Kinetic Intermediate in the Folding of Human Prion Protein*

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Transmissible spongiform encephalopathies are associated with the conversion of cellular prion protein, PrP<sub>Sc</sub>, into a misfolded oligomeric form, PrP<sub>Sc</sub>. Here we have examined the kinetics of folding and unfolding reactions for the recombinant human prion protein C-terminal fragment 90–231 at pH 4.8 and 7.0. The stopped-flow data provide clear evidence for the population of an intermediate on the refolding pathway of the prion protein as indicated by a pronounced curvature in chevron plots and the presence of significant burst phase amplitude in the refolding kinetics. In addition to its role in the normal prion protein folding, this intermediate likely represents a crucial monomeric precursor of the pathogenic PrP<sub>Sc</sub> isofom.

Transmissible spongiform encephalopathies, or prion diseases, are a group of intriguing neurodegenerative disorders that include scrapie in sheep and goat, bovine spongiform encephalopathy in cattle, chronic waste disorder in deer and elk, and Creutzfeldt-Jacob disease in humans (1). These diseases are associated with conformational conversion of a normal prion protein, PrP<sub>C</sub>, into a misfolded, oligomeric isofom, PrP<sub>Sc</sub>. According to the “protein-only” hypothesis (1), the transmission of the disease does not require nucleic acids, and the prion pathogen consists solely of PrP<sub>Sc</sub>. The latter conformer is believed to act as an infectious agent by catalyzing self-propagating conversion of endogenous PrP<sub>C</sub> into the pathogenic PrP<sub>Sc</sub> isofom. Although the ultimate proof for the protein-only hypothesis is still missing (2), the central role of prion protein in the pathogenesis of the disease is documented by a wealth of biochemical and genetic data (1).

Cellular human prion protein, PrP<sub>C</sub>, is a glycoprotein that contains a disulfide bond, is N-glycosylated, and is attached to the plasma membrane by a glycosyl phosphatidylinositol anchor (1). NMR studies have shown that the recombinant prion protein in solution consists of a largely unordered N-terminal region and the folded C-terminal domain encompassing three α-helices and a short β-sheet (3–5). Recent crystallographic studies have captured the C-terminal part of PrP as a domain-swapped dimer (6). This dimer, which is only marginally populated in solution and selectively crystallizes, is also α-helical, and its overall fold is similar to that of the monomer. The PrP<sub>C</sub> → PrP<sub>Sc</sub> conversion, which appears to occur without any conformational modifications, is believed to involve a major conformational change in the prion protein (1). In contrast to a largely α-helical PrP<sub>C</sub>, the pathogenic PrP<sub>Sc</sub> isoform is characterized by a high content of β-sheet structure (7, 8), partial resistance to proteolytic digestion, and a propensity to aggregate into insoluble amyloid-like fibrils and plaques (1). Despite extensive research, the molecular mechanism underlying the PrP<sub>C</sub> → PrP<sub>Sc</sub> conversion and prion propagation remains enigmatic.

The key to understanding this mechanism is to elucidate the folding pathway(s) of the prion protein. Here we present evidence for a population of a kinetic intermediate during the folding of the human prion protein. This species may represent a crucial monomeric precursor on the pathway of prion protein conversion to the pathogenic PrP<sub>Sc</sub> isofom.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The plasmid encoding huPrP-(90–231) with an N-terminal linker containing a His<sub>6</sub> tail and a thrombin cleavage site was described previously (9, 10). The Y218W and F175W variants were constructed by site-directed mutagenesis using appropriate primers and the QuikChange kit (Stratagene). The proteins were expressed, cleaved with thrombin, and purified according to the previously described protocol (9).

Equilibrium Unfolding in Urea—The equilibrium unfolding curves were obtained using a Jasco J-810 spectropolarimeter equipped with an automated titrator and a temperature control system. In these experiments, native protein (1.4 μM) in an appropriate buffer was titrated with a 9 M buffered urea solution containing protein at the same concentration. The extent of protein unfolding was monitored by ellipticity at 222 nm or fluorescence intensity above 320 nm (excitation wavelength of 296 nm). The unfolding curves were analyzed using a two-state transition model (11).

Stopped-flow Measurements—The kinetics of unfolding and refolding reactions were studied by diluting the native protein or protein fully unfolded in 8 M urea into the buffer (50 mM phosphate, pH 7.0 or 50 mM sodium acetate, pH 4.8) containing urea at a desired concentration. In most experiments, the final protein concentration was 5.0 μM. The reactions were monitored by fluorescence above 320 nm (excitation at 296 nm) using the Applied Photophysics π* stopped-flow instrument equipped with a 5-μl cell and operating at a 1:10 mixing ratio. Depending on urea concentration, the mixing dead time of this instrument was 0.8–1.1 ms. The dead times were determined by a standard protocol for the reduction of 2,6-dichlorophenolindophenol by l-ascorbate (12). Each protein folding/unfolding reaction was measured at least eight times. The kinetic traces were averaged and analyzed using the software provided by Applied Photophysics.

RESULTS

In the present study, we have focused on folding of the recombinant protein corresponding to human PrP fragment 90–231 (huPrP<sub>Sc</sub>-[90–231]). This region of PrP is of special importance because it encompasses the entire sequence of proteinase-resistant protein found in prion-infected brain, contains all known point mutations associated with familial prion disorders, and is sufficient for the propagation of the disease. The folding mechanism of huPrP<sub>Sc</sub>-[90–231] was studied by the kinetic stopped-flow method with tryptophan fluorescence detection. Since the fluorescence of the sole native Trp at position 99 changes very little upon protein unfolding, these studies required preparation of huPrP<sub>Sc</sub>-[90–231] variants with a single Trp engineered into the folded domain of the protein. This was accomplished by replacing Trp<sup>99</sup> with Phe and, on that back-
ground, substituting Phe or Tyr at different positions with tryptophan. Preliminary characterization of these single Trp variants has identified the Y218W mutant (Fig. 1) as potentially the most suitable candidate for stopped-flow experiments. Both at neutral and acidic pH, the Y218W substitution is essentially non-perturbing as indicated by far-UV circular dichroism spectra and equilibrium urea unfolding curves (data not shown). Furthermore, the fluorescence of the Trp319 probe is very sensitive to protein conformation. It is quenched in the native state, increasing sharply upon huPrP-(90–231) unfolding in urea.

The stopped-flow method was used to follow kinetics of Y218W huPrP-(90–231) folding and unfolding at both acidic and neutral pH. The experiments were performed at 5°C because, as shown previously (13), at room temperature the refolding and unfolding reactions for the prion protein are very fast, occurring within the dead time of the stopped-flow instrument. Representative kinetic traces for the fluorescence-detected folding and unfolding reactions of Y218W huPrP-(90–231) at pH 4.8 are shown in Fig. 2. At each denaturant concentration, the kinetic traces could be fitted by a single exponential function, yielding the apparent rate constants. The urea dependence of these rate constants ("chevron plot") is shown in Fig. 3A. The data do not fit a two-state transition model since the plot of the logarithm of the refolding rate versus [denaturant] at low urea concentrations (below ~3.5 M) clearly deviates from linearity. The unfolding branch of the curve does not show any detectable curvature; however, the urea concentration range available for unfolding measurements is too narrow to allow any definitive conclusions. A non-linearity of the refolding part of the chevron plot (below 3 M urea) was also observed at pH 7 (Fig. 3B). Similar downward curvatures of the rate constant versus [denaturant] plots have been reported for a number of other proteins (14–16). This effect is usually attributed to the presence of an early folding intermediate that forms during the dead time of the instrument and becomes increasingly populated under stabilizing conditions (i.e. at low denaturant concentration) (14–16). However, recent studies indicate that deviation from linearity in chevron plots could also result from the transient protein aggregation during the refolding reaction (17). To test the latter possibility, the measurements of refolding kinetics at a few urea concentrations (1.2, 1.8, 2.3, and 2.9 M) were repeated using protein concentration of 1.2, 2.7, 5.0, and 25 μM. The rate constants did not show any appreciable protein concentration dependence, indicating that, under the conditions used, the folding reaction is monomolecular and does not involve any transient aggregates.

The presence of a kinetic folding intermediate at pH 4.8 was further corroborated by the analysis of the refolding amplitudes. At low urea concentrations, the initial fluorescence intensities obtained by extrapolation of the kinetic curves to time 0 are markedly smaller than the amplitudes expected assuming that the fluorescence intensity of the unfolded state changes linearly with denaturant concentration (Fig. 3C). This indicates that a significant fraction of the total fluorescence change occurs within the dead time of the instrument (burst phase), suggesting rapid formation of a folding intermediate (14–16). At pH 7, the unfolding curve was shifted to very high urea concentrations. The unfolding baseline for Y218W huPrP-(90–231) under these conditions was poorly defined (Fig. 3D), precluding application of the "amplitude test."

A non-linearity of chevron plots and the observation of a burst phase strongly indicate the presence of at least one intermediate on the Y218W huPrP-(90–231) folding pathway. It has been recently argued that curved chevron plots could also result from changes in the transition state (18). However, this is unlikely to be a significant factor in the present study, especially since transition state movement could not account for the observed burst phase in the refolding reaction. Indeed,
Thermodynamic and kinetic parameters for the folding of human prion protein

$\Delta G_{\text{unf}}^{\text{kin}}$ represents the free energy obtained from the best fit of the equilibrium unfolding data to a two-state model (11). The remaining parameters are from the best fit of data of Figs. 3 and 4 to a three-state sequential model as shown in Scheme I according to the equation (16): \n
$$
\ln k_{\text{obs}} = \ln \left( k_{\text{on}} \cdot \frac{m_{\text{U}} - m_{\text{NI}}}{m_{\text{NI}} - m_{\text{I}}} \right) \frac{k_{\text{U}}}{k_{\text{N}}} + k_{\text{I}} \cdot \frac{m_{\text{U}} - m_{\text{NI}}}{m_{\text{NI}} - m_{\text{I}}} + k_{\text{I}} \cdot \frac{m_{\text{NI}} - m_{\text{I}}}{m_{\text{I}}},
$$

where $k_{\text{obs}}$ is the observed rate constant, $k_{\text{on}}$ and $k_{\text{I}}$ are microscopic rate constants corresponding to the $I \rightarrow N$ and $N \rightarrow I$ transitions, respectively, and $K_{\text{U}}$ is the equilibrium constant between the unfolded and intermediate states. The parameters $m_{\text{U}}$, $m_{\text{IC}}$, and $m_{\text{NI}}$ represent the denaturant concentration dependence of $K_{\text{U}}$, $k_{\text{I}}$, and $k_{\text{NI}}$, respectively. $\Delta G_{\text{unf}}^{\text{kin}}$ was calculated from kinetic data according to the equation: $\Delta G_{\text{unf}}^{\text{kin}} = \Delta G_{\text{U}}^{\text{kin}} + \Delta G_{\text{N}}^{\text{kin}}$, where $\Delta G_{\text{U}}^{\text{kin}} = RT \ln \left( k_{\text{on}} \right)$ and $\Delta G_{\text{N}}^{\text{kin}} = RT \ln \left( m_{\text{U}} / m_{\text{N}} \right)$. The parameter $a_1$, related to the compactness of the I state, was calculated as $a_1 = m_{\text{U}} / (m_{\text{IC}} + m_{\text{NI}} + m_{\text{N}})$.

| Probe | pH | $\Delta G_{\text{U}}^{\text{kin}}$ | $\Delta G_{\text{N}}^{\text{kin}}$ | $K_{\text{U}}$ | $m_{\text{U}}$ | $k_{\text{U}}$ | $m_{\text{IC}}$ | $k_{\text{I}}$ | $m_{\text{NI}}$ | $k_{\text{NI}}$ | $m_{\text{N}}$ | $a_1$ |
|-------|----|-----------------|-----------------|----------|----------|----------|----------|----------|----------|----------|----------|--------|
| Trp$^{318}$ | 4.8 | 17.4 ± 0.8 | 19.3 | 67.2 | 3.6 | 1165 | 0.24 | 18.2 | 1.0 | 0.74 |
| | 7.0 | 29.4 ± 1.9 | 12.2 | 21.1 | 7.0 | 1290 | 0.20 | 0.03 | 2.4 | 0.45 |
| Trp$^{375}$ | 7.0 | 27.7 ± 0.5 | 29.6 | 11.3 | 2.1 | 1340 | 0.24 | 0.04 | 2.6 | 0.43 |

Scheme I

where U and N represent the unfolded and native states, respectively, and I represents an early folding intermediate formed during the dead time of the instrument (16). The fitting parameters (Table I) indicate that, relative to the unfolded state, the intermediate is significantly more stable under acidic conditions than at neutral pH ($\Delta G_{\text{U}}$ of 5.7 and 9.6 kJ mol$^{-1}$ at pH 7 and 4.8, respectively). In either case, the intermediate rapidly folds to the native state at a rate of $\approx$ 1200–1300 s$^{-1}$. The fit also allows the calculation of parameter $a_1$, $f$, the ratio of $m$ values for the pre-equilibrium transition, $m_{\text{UIC}}$, relative to the equilibrium $m$ value, $m_{\text{NI}}$, $m_{\text{UIC}} = m_{\text{U}} + m_{\text{NI}} + m_{\text{IC}}$. The $a_1$ parameter, which provides a quantitative measure of the solvent-accessible area of U that becomes buried upon formation of I (14), indicates that the folding intermediate is more compact (i.e. characterized by a smaller solvent-accessible area) under acidic conditions than at neutral pH.

The present results are at odds with those previously reported for mouse prion protein domain 121–231. Based on limited data for the F175W variant of mouse PrP-(121–231), it was concluded that at neutral pH prion protein folds according to the two-state mechanism without any kinetic intermediates (13). In view of this apparent discrepancy, we have repeated our measurements at pH 7 for F175W huPrP-(90–231), i.e. using the same reporter group as in the previous study with mouse prion protein. The rate profile for F175W huPrP-(90–231) was very similar to that of the Y218W variant, showing a well defined curvature at low denaturant concentrations (Fig. 4A). Furthermore, a clear departure from a linear baseline was observed for initial fluorescence intensities (refolding amplitudes), indicating the presence of a burst phase in the refolding reaction (Fig. 4B). The fit of kinetic data according to a three-state model yielded, for F175W huPrP-(90–231), folding parameters very similar to those derived for the Y218W variant (Table I). The close similarity of the results obtained using the reporter groups in different parts of the protein molecule (helix 3 and helix 2 in Y218W and F175W variants, respectively (Fig. 1)) indicates that the single Trp variants used in this study faithfully represent the folding of the native human prion protein.

A number of factors could contribute to the discrepancy between the present data and the apparent lack of a folding intermediate in the previous study with mouse prion protein. It is conceivable that the human and mouse PrP fold through different pathways and/or that the N-terminal fragment 90–120 affects the folding of the C-terminal domain 121–231 (experiments with mouse PrP were performed using the 121–231 domain, whereas the present study with human protein used a longer 90–231 fragment). However, it should also be noted that data reported for mouse PrP-(121–231) refolding are limited to a relatively narrow urea concentration range above 3.2 M urea. The missing data below 3 M urea are of potentially critical importance since folding intermediates (and curvatures in chevron plots) usually become detectable only under stabilizing conditions, i.e. at low denaturant concentrations (14, 15). Indeed, at high urea concentrations the rate constants for huPrP-(90–231) refolding at pH 7 are very similar to those reported for mouse PrP-(121–231), and there is a linear dependence of the logarithm of these rates on denaturant concentration. A departure from this linearity, indicative of a folding intermediate, becomes detectable only at urea concentrations below 3 M. Experiments at low urea concentrations became feasible in the present study due to the recent improvements in stopped-flow instrumentation. The use of a 5-$\mu$l cell reduced the dead time of our instrument to 1 ms, allowing reliable measurements of rate constants in excess of 1500 s$^{-1}$. In a recent study with ubiquitin, it has been noted that the stopped-flow measurements of reactions that are fast relative to the instrumental dead time may be prone to experimental error (19). However, this concern applies mainly to complex folding reactions in which the fastest detectable phase is followed by poorly resolved slower phase(s). The present studies with prion protein should be largely immune to these problems since the observed kinetic traces are clearly monophasic.
Within the context of the protein-only hypothesis, the key event in the pathogenesis of prion diseases is the conversion of prion protein from α-helical form, PrPα, to a β-sheet-rich conformation, PrPSc (1). One of the central questions related to the mechanism of this conversion pertains to the nature of the PrP monomer that is a direct precursor of the oligomeric PrPSc. Is it a native α-helical conformer, a fully unfolded form, or a monomeric folding intermediate? Some theoretical models point to a potential involvement in the PrPα → PrPSc conversion of partially structured folding intermediates (20). Such intermediate structures are also believed to play a central role in amyloid formation by other proteins. However, unlike for classical amyloidogenic proteins such as transthyretin and lysozyme variants (21, 22), putative folding intermediates for prion protein have proved very difficult to detect and characterize (13, 23, 24).

Early studies with the recombinant prion protein have identified an acid-induced β-sheet-rich form of PrP that was claimed to represent a “monomeric equilibrium intermediate” (25, 26). However, more recent data have invalidated this claim, showing that the β-sheet-rich species is not an equilibrium folding intermediate but, in fact, represents an aggregated form of prion protein with physicochemical properties similar to those of PrPSc (10, 27). Given the later finding, the involvement of intermediate(s) in prion protein folding remains a matter of speculation.

The present kinetic stopped-flow study provides, for the first time, conclusive experimental evidence that prion protein folds by a three-state mechanism involving a monomeric intermediate. This early intermediate, which precedes the rate-limiting formation of the native state, appears to be relatively compact and especially stable under acidic conditions. In addition to its role in normal prion protein folding, the newly discovered folding intermediate is also likely to be of major importance in the PrPα → PrPSc conversion. In the absence of experimental evidence for an intermediate, some authors have argued that the mechanism of prion protein conversion is fundamentally different from that for other amyloid-forming proteins and that it is the unfolded state of PrP that is directly converted into PrPSc (13, 23). However, the present finding of a stable monomeric intermediate in prion protein folding calls for a critical reassessment of this view. Compared with the fully unfolded protein, the partially structured PrP intermediate is much more stable. This, combined with a relatively high hydrophobicity and aggregation propensity of typical folding intermediates, renders the I state of PrP as a better candidate than the unfolded state for a direct monomeric precursor of PrPSc. The role of a folding intermediate in the PrP conversion is in line with the recent finding that the transition of the recombinant prion protein to a scrapie-like form is strongly promoted in the presence of relatively low concentrations of urea, i.e., under conditions that would increase the population of an intermediate state (10). In contrast, conditions favoring the native state (no denaturant), or those that shift equilibrium toward the fully unfolded state (high concentration of urea), are not conducive to the conversion reaction (10). It should also be noted that the in vitro transition of the recombinant PrP to a scrapie-like form appears to be especially effective at low pH (27, 28). This correlates well with the present observation that acidophilic pH stabilizes the intermediate populated during the prion protein folding reaction. Mildly acidic conditions are of potential relevance to prion disease pathogenesis since previous reports indicate that the PrPα → PrPSc conversion may take place in acidic compartments (29).

The evidence of an intermediate in prion protein folding also has potentially important implications for understanding the mechanism of familial diseases that segregate with specific mutations in human prion protein (1). In these disorders, the PrPα → PrPSc conversion appears to occur spontaneously, i.e., without involving a preexisting prion agent. It has been postulated that familial mutations promote the spontaneous conversion of the prion protein by destabilizing the native structure of PrPSc (20). However, recent data indicate that some of these mutations produce negligible thermodynamic destabilization of the native state of prion protein relative to the unfolded state (30, 31), a conundrum for the model assuming that a fully unfolded prion protein is a direct precursor of PrPSc.

The finding of an intermediate in prion protein folding may explain this conundrum. Based on the present data, we propose that familial mutations facilitate the conversion reaction not necessarily by increasing the population of an unfolded protein but rather by affecting the conformation and/or increasing the stability of the partially folded intermediate species. This work provides a foundation for studying the effect of disease-causing mutations on the folding pathway of the prion protein.

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REFERENCES

1. Prusiner, S. B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13363–13383
2. Caughey, B. (2000) Nat. Med. 6, 751–754
3. Rick, E., Hornemann, S., Wider, G., Billette, M., Glocshuber, R., and Wuthrich, K. (1996) Nature 382, 180–182
4. 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, 13452–13457
5. Zahn, R., Liu, A., Luhrs, T., Rick, R., Von Scooter, C., Garcia, F. L., Billette, M., Zacaloiu, L., Wider, G., and Wuthrich, K. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 145–150
6. Krantz, B. A., and Sosnick, T. R. (2000) Structure and Mechanism in Protein Science. A Guide to the Molecular Biology (construct preparation) part of this work.
