The Effect of Disease-associated Mutations on the Folding Pathway of Human Prion Protein

Adrian C. Apetri, Krystyna Surewicz and Witold K. Surewicz‡

Department of Physiology and Biophysics and Department of Chemistry, Case Western Reserve University, 2109 Adelbert Road, Cleveland, Ohio 44106

‡To whom correspondence should be addressed: Department of Physiology and Biophysics, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106. Tel.: 216-368-0139; Fax: 216-368-1693; E-mail: wks3@po.cwru.edu

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SUMMARY

Propagation of transmissible spongiform encephalopathies is believed to involve the conversion of cellular prion protein, PrP$^C$, into a misfolded oligomeric form, PrP$^{Sc}$. An important step towards understanding the mechanism of this conversion is to elucidate the folding pathway(s) of the prion protein. We recently reported that the folding of wild-type prion protein can be best described by a three-state sequential model involving a partially folded intermediate (Apetri, A. C. and Surewicz, W. K. (2002) J. Biol. Chem. 277, 44589-44592). Here, we have performed kinetic stopped flow studies for a number of recombinant prion protein variants carrying mutations associated with familial forms of prion disease. Analysis of kinetic data clearly demonstrates the presence of partially structured intermediates on the refolding pathway of each PrP variant studied. In each case, the partially folded state is at least one order of magnitude more populated than the fully unfolded state. The present study also reveals that, for the majority of PrP variants tested, mutations linked to familial prion diseases result in a pronounced increase in the thermodynamic stability – and thus the population – of the folding intermediate. These data strongly suggest that partially structured intermediates of PrP may play a crucial role in prion protein conversion, serving as direct precursors of the pathogenic PrP$^{Sc}$ isoform.
Prions are infectious pathogens that cause a group of fatal neurodegenerative diseases known as transmissible spongiform encephalopathies (1-4). These diseases can be sporadic, inherited or acquired by infection. The human forms of prion disorders include Creutzfeldt-Jacob disease, Gerstmann-Straussler-Scheinker disease, fatal familial insomnia and kuru, whereas the animal diseases encompass bovine spongiform encephalopathy in cattle, chronic wasting disorders in deer and elk, and scrapie in sheep. The histopathological marker of all prion diseases is the accumulation in the brain of an abnormal isoform of prion protein, PrP\textsuperscript{Sc}. This protein is derived by a post-translational mechanism from the normal (cellular) prion protein, PrP\textsuperscript{C}. According to the protein-only hypothesis, PrP\textsuperscript{Sc} is the sole component of the infectious prion agent (1, 5). It is also believed that when introduced into a normal host - PrP\textsuperscript{Sc} induces the conversion of endogenous PrP\textsuperscript{C} into PrP\textsuperscript{Sc}, and that this conversion is the central event in the propagation of the disease. Although the ultimate proof for the protein only model is still missing (6), the central role of prion protein in the propagation of transmissible spongiform encephalopathies is documented by a wealth of biochemical data as well as animal studies (1-7). Furthermore, the notion that proteins can act as infectious agents is supported by studies on prion-like phenomena in yeast and fungi (8-10).

Human PrP\textsuperscript{C} is a 209 residue glycoprotein that contains a single disulfide bond and is attached to the plasma membrane via a glycosyl phosphatidylinositol anchor. NMR structural studies have shown that the folded C-terminal domain of PrP\textsuperscript{C} consists of three \(\alpha\)-helices and two very short \(\beta\)-strands, whereas the entire N-terminal part encompassing residues 23 to \(\sim\)120 is highly flexible and largely unordered (11-14). Although PrP\textsuperscript{C} and PrP\textsuperscript{Sc} have identical covalent structures (15), their biophysical properties are profoundly different. PrP\textsuperscript{C} is monomeric, highly \(\alpha\)-helical and readily degradable by proteinase K, whereas PrP\textsuperscript{Sc} is
characterized by an oligomeric β-sheet rich structure, partial resistance to proteinase K digestion and a pronounced tendency to aggregate into insoluble plaques (1-4, 7, 16-19). In some cases, these insoluble aggregates have characteristics of amyloid fibrils. Given the conformational differences between the normal and pathogenic prion protein isoforms, transmissible spongiform encephalopathies are often classified as diseases of protein misfolding.

One of the key arguments in support of the protein only hypothesis is the evidence linking familial prion diseases with mutations in the gene coding for human prion protein (1-3, 20). Over 20 mutations in this gene have been shown to segregate with inherited forms of Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker disease or fatal familial insomnia. Since the pathogenic conversion process in individuals carrying these mutations appears to develop spontaneously, the mutant proteins provide an invaluable model for studying molecular mechanisms of the PrP\textsuperscript{C}→PrP\textsuperscript{Sc} conversion \textit{in vitro}. An important step towards understanding this mechanism is to dissect the folding pathway of the prion protein and identify the monomeric precursor of the oligomeric PrP\textsuperscript{Sc} species. In a recent kinetic stopped-flow study, we demonstrated that the wild-type human prion protein folds by a three-state mechanism involving a partially structured intermediate (21). Here, we have extended these studies to a number of PrP variants carrying mutations corresponding to inherited prion diseases. Our data indicate that, in the vast majority of cases, familial mutations result in a pronounced stabilization of the folding intermediate. This strongly supports the notion that these intermediates may play a crucial role in prion protein conversion, serving as direct precursors of the pathogenic PrP\textsuperscript{Sc} isoform.
EXPERIMENTAL PROCEDURES

*Plasmid Construction and Protein Purification* - The plasmid encoding huPrP90-231 with an N-terminal linker containing His$_6$ tail and a thrombin cleavage site was described previously (22, 23). All variants containing mutations linked to familial prion diseases (P102L, D178N with 129M and 129V polymorphism, V180I, F198S, E200K, R208H, V210I, and Q217R) were constructed on the background of W99F/Y218W huPrP90-231 (21) 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 (22, 24). The concentration of purified protein was determined spectrophotometrically using the molar extinction coefficient, $\varepsilon_{276}$, of 21,640 M$^{-1}$cm$^{-1}$.

*Circular Dichroism Spectroscopy* - The far-UV circular dichroism spectra were obtained on a Jasco J-810 spectropolarimeter. The measurements were performed at 25 °C using a 1 mm path-length cell at a protein concentration of 15 µM. Typically, five spectra were averaged to improve the signal-to-noise ratio, and the spectra were corrected for small buffer contributions.

*Equilibrium Unfolding in Urea* - The equilibrium unfolding curves were obtained using an AVIV 215 series circular dichroism spectrometer equipped with a fluorescence detection unit, an automatic titrator and a Peltier temperature control system. The measurements were carried out at 5 °C in 50 mM potassium phosphate buffer, pH 7. In these experiments, native protein (1.4 µM) in the above buffer was titrated – using urea increments of approximately 0.1 M - with a 9 M buffered urea solution of identical protein concentration. Upon each addition of urea, the mixture was incubated for 15 seconds and the extent of protein unfolding was monitored by ellipticity at 222 nm and tryptophan fluorescence intensity above 320 nm.
(excitation wavelength of 296 nm). The unfolding curves were analyzed using a two-state transition model as described previously (25). In the control experiments, it was verified that an incubation time of less than 1 sec is sufficient for the system to reach equilibrium [the unfolding/refolding of the prion protein is very fast, occurring on the millisecond time scale (21, 26)], and that the unfolding and refolding reactions are fully reversible. Urea used in this study was deionized by treatment with a mixture of anion exchange (trimethylbenzylammonium) and cation exchange (Dowex MR-3) resins. The concentration of the denaturant was determined by measuring the refractive index. All reagents were of the highest analytical grade commercially available.

**Stopped-flow Measurements** - The kinetic measurements were performed under conditions identical to those used in equilibrium experiments (5 °C, 50 mM potassium phosphate buffer, pH 7). In the unfolding reaction, the native protein was rapidly diluted into 10 volumes of a buffer containing different concentrations of urea. For the refolding experiments, the protein was first completely unfolded in a concentrated solution of buffered urea (8 M) and then diluted elevenfold to the desired concentration of urea. In most experiments, the final protein concentration was 5.0 µM. The changes in fluorescence above 320 nm (excitation at 296 nm) were monitored using an Applied Photophysics π* stopped-flow instrument equipped with a 5-µl cell and operating at the 1:10 mixing ratio. The mixing dead time of this instrument was determined by a standard protocol for the reduction of 2, 6 dichlorophenolindophenol by L-ascorbate (27). Depending on the urea concentration, this dead time was between 0.8 and 1.1 ms. Each protein folding/unfolding reaction was measured at least fifteen times. The kinetic traces were averaged and analyzed using the software provided by Applied Photophysics.
RESULTS

Our recent kinetic stopped-flow experiments revealed that human prion protein fragment 90-231 (huPrP90-23) refolds from urea according to a three-state transition model with an intermediate that accumulates at low denaturant concentrations (21). Here, we have extended the folding studies to a number of PrP variants containing amino acid substitutions associated with familial prion diseases. The 90-231 fragment of prion protein is especially suitable for such studies since, in addition to containing the folded domain, it encompasses the entire proteinase K resistant sequence of PrP\textsuperscript{Sc}, contains all point mutations that are known to segregate with the familial forms of prion disease, and is sufficient for the propagation of the disease. The specific mutations considered in the present study, along with their location within the structure of PrP\textsuperscript{C}, are shown in Fig. 1. All these mutations were introduced on the background of an engineered single Trp variant Y218W/W99F of huPrP90-231. The latter protein was previously identified as especially useful for stopped-flow experiments since, unlike Trp99, the fluorescence of Trp218 is very sensitive to protein conformation. Furthermore, the Y218W substitution does not significantly perturb the structure or thermodynamic stability of PrP (21).

Equilibrium Unfolding Studies - Prior to kinetic stopped-flow experiments, the effect of individual disease-associated point mutations on the global thermodynamic stability of Y218W/W99F huPrP90-231 was probed by equilibrium unfolding in urea. Essentially identical curves were obtained when equilibrium unfolding was monitored by circular dichroism spectroscopy (ellipticity at 222 nm) or Trp218 fluorescence. Figure 2 shows representative equilibrium unfolding curves for the wild type huPrP90-231 and the F198S variant. The free energies of unfolding, $\Delta G_{\text{UN}}^{\text{eq}}$, obtained from the best fits of equilibrium unfolding data for
individual mutant proteins are shown in Table I. These data show that while some of the
disease-associated mutations produce a pronounced decrease in the global stability of
Y218W/W99F huPrP90-231, the effect of others (e.g. P102L, E200K) is essentially negligible.
Importantly, the destabilization induced by familial mutations found here for Y218W/W99F
huPrP90-231 corresponds closely to the values reported in similar studies using unmodified
mouse (28) or human prion proteins (29). This indicates that the introduction of Trp218 as a
fluorescent probe for stopped-flow experiments is essentially inconsequential with respect to
the effect of familial mutations on the thermodynamic properties of the prion protein. It should
also be noted that all mutant proteins studied here have essentially identical far-UV circular
dichroism spectra, indicative of similar global secondary structures.

Stopped-flow Experiments - The effect of mutations corresponding to inherited prion
diseases on the folding pathway of the recombinant human PrP90-231 was studied by kinetic
stopped-flow measurements. These experiments were performed at 5 °C because at room
temperature the unfolding/refolding reactions for the prion protein are extremely fast, occurring
within the dead time of stopped-flow instrumentation (21, 26). For all PrP variants studied, both
the refolding and unfolding kinetic curves at each denaturant concentration could be fit by a
single exponential function, yielding the apparent rate constants. Figure 3 shows representative
kinetic traces for the refolding and unfolding of the wild type huPrP90-231 and the V210I
variant. These data were used to construct the “chevron plots”, in which the logarithm of the
rate constant is plotted as a function of urea concentration. Consistent with our previous report
(21), the refolding branch of this plot for the “wild-type” protein sharply deviates from
linearity at low urea concentrations (Fig. 4 A). Similar inflections in the refolding sections of
the chevron plots were found for all huPrP90-231 mutants studied (Fig. 4). The unfolding (high
urea concentration) branches showed no well-defined curvature. However, for most huPrP90-231 variants, the urea concentration range available for unfolding measurements was too narrow for any conclusions to be drawn.

Downward curvatures (“rollovers”) in the refolding branches of chevron plots have been reported for a number of other proteins. These effects are usually attributed to accumulation of partially folded intermediates that form during the dead time of the instrument and become increasingly populated under stabilizing conditions (i.e. at low denaturant concentrations) (30, 31). Recent studies suggest, however, that deviations from linearity in chevron plots could also be caused by other factors (32-34). Therefore, we performed a series of control experiments to test alternative interpretations of curvatures in chevron plots for huPrP90-231 variants.

The most common experimental problem that could lead to nonlinearities in chevron plots is transient aggregation of protein during the refolding reaction (32). In a previous study, we showed that aggregation does not occur upon refolding of the wild-type huPrP90-231 (21). Here, we performed control experiments to test the possibility of aggregate formation during refolding of huPrP90-231 variants used in this study. To this end, the measurements of refolding kinetics at several urea concentrations (1.1, 1.6, 2.2, 2.6 and 3.1 M) were repeated using mutant proteins at concentrations ranging between 3 and 50 µM. The results of such experiments for two huPrP90-231 variants, F198S and V210I, are shown in Fig. 5. Clearly, in both cases the apparent rate constants show no detectable dependence on protein concentration, indicating that - as for the wild-type protein - the refolding reactions for huPrP90-231 variants are monomolecular and do not involve transient protein aggregates. The control experiments described above were especially important for the F198S variant since the latter protein was reported to undergo time-dependent conversion to an oligomeric scrapie-like form (35).
However, the kinetics of this conversion is many orders of magnitude slower than the time scale of the present refolding experiments.

Other factors considered as potential sources of error in stopped-flow measurements include poor resolution of individual phases in multiphasic reactions, mixing artifacts and/or unspecified “trivial interactions” that could lead to transient changes in fluorescence intensity of Trp residues (33, 34). The first problem does not apply to the present situation because the kinetic traces for prion protein folding are monophasic. To test the possibility that changes in Trp fluorescence observed in our stopped-flow experiments could be caused by factors other than the actual protein conformational transition, we performed control measurements using N-acetyl-Trp-amide (NATA). In these control experiments, huPrP90-231 and NATA were dissolved in 8 M urea and then individually mixed in the stopped flow instrument with solutions of lower urea concentration. Figure 6 shows representative kinetic traces for fluorescence of huPrP90-231, along with those for NATA. Clearly, the fluorescence signal for prion protein refolding follows an exponential decay, whereas the fluorescence of NATA remains constant. This strongly indicates that changes in fluorescence observed in our refolding experiments are exclusively due to protein conformational transition, and not due to any mixing artifacts or trivial interactions of Trp with the bulk aqueous solvent or co-solutes.

Given the results of the control experiments, the curvature of the refolding branches of the chevron plots strongly indicates the presence of at least one intermediate on the refolding pathway of all prion protein variants tested in the present study. Indeed, the denaturant dependence of the folding rates for each protein could be fit according to the three-state sequential model:
where U and N represent the unfolded and native states, respectively, and I represents an early intermediate that accumulates within the dead time of the instrument. The best-fit parameters for individual huPrP90-231 variants are shown in Table I. Inspection of these data reveals that the folding pathway of each protein involves a rapid (i.e. occurring within instrumental dead time) collapse of the unfolded state to an unstable intermediate. This initial step, characterized by an equilibrium constant $K_{UI}^{0}$, is followed by a slower step representing folding of I to the native state N. The rate constant of the latter reaction, $k_{IN}$, varies between approximately 1000 and 1300 s$^{-1}$, depending on the particular mutation. The $\Delta G_{IN}^{0,\text{kin}}$ values derived from the kinetic data represent the free energy difference between the I and N states. The lower the $\Delta G_{IN}^{0,\text{kin}}$ value, the higher the stability of the intermediate relative to the native state. Data of Table I clearly show that, in the vast majority of cases, familial mutations induce a significant increase in the relative stability of the I state. Two notable exceptions are the E200K and P102L variants, in which cases mutations have very little effect on either the global protein stability (as measured by $\Delta G_{UN}^{0,\text{kin}}$) or the stability of the folding intermediate.

In addition to providing information about the relative thermodynamic stabilities of the native and intermediate states, the fit of the kinetic data also allows the calculation of the parameter $\alpha_I$, the ratio of the m value for the pre-equilibrium transition, $m_{UI}$, relative to the equilibrium $m_{eq}$ value, where $m_{eq} = m_{UI} + m_{IN} + m_{NI}$. The latter parameter represents a measure of the surface of U that becomes buried upon formation of I. As shown in Table I, the $\alpha_I$ values for mutant proteins are larger than for wild-type PrP, indicating a somewhat more
compact (i.e., characterized by a smaller solvent accessible area) structure of the folding intermediate for the mutant proteins.

Apart from potential experimental problems such as transient protein aggregation or mixing artifacts (see above), it has been argued that rollovers in chevron plots could also be related to the movement of the transition state along the reaction coordinate as the denaturant concentration changes (36, 37). However, this hypothetical transition state movement would result in shallow and gradual curvatures that are clearly different from the well-defined inflections observed in the chevron plots for prion protein variants. Recent evaluation of stopped-flow data for a number of proteins has unambiguously demonstrated that inflections in chevron plots - such as those observed in the present study - are almost always the result of changes in the rate limiting step with denaturant concentration, providing conclusive evidence for the presence of on-path folding intermediates (37-39). It should also be noted that the $\Delta G_{UN}^{0}$ and $m_{UN}$ values derived from the best fit of the kinetic data ($\Delta G_{UN}^{0,kin} = \Delta G_{UI}^{0,kin} + \Delta G_{IN}^{0,kin}$, $m_{UN} = m_{NI} + m_{IN} + m_{UI}$) correspond closely to the $\Delta G_{UN}^{0,eq}$ and $m_{UN}^{eq}$ values obtained from equilibrium measurements. The intermediate detected by the kinetic method is populated at most at the level of one per several hundreds of molecules. This is well below the detection limit of the equilibrium experiments. Therefore, as in similar studies with other proteins (31, 40, 41), the equilibrium data were analyzed according to a two-state model, yielding information about the global stability of the prion protein (free energy difference between the native and fully unfolded states). Nevertheless, the close correspondence of $\Delta G_{UN}^{0,kin}$ and $\Delta G_{UN}^{0,eq}$ provides additional evidence that the sequential three-state model used to analyze kinetic data accurately describes the folding of the wild type huPrP90-231 and pathogenic variants thereof.
DISCUSSION

It is generally believed that the pathogenesis of transmissible spongiform encephalopathies is associated with the conversion of PrP$^C$ into an abnormally folded PrP$^{Sc}$ form (1-7). However, despite extensive research, the molecular mechanism of this conformational conversion remains enigmatic. The key to understanding this mechanism is to determine the folding pathway and energy landscape of the prion protein. Initial experiments with recombinant PrP suggested that the protein folds into the native $\alpha$-helix-rich structure by a simple two-state mechanism that involves only the fully unfolded and native states (26). However, recent kinetic stopped-flow studies demonstrated that the folding pathway of this protein is more complex and can be best described as a sequential three-state reaction involving a relatively stable monomeric intermediate (21). The existence of a prion protein folding intermediate is also indicated by hydrogen exchange experiments (43) as well as high-pressure NMR and fluorescence spectroscopy studies (44, 45).

The evidence for an intermediate on the folding pathway of the wild-type prion protein has prompted us to extend the stopped-flow experiments to PrP variants with mutations corresponding to familial prion disorders. Studies with mutant proteins are of considerable interest since inherited prion diseases develop spontaneously, without infection by an exogenous prion agent. Therefore, understanding how familial mutations affect the structure, folding pathway and energetics of the prion protein could provide fundamentally important clues regarding the mechanism of prion propagation in general, and initial events associated with the PrP$^C$$\rightarrow$PrP$^{Sc}$ conversion in particular.

The present data clearly demonstrate that, as for the wild-type protein, the folding of all PrP variants studied may be approximated by a three-state model with a folding intermediate. In
each case, this intermediate is relatively compact and becomes stabilized at low denaturant concentrations. To obtain a quantitative measure of a fraction of protein molecules that populate each state under native-like conditions (i.e., in the absence of the denaturant), we have calculated from the thermodynamic data the ratios $[U]/[N]$ and $[I]/[N]$, where $[U]$, $[I]$ and $[N]$ represent the concentration of protein in the unfolded, intermediate and native state, respectively (see Table II). Since in the absence of the denaturant N is always much more populated than either U or I, these ratios provide a good approximation of the population of the unfolded and intermediate states relative to total protein concentration.

Inspection of the thermodynamic data shows that, for all proteins studied, the partially structured intermediate is much more stable as compared with the fully unfolded state (Table I). When translated into the $[U]/[N]$ and $[I]/[N]$ ratios (Table II), this higher thermodynamic stability indicates that, at a given point of time, the number of protein molecules in the I state is at least one order of magnitude (8 to 80-fold) larger than the number of molecules populating the fully unfolded state. The above finding has important implications for understanding the molecular basis of the $\text{PrP}^C \rightarrow \text{PrP}^\text{Sc}$ conversion. One of the central questions in this regard relates to the nature of the PrP monomer that is a direct precursor of the oligomeric $\text{PrP}^\text{Sc}$. While some early models hypothesized that the conversion reaction is mediated by a partially structured folding intermediate (46), other authors proposed that it is the fully unfolded state of PrP that is directly converted to $\text{PrP}^\text{Sc}$ (26, 47). The latter scenario is not impossible. However, the present thermodynamic data clearly indicate that, in all cases studied, the population of the I state is much higher than that of the U state. This, combined with the relatively high aggregation propensity of typical I states, strongly argues in favor of the conversion model involving partially structured folding intermediate(s). This model is also supported by a recent
finding that the conversion at acidic pH of the recombinant prion protein to an oligomeric β-sheet structure is promoted in the presence of low concentrations of urea (i.e., under conditions expected to increase the population of an intermediate), whereas conditions favoring the native or fully unfolded states are less conducive to this transition (23). Partially destabilizing conditions are also known to stimulate the PrPSc-templated conversion of PrPc to a proteinase K-resistant form (48, 49). Furthermore, the role of folding intermediates in prion protein conversion is in line with recent hydrogen exchange NMR experiments (43). The latter study indicates the existence of a partially folded state for the prion protein, whereas no such state could be detected for Doppel, a homolog of PrP that does not form infectious prions.

The present study also reveals that, for the vast majority of PrP variants tested (seven out of nine), mutations linked to familial prion diseases result in a pronounced increase in the stability - and thus population - of the folding intermediate. Under the present experimental conditions, the intermediate state of the wild-type PrP is populated at a level of approximately one per 42,000 molecules. For most mutant proteins, this population increases by at least one order of magnitude, to the level of one per several hundred to one per several thousand molecules. In the case of the F198S variant, the estimated population of the I state is as high as 1:350. Interestingly, the recombinant protein corresponding to the latter variant - associated with Gerstmann-Straussler-Scheinker disease - was recently shown to undergo a spontaneous conversion to the scrapie-like form (35). A dramatic increase in the population of a folding intermediate is observed even in those cases when mutations have only modest effect on the global stability of the protein. For example, the V210I mutation results in only a minor decrease in ΔGU0,k, whereas the intermediate for this protein is one of the most stable among all PrP
variants tested. The reaction coordinate diagram for the V210I variant, together with that for the wild-type PrP, is shown schematically in Fig. 7.

Given the present data, we propose that most familial prion diseases may be rationalized by a simple model based on a mutation-induced increase in the population of partially folded intermediate(s) of the prion protein. The specific structural features of these intermediates are at present unknown. However, protein folding intermediates are usually characterized by a significant exposure of the polypeptide backbone to solvent, enabling increased intermolecular interactions (50). For the wild-type PrP, the concentration of these partially folded monomeric species is very low. Therefore, except for extremely rare sporadic cases, these species may be recruited into the aggregated state - and converted to intermolecular β-sheet structure - only in the presence of externally provided PrP Sc seeds. In contrast, for mutant proteins the concentration of partially structured intermediates may become sufficient to initiate the aggregation process even in the absence of preexisting seeds, leading to de novo formation of the pathogenic PrP Sc isoform. Folding intermediates have also been implicated as crucial monomeric precursors of fibrils formed by classical amyloidogenic proteins such as transthyretin or human lysozyme variants (51-53). In a recent study with the amyloidogenic lysozyme variant D67H, it was estimated that approximately one molecule in 1,500 is in the partially folded state (53). Interestingly, this number is of the same order of magnitude as the population of the intermediate state found here for the majority of disease-associated prion protein variants.

While our model based on mutation-induced stabilization of the partially folded state is applicable to a majority of inherited prion diseases, this model is clearly not universal. Data of Table II indicate that at least for two PrP variants - P102L and E200K - the population of an
intermediate is similar to that for the wild-type prion protein, even though the intermediate state is always much more populated than the fully unfolded state. Clearly, in these cases additional factor(s) must be involved to facilitate a spontaneous PrP$^C \rightarrow$PrP$^{Sc}$ conversion. For the E200K variant, these factors may include an alteration of surface electrostatic potential, as observed in a recent NMR structural study (54). Mutation-dependent changes in surface properties could promote the conversion reaction by inducing abnormal interactions of PrP with other molecules present in the cellular environment. As far as the P102L mutation is concerned, the lack of significant changes in the energy landscape is not surprising since residue 102 maps to a region outside the folded domain. A similar behavior would be expected for prion protein variants P105L and, likely, A117V. In these cases, the pathogenic process could potentially be facilitated by mutation-dependent changes in the amyloidogenic propensity of the unstructured PrP$^C$ region. The role of this region in prion protein conversion is in line with a number of observations (55, 56), including the recent finding that a polypeptide corresponding to residues 23-144 of PrP undergoes a self-propagating conversion to the amyloid state (57).
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FOOTNOTES (p. 2 and 7)

1The abbreviations used are: PrP, prion protein; PrP\textsuperscript{C}, cellular PrP isoform; PrP\textsuperscript{Sc}, scrapie PrP isoform; huPrP90-231, recombinant human prion protein fragment 90-231; NATA, N-acetyl-tryptophan-amide.

2In the remainder of the text, the wild-type protein refers to W99F/Y218W huPrP90-231 in the absence of any disease-associated mutations.
FIG. 1. Ribbon representation of the NMR structure of human prion protein (14) showing the location of disease-related amino acids considered in the present study (blue dots). α-helices are shown in red, the fluorescent probe, Trp218, is shown in green, the position of the W99F substitution is represented as a black dot, and the disulfide bond between Cys179 and Cys214 is indicated in yellow.

FIG. 2. Urea-induced equilibrium unfolding for wild type huPrP90-231 (circles) and the F198S variant (triangles). The unfolding curves were obtained in 50 mM potassium phosphate, pH 7.0, at 5 °C. The degree of unfolding was monitored by Trp fluorescence (closed symbols) and far-UV circular dichroism at 222 nm (open symbols). The wild-type protein refers to W99F/Y218W huPrP90-231 in the absence of any disease-associated mutations.

FIG. 3. Representative kinetic traces for the refolding (left panels) and unfolding (right panels) of the wild type huPrP90-231 and the V210I variant. Experiments were performed in 50 mM phosphate buffer, pH 7, at 5 °C, and a protein concentration of 5 µM. The solid lines represent best fits of kinetic traces to a single exponential function. Points collected during the dead time of the instrument are not shown. Numbers at each curve indicate final concentrations of urea. The wild-type protein refers to W99F/Y218W huPrP90-231 in the absence of any disease-associated mutations.
FIG. 4. Kinetic data for the folding and unfolding of huPrP90-231 variants with amino acid substitutions corresponding to inherited prion diseases. Each panel represents urea concentration dependence of the observed refolding (low denaturant concentrations) and unfolding (high denaturant concentration) rate constants. Experiments were performed in 50 mM phosphate buffer, pH 7, at 5 °C, and a protein concentration of 5 µM. Solid lines represent best fits of kinetic data to a three state sequential folding model $U \leftrightarrow I \leftrightarrow N$.

FIG. 5. The effect of protein concentration on refolding kinetics of huPrP90-231 variants F198S and V210I. Full Chevron plots obtained at 5 µM protein (●) are shown together with selected rate constants at protein concentration of 50 µM (O). Final concentrations of urea in the refolding experiments at 50 µM protein are 1.13, 1.66, 2.10, 2.53, and 3.03 M. Solid lines represent the best fit of the kinetic data to a three state sequential folding model.

FIG. 6. Representative kinetic traces for the refolding of V210I huPrP90-231 (O), together with control stopped flow data for N-acetyl-Trp-amide (NATA) (●). The protein and NATA were dissolved in a buffer containing 8 M urea and then mixed in the stopped-flow instrument with a buffer containing final urea concentration of 2.0 M (A) and 3.1 M (B). Measurements with NATA were performed employing same experimental protocol as that used for the protein. The final concentration of NATA or protein was 5 µM. The solid lines represent best fits of kinetic traces for the protein to a single exponential function. Points collected during the dead time of the instrument (first one millisecond) are not shown.
FIG. 7. The reaction coordinate diagram for the refolding of the wild-type huPrP90-231 (red) and the V210I variant (blue). The diagram shows free energy levels of the native (N) and intermediate (I) states relative to the unfolded state (U). TS represents the transition state between I and N. The unfolded states for the wild-type and mutant proteins are assumed to be identical. The $\Delta G_{\text{IN}}^{0,\text{kin}}$ value for the V210I variant is lower than that for the wild type huPrP90-231, indicating higher population of the intermediate for the mutant protein.
TABLE I

Thermodynamic and kinetic parameters for the folding of huPrP variants associated with inherited prion diseases

\( \Delta G_{UN}^{0,eq} \) represents the free energy obtained from the best fit of the equilibrium unfolding data to a two-state model (25). The remaining parameters were obtained from the best fit of the kinetic data from Fig. 4 to a three state sequential model (31) according to the following equation:

\[
\ln k_{\text{obs}} = \ln\left[\left(K_{UI}^0 e^{(-m_{UI}/RT)\text{[urea]}} / \left[1 + K_{UI}^0 e^{(-m_{UI}/RT)\text{[urea]}}\right]\right) \times k_{IN}^0 e^{(-m_{IN}/RT)\text{[urea]}} + k_{NI}^0 e^{(-m_{NI}/RT)\text{[urea]}}\right],
\]

where \( k_{\text{obs}} \) is the measured rate constant, \( k_{IN}^0 \) and \( k_{NI}^0 \) are microscopic rate constants corresponding to the I \( \rightarrow \) N and N \( \rightarrow \) I transitions, respectively, and \( K_{UI}^0 \) is the equilibrium constant between the unfolded and intermediate states. The parameters \( m_{UI} \), \( m_{IN} \) and \( m_{NI} \) represent the denaturant concentration dependence of \( K_{UI} \), \( k_{IN} \) and \( k_{NI} \), respectively. \( \Delta G_{UN}^{0,\text{kin}} \) was calculated from kinetic data according to the equation: \( \Delta G_{UN}^{0,\text{kin}} = \Delta G_{IN}^{0,\text{kin}} + \Delta G_{UI}^{0,\text{kin}} \), where \( \Delta G_{IN}^{0,\text{kin}} = RT \ln(k_{IN}^0/k_{NI}^0) \) and \( \Delta G_{UI}^{0,\text{kin}} = RT \ln K_{UI}^0 \). The parameter \( \alpha_{I} \), related to the compactness of the I state, was calculated as \( \alpha_{I} = m_{UI}/(m_{UI} + m_{IN} + m_{NI}) \).

| Protein   | \( K_{UI}^0 \) | \( k_{IN}^0 \) | \( k_{NI}^0 \) | \( m_{UI} \) | \( m_{IN} \) | \( m_{NI} \) | \( \Delta G_{UI}^{0,\text{kin}} \) | \( \Delta G_{IN}^{0,\text{kin}} \) | \( \Delta G_{UI}^{0,\text{kin}} \) | \( \Delta G_{UN}^{0,\text{eq}} \) | \( \alpha_{I} \) |
|------------|----------------|----------------|----------------|------------|------------|------------|----------------|----------------|----------------|----------------|------------|
| Wild type  | 12.2 s^{-1}    | 1290 M^{-1}    | 0.03 M^{-1}    | 2.1 kJ mol^{-1} | 0.24 kJ mol^{-1} | 2.4 kJ mol^{-1} | 5.8 kJ mol^{-1} | 24.6 kJ mol^{-1} | 30.4 kJ mol^{-1} | 29.4 \( \pm \) 1.4 kJ mol^{-1} | 0.45        |
| F198S      | 20.6 s^{-1}    | 1150 M^{-1}    | 3.3 M^{-1}     | 2.5 kJ mol^{-1} | 0.12 kJ mol^{-1} | 1.4 kJ mol^{-1} | 7.0 kJ mol^{-1} | 13.5 kJ mol^{-1} | 20.5 kJ mol^{-1} | 21.6 \( \pm \) 1.1 kJ mol^{-1} | 0.66        |
| Q217R      | 24.4 s^{-1}    | 1015 M^{-1}    | 1.4 M^{-1}     | 2.6 kJ mol^{-1} | 0.27 kJ mol^{-1} | 1.7 kJ mol^{-1} | 7.4 kJ mol^{-1} | 15.2 kJ mol^{-1} | 22.6 kJ mol^{-1} | 22.2 \( \pm \) 1.0 kJ mol^{-1} | 0.64        |
| R208H      | 8.0 s^{-1}     | 1150 M^{-1}    | 0.17 M^{-1}    | 2.4 kJ mol^{-1} | 0.60 kJ mol^{-1} | 2.1 kJ mol^{-1} | 4.8 kJ mol^{-1} | 20.3 kJ mol^{-1} | 25.1 kJ mol^{-1} | 25.3 \( \pm \) 1.1 kJ mol^{-1} | 0.62        |
| D178N/129M | 35.6 s^{-1}    | 1023 M^{-1}    | 0.58 M^{-1}    | 2.6 kJ mol^{-1} | 0.11 kJ mol^{-1} | 1.8 kJ mol^{-1} | 8.2 kJ mol^{-1} | 17.3 kJ mol^{-1} | 25.7 kJ mol^{-1} | 26.3 \( \pm \) 1.2 kJ mol^{-1} | 0.61        |
| D178N/129V | 31.4 s^{-1}    | 970 M^{-1}     | 0.27 M^{-1}    | 2.7 kJ mol^{-1} | 0.19 kJ mol^{-1} | 1.9 kJ mol^{-1} | 8.0 kJ mol^{-1} | 18.6 kJ mol^{-1} | 26.6 kJ mol^{-1} | 26.7 \( \pm \) 1.5 kJ mol^{-1} | 0.61        |
| V180I      | 35.7 s^{-1}    | 1305 M^{-1}    | 0.60 M^{-1}    | 2.4 kJ mol^{-1} | 0.15 kJ mol^{-1} | 1.5 kJ mol^{-1} | 8.3 kJ mol^{-1} | 17.8 kJ mol^{-1} | 26.1 kJ mol^{-1} | 26.8 \( \pm \) 1.2 kJ mol^{-1} | 0.60        |
| V210I      | 73.6 s^{-1}    | 1100 M^{-1}    | 0.75 M^{-1}    | 2.5 kJ mol^{-1} | 0.04 kJ mol^{-1} | 1.6 kJ mol^{-1} | 10.1 kJ mol^{-1} | 16.9 kJ mol^{-1} | 27.0 kJ mol^{-1} | 27.3 \( \pm \) 1.1 kJ mol^{-1} | 0.63        |
| E200K      | 6.4 s^{-1}     | 1216 M^{-1}    | 0.03 M^{-1}    | 2.2 kJ mol^{-1} | 0.46 kJ mol^{-1} | 2.4 kJ mol^{-1} | 4.6 kJ mol^{-1} | 24.6 kJ mol^{-1} | 29.2 kJ mol^{-1} | 28.6 \( \pm \) 1.2 kJ mol^{-1} | 0.52        |
| P102L      | 12.1 s^{-1}    | 1058 M^{-1}    | 0.03 M^{-1}    | 2.2 kJ mol^{-1} | 0.34 kJ mol^{-1} | 2.2 kJ mol^{-1} | 5.8 kJ mol^{-1} | 23.6 kJ mol^{-1} | 29.4 kJ mol^{-1} | 30.1 \( \pm \) 1.4 kJ mol^{-1} | 0.53        |
TABLE II
Population of intermediate (I) and unfolded (U) states relative to the native (N) state for huPrP90-231 variants at zero denaturant concentration

The \([I]:[N]\) ratio was calculated using the formula: \([I]/[N] = \exp(-\Delta G_{IN}^{0, \text{kin}}/RT)\), whereas the \([U]:[N]\) ratio was calculated as: \([U]/[N] = \exp(-\Delta G_{UN}^{0, \text{kin}}/RT)\).

| huPrP90-231 variant | [I]:[N]   | [U]:[N]   |
|---------------------|-----------|-----------|
| Wild type           | 1:42000   | 1:520000  |
| F198S               | 1:350     | 1:7200    |
| Q217R               | 1:720     | 1:18000   |
| R208H               | 1:6500    | 1:52000   |
| D178N/129M          | 1:1800    | 1:62000   |
| D178N/129V          | 1:3100    | 1:99000   |
| V180I               | 1:2200    | 1:81000   |
| V210I               | 1:1500    | 1:120000  |
| E200K               | 1:42000   | 1:320000  |
| P102L               | 1:27000   | 1:340000  |

\(^1\)The wild-type protein refers to W99F/Y218W huPrP90-231 in the absence of any disease-associated mutations.
FIGURE 1
Figure 2: Fraction of Native Molecules vs. [urea], M for WT and F198S.
FIGURE 3
FIGURE 4
FIGURE 5
FIGURE 6
FIGURE 7
The effect of disease-associated mutations on the folding pathway of human prion protein
Adrian C. Apetri, Krystyna A. Surewicz and Witold K. Surewicz

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