^{160} \), Tyr\(^{161} \), and Arg\(^{163} \) in segment 154–167 exchange in a denaturant-dependent manner with an \( m_{op} \) value of 0.8 kcal mol\(^{-1} \)M\(^{-1} \). Despite being in \( \beta_2 \) and surrounded by slow exchanging residues, Tyr\(^{162} \) was observed to exchange fast, whereas previous studies indicate that it has a high \( \Delta G_{op} \) value at pH 5.5 (19, 20).

In \( \alpha_2 \), residues Val\(^{175} \), Val\(^{179} \), Asn\(^{180} \), and Ile\(^{181} \) surrounding the disulfide bond between Cys\(^{178} \) and Cys\(^{213} \) were observed to...
Evidence for Partially Unfolded Forms—PUFs are energetically placed between the native and unfolded states (13, 15, 56, 61). A PUF is typically characterized by an enhanced solvent-accessible surface area that is buried in the native state and a stability ($\Delta G_{op}$) lower than that of the unfolded state relative to the N state. PUFs may be either crucial on-pathway intermediates or off-pathway intermediates populated as a result of fluctuations of the native structure (62–64). Partially unfolded forms have been indicated to be key molecular species in fibril formation (7, 65, 66). It has been seen that mutations or solvent conditions that destabilize the native protein and increase the population of these intermediate forms result in increased amyloidogenicity (67).

The results from native state HDX measurements on moPrP clearly indicate that at least two PUFs are present in equilibrium with the native state. In PUF1 characterized by an $m_{op}$ value of 0.4 $\pm$ 0.03 kcal mol$^{-1}$ M$^{-1}$ and a stability ($\Delta G_{op}$) of 2.2 $\pm$ 0.2 kcal mol$^{-1}$, $\beta_1$, the loop region between $\alpha$1 and $\alpha$1, and the C-terminal region of $\alpha$3 appear to be disordered. PUF2 has an $m_{op}$ value of 0.8 $\pm$ 0.1 kcal mol$^{-1}$ M$^{-1}$ and a stability ($\Delta G_{op}$) of 3.1 $\pm$ 0.5 kcal mol$^{-1}$ and is characterized by a disruption or detachment of $\alpha$1 and $\beta_2$ from the $\alpha_2$-$\alpha_3$ core subdomain of the protein. It has been suggested that PUFs are produced by stepwise unfolding and refolding of the cooperative structural units of a protein (64). If PUF1 and PUF2 are indeed intermediates populated sequentially on the unfolding pathway of moPrP, PUF1 would then be characterized by a disordered $\beta_1$, disordered $\beta_1$-$\alpha_1$ loop, and disordered C-terminal region of $\alpha_3$. PUF2 additionally would have the $\alpha_1$ and $\beta_2$ regions either structurally disordered or displaced from the intact core subdomain comprising $\alpha_2$ and $\alpha_3$. However, in this study, native state HDX occurred in the EX2 limit in which only thermodynamic information on intermediates and not kinetic information on the pathway of unfolding can be obtained.

A recent study of the effects of destabilizing pathogenic mutations on the structural dynamics of moPrP has suggested that the unraveling or displacement of $\alpha_1$ from the stable core of the protein is a key initial event in conformational conversion (35). Such a structural perturbation also appears to have occurred in a molten globule-like kinetic intermediate that is also populated at equilibrium under highly acidic conditions, which has been implicated as a key precursor to oligomerization (68, 69). Molecular dynamics studies on the prion protein (34, 70) support these results. Such structural perturbations are identical to those that led to the formation of PUF2. Hence, these studies suggest a potential role of PUF2 in initiating the conversion of PrP$^{C}$ to PrP$^{SC}$.

No Evidence for Residual Structure in the Unfolded State under Native Conditions—The residues in $\alpha_2$ and $\alpha_3$ around the disulfide bond linking residue Cys$^{213}$ in $\alpha_2$ with residue Cys$^{178}$ in $\alpha_2$ have a much higher stability than the rest of the
residues in the protein. Their stability is similar to albeit a bit lower than the global stability as shown in Fig. 6a. In earlier studies carried out at pH 5.5, the same region was reported to be more stable than the globally unfolded state by 1.1 kcal mol\(^{-1}\), and as a result it was referred to as a superprotected region (19, 20). Hence, it was suggested that the unfolded state of human PrP and Syrian hamster PrP at pH 5.5 might have residual structure around the disulfide bond. In this study carried out at pH 4, the stability of this region was found to be less than that of the globally unfolded state: with the exception of Cys213, the other residues in this region exchange with an average value of \(\Delta G_{\text{op}}\) that is 1 kcal mol\(^{-1}\) lower than that of the globally unfolded state. This may be the result of pH-induced destabilization of this region in the native ensemble or the stabilization of the unfolded state ensemble. However, studies of pH-induced changes in the conformational dynamics of the native protein have not indicated that structural changes occur in this region of the N state of the protein (55, 59, 60).

In summary, native state HDX of moPrP(23–231) at pH 4 where the protein is destabilized and has a higher propensity to misfold into amyloidogenic forms revealed the presence of partially unfolded forms that were not detected earlier at pH 5.5 for the human or Syrian hamster prion proteins (19, 20). At pH 4, the N state of moPrP is in equilibrium with at least two PUFs that differ in stability by \(\sim 0.9\) kcal mol\(^{-1}\). One of these PUFs appears to resemble a conformation that has been shown to be a key initial intermediate in misfolding and oligomerization (35) and is therefore the likely a monomeric precursor to aggregated forms.

**Author Contributions**—J. B. U. and R. M. designed the study and wrote the paper. R. M. and R. D. performed the experiments and analyzed the results. The final version of the manuscript was approved by all authors.

**Acknowledgments**—We thank the NMR facility at the National Centre for Biological Sciences and Purushottam Reddy Pothula for assistance in carrying out the experiments on the NMR instrument. We thank M. K. Mathew, R. Varadarajan, and our laboratory members for discussions.

**References**

Partially Unfolded Forms of Prion in Misfolding Conditions


Partially Unfolded Forms of Prion in Misfolding Conditions


Protein Structure and Folding:
Partially Unfolded Forms of the Prion
Protein Populated under
Misfolding-promoting Conditions:
CHARACTERIZATION BY HYDROGEN
EXCHANGE MASS SPECTROMETRY
AND NMR

Roumita Moulick, Ranabir Das and Jayant B. Udgaonkar

doi: 10.1074/jbc.M115.677575 originally published online August 25, 2015

Access the most updated version of this article at doi: 10.1074/jbc.M115.677575

Find articles, minireviews, Reflections and Classics on similar topics on the JBC Affinity Sites.

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2015/08/25/M115.677575.DC1.html

This article cites 70 references, 25 of which can be accessed free at
http://www.jbc.org/content/290/42/25227.full.html#ref-list-1