Familial Mutations and the Thermodynamic Stability of the Recombinant Human Prion Protein*

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Wieslaw Swietnicki, Robert B. Petersen, Pierluigi Gambetti, and Witold K. Sureswicz

From the Department of Pathology, Case Western Reserve University, Cleveland, Ohio 44106

Hereditary forms of human prion disease are linked to specific mutations in the PRNP gene. It has been postulated that these mutations may facilitate the pathogenic process by reducing the stability of the prion protein (PrP). To test this hypothesis, we characterized the recombinant variants of human PrP(90–231) containing point mutations corresponding to Gerstmann-Straussler-Scheinker disease (P102L), Creutzfeldt-Jakob disease (E200K), and fatal familial insomnia (M129/D178N). The two first of these mutants could be recovered from the periplasmic space of Escherichia coli in a soluble form, whereas the D178N variant aggregated into inclusions bodies. The secondary structure of the two soluble variants was essentially identical to that of the wild-type protein. The thermodynamic stability of these mutants was assessed by unfolding in guanidine hydrochloride and thermal denaturation. The stability properties of the P102L variant were indistinguishable from those of wild-type PrP, whereas the E200K mutation resulted in a very small destabilization of the protein. These data, together with the predictive analysis of other familial mutations, indicate that some hereditary forms of prion disease cannot be rationalized using the concept of mutation-induced thermodynamic destabilization of the cellular prion protein.

Prion diseases, also known as spongiform encephalopathies, are disorders of the central nervous system. Originally described in sheep under the name of scrapie, they affect both humans and animals. These diseases are unique in that they may arise sporadically, may be inherited, or may be acquired by transmission of an infectious agent (1–4).

The molecular nature of the prion pathogen is a matter of controversy (5, 6). According to the "protein only" hypothesis (7, 8), the key factor in the disease is an abnormal protein, designated PrP 

PrP 

PrP is highly soluble and easily degraded by proteinase K, PrPRES exists as an insoluble aggregate that is resistant to proteinase K digestion and often has the characteristics of an amyloid (10, 11). These differences in physical properties most likely reflect different conformations of the two isoforms: PrP 

PrPRES is highly α-helical, whereas PrPRES appears to contain a large proportion of β-sheet structure (12–18). The current view is that spongiform encephalopathies may be classified as disorders of protein folding and that the key event in the pathogenic process is the transition between the "benign" fold of PrP 

PrPRES and the "pathological" fold of PrPRES (4, 19–22). However, the molecular mechanism of this conformational transition remains unknown.

Hereditary human spongiform encephalopathies include Creutzfeldt-Jakob disease, fatal familial insomnia, and Gerstmann-Straussler-Scheinker disease. All these disorders have been linked to specific mutations in the PRNP gene (3, 4). It has been hypothesized that these mutations facilitate the conformational conversion of the prion protein by destabilizing the native structure of PrP 

PrP 

PrP mutants were expressed in Escherichia coli and purified as described previously (23). Protein concentration was determined spectrophotometrically using a molar extinction coefficient of 21,640 M–1 cm–1.

Equilibrium Unfolding in GdnHCl—For equilibrium unfolding, individual proteins (approximately 0.1 mg/ml) were incubated for 10 h at 30 °C in an appropriate buffer in the presence of increasing concentrations of GdnHCl. Ellipticity at 222 nm (θ222) was then measured in a 1-mm cell by averaging the signal over 2 min. The θ222 versus GdnHCl concentration plots were analyzed as described by Santoro and Bolen (25), yielding the free energy of unfolding extrapolated to zero denaturant concentration (ΔGf0) and the m parameter. The ΔGf0 value is subject to considerable error due to data extrapolation from the transition region to water. Therefore, to obtain more reliable measure of small differences between the stabilities of mutant proteins, we have used the procedure described by Clarke and Fersht (26). In this approach, the individual unfolding curves are analyzed in terms of the midpoint unfolding concentration of the denaturant [D]f0 and the m values. These parameters are then used to obtain ΔG[D]f0, the difference in the free energy of denaturation for the wild-type and mutant proteins at the denaturant concentration at which 50% of the protein is unfolded. The ΔG[D]f0 values were calculated according to the formula: ΔG[D]f0 = <m>D[D]f0, where <m>D[D]f0 is the average of the value of [D]f0 for the wild-type and the mutant and <m> is the average value of m for the wild-type and mutant proteins. This

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† To whom correspondence should be addressed: Dept. of Pathology, Case Western Reserve University, 2085 Adelbert Rd., Cleveland, OH 44106. Tel.: 216-368-0139; Fax: 216-368-2546; E-mail: wks3@pop.cwr.edu.

‡ The abbreviations used are: PrP, prion protein; PrPRES, cellular form of prion protein; PrPRES, proteinase K-resistant form of prion protein; GdnHCl, guanidine hydrochloride; huPrP(90–231), human prion protein domain 90–231.

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procedure is applicable to our system, since the m values for all PrP variants studied are very similar.

**Thermal Denaturation Experiments**—Thermal unfolding experiments were performed at a protein concentration of 0.1 mg/ml by following changes in ellipticity at 222 nm as a function of temperature. The heating rate in most experiments was 1 degree/min. Control measurements at a lower heating rate (0.2 degree/min) gave essentially identical results. The thermal unfolding curves were analyzed as described previously (27), yielding the midpoint denaturation temperature, $T_m$, and the van’t Hoff enthalpy of denaturation, $\Delta H_{vH}$. Thermal denaturation data may be used to calculate the free energy of unfolding, but only if the heat capacity change function ($\Delta C_p$) is known (28). Attempts to obtain $\Delta C_p$ from the temperature dependence of van’t Hoff enthalpy at different pH did not yield consistent results, presumably due to the departure of the unfolding at low pH from the two-state model. An estimate of $\Delta C_p$ was obtained from the m values derived from GdnHCl denaturation experiments as described previously (29). Using this estimate, we have calculated $\Delta G^{\text{m}}$, the difference in the free energy of unfolding between the wild-type and mutant protein, evaluated at $T_m$ for the wild-type protein, by means of the Gibbs-Helmholz equation: $\Delta G^{\text{m}} = \Delta H_{vH} (T_2 - T_1)/T_2 - \Delta C_p \ln(T_2/T_1)$, where $T_1$ and $T_2$ represent $T_m$ values for the wild-type and mutant protein and $\Delta H_{vH}$ is the denaturational enthalpy of the mutant at $T_m$ (30).

The measurements were performed using Jasco-600 spectropolarimeter equipped with a 1-mm water-jacketed cell and a computer-controlled water bath.

**RESULTS**

Following our initial studies with the wild-type huPrP(90–231) (23), we characterized three protein variants with the following point mutations: P102L, E200K, and D178N. The wild-type huPrP(90–231) and the P102L and E200K mutants were secreted in a soluble form into the periplasmic space of E. coli. These proteins were purified to homogeneity (95% or better according to SDS-polyacrylamide gel electrophoresis) and their identities confirmed by Western blotting and mass spectrometry. In contrast, the D178N huPrP(90–231), which was aggregated in inclusion bodies and very little protein was recovered, was expressed at a level similar to that of the other variants, aggregated in inclusion bodies and very little protein was recovered in a soluble form.

The far-UV circular dichroism spectra of the P102L and E200K variants at pH 7.2–3.6 were very similar to those of the wild-type protein (Fig. 1A; only data at pH 7.2 are shown for brevity), indicating in all cases a high content of $\alpha$-helical structure. Furthermore, the near-UV CD spectra of the P102L and E200K mutants were essentially indistinguishable from that of the wild-type huPrP(90–231) (spectra not shown for brevity). Clearly, these mutations do not result in any significant perturbation of the protein secondary and tertiary structure.

The effect of individual mutations on the thermodynamic stability of PrP(90–231) was probed by equilibrium unfolding in GdnHCl. At neutral pH, the unfolding of both the wild-type protein as well as the P102L and E200K variants can be described as a two-state transition (Fig. 2). The thermodynamic parameters derived from the unfolding curves are shown in Table I. Analysis of these data show that the P102L mutation does not have any measurable effect on the protein thermodynamic stability. However, comparison of the free energy of denaturation ($\Delta G_{\text{HCl}}$) for the wild-type and E200K PrP(90–231) indicates a small destabilization of the latter mutant. The main source of error in calculating $\Delta G_{\text{HCl}}$ is the extrapolation of the data from the transition region of the unfolding curve to zero denaturant concentration (26). As shown previously, the relative stabilities of a series of mutant proteins may be determined with higher precision by calculating the difference in free energy (relative to the wild-type protein) at the denaturant concentration corresponding to the midpoint unfolding (26). The results of such calculations (shown in column five of Table I) confirm a small reduction in the stability of the E200K mutant.

**FIG. 1.** Far-UV circular dichroism spectra of huPrP(90–231) variants. A, spectra recorded in 50 mM phosphate buffer, pH 7.2; B, spectra in 50 mM acetate buffer, pH 3.6, in the presence of 1 M guanidine hydrochloride. Solid line, wild-type protein; dashed line, P102L variant; dotted line, E200K variant.

**FIG. 2.** Unfolding of huPrP(90–231) variants in guanidine hydrochloride at pH 7.2. Squares (solid line) represent the wild-type protein, and circles (dashed line) represent the E200K mutant. The data for the P102L mutant are essentially identical to those for the wild-type protein and are not shown for clarity of the figure. Lines represent best fits according to the two-state model.

The GdnHCl denaturation of huPrP(90–231) at acidic pH is more complex, indicating the presence of a stable folding intermediate (23). Very similar unfolding curves were obtained for the wild-type protein and the P102L and E200K mutants (Fig. 3). These curves may be analyzed according to the three state
The reported values represent the average of two to three experiments. \( |D|_{0.50} \) is the concentration of GdnHCl at the midpoint of denaturation, and the parameter \( m \) represents the slope of free energy of unfolding versus denaturant concentration plot. \( \Delta G_{H2O} \) is the free energy of unfolding extrapolated to zero GdnHCl concentration. The value of \( \Delta G_{H2O} \) for the wild-type protein is somewhat higher than reported in our previous study (23). The present value is based on a larger number of experiments. \( \Delta G(D)_{0.50} \) represents the difference in the free energy of GdnHCl denaturation for the wild-type and mutant protein at the denaturant concentration at which 50% of the protein is unfolded (26). \( T_m \) and \( \Delta H_{H2O} \) represent the midpoint temperature and the van’t Hoff enthalpy of the thermal denaturation, respectively.

### Thermodynamic parameters for equilibrium unfolding of huPrP(90–231) variants

| Protein       | \( |D|_{0.50} \) | \( m \) \( \text{kJ mol}^{-1} \text{M}^{-1} \) | \( \Delta G_{H2O} \) \( \text{kJ mol}^{-1} \) | \( \Delta G(D)_{0.50} \) \( \text{kJ mol}^{-1} \) | \( T_m \) °C | \( \Delta H_{H2O} \) \( \text{kJ mol}^{-1} \) |
|---------------|--------------|-----------------|-----------------|-----------------|-------|-----------------|
| Wild-type     | 2.19 ± 0.02  | 12.0 ± 0.3      | 26.3 ± 1.7      | 0.1 ± 0.1       | 70.2 ± 0.1 | 294 ± 6         |
| P102L         | 2.18 ± 0.02  | 11.4 ± 1.0      | 24.9 ± 2.4      | 0.1 ± 0.1       | 70.3 ± 0.1 | 306 ± 6         |
| E200K         | 2.04 ± 0.01  | 10.9 ± 0.3      | 22.2 ± 0.9      | 1.7 ± 0.2       | 67.0 ± 0.9 | 300 ± 8         |

Fig. 3. Unfolding of huPrP(90–231) variants in the presence of 1 M guanidine hydrochloride at pH 3.6 (50 mM acetate buffer). A, wild-type protein; B, P102L mutant; C, E200K mutant. \( \theta_{222} \) represents the molar ellipticity at 222 nm.

model (23, 31). However, due to a large number of parameters and poor baseline definition, the accuracy of such an analysis is insufficient to allow reliable determination of small differences between the protein variants. The far-UV CD spectra measured under conditions corresponding to the presence of a folding intermediate (pH 3.6, 1 M GdnHCl) are clearly different from those of the native protein at pH 7.2 (Fig. 1). These spectra display a single minimum around 215 nm, indicating that the intermediate contains a large proportion of a \( \beta \)-sheet conformation. While the high resolution structure of this folding intermediate is at present unknown, its properties appear to be different from those of a molten globule state, since molten globules are usually characterized by native-like secondary structure. Importantly, there are no significant differences between the far-UV CD spectra of the equilibrium intermediates formed by the wild-type huPrP(90–213) and the P102L and E200K mutants.

The effect of hereditary pathogenic mutations on the stability of the recombinant prion protein was further probed by thermal denaturation. Under the present experimental conditions, the thermal unfolding of both wild-type and mutated proteins was fully reversible and did not result in protein aggregation. The CD spectra recorded at room temperature after the heating-cooling cycle as well as the elution patterns on size exclusion column (Superose 6) were identical to those measured before the heat treatment. Furthermore, the unfolding (heating) and refolding (cooling) curves were superimposable. These findings contrast with the apparent irreversibility of thermal denaturation reported for hamster PrP(90–231) (32). The above discrepancy may be partly due to the higher protein concentration used in the previous study. The thermal unfolding curve (pH 7.2) for the P102L mutant was indistinguishable from that for the wild-type protein, while the unfolding of E200K huPrP(90–231) occurred at somewhat lower temperature (Fig. 4). Analysis of these curves according to a two-state model yielded the midpoint unfolding temperatures (\( T_m \)) and van’t Hoff enthalpies of denaturation shown in Table I. Using these data, we calculated \( \Delta G(T_m) \), the standard free energy of denaturation of the E200K mutant corrected to \( T_m \) of the wild type protein. This parameter, which provides a measure of the energy of destabilization of the mutant relative to the wild-type protein (30), amounts to 2.9 kJ/M. Overall, both the chemical and thermal denaturation data indicate an essentially unchanged thermodynamic stability of the P102L variant and a slight reduction in the stability of the E200K mutant.

**Fluorescence Spectroscopy**—The substitution of PrP\textsuperscript{102} with Leu is nonconservative. While circular dichroism data indicate that this substitution has a negligible effect on the overall secondary structure, the question remains whether the P102L mutation could affect the local conformation of the protein. Such a local perturbation would not necessarily be reflected in the thermodynamic stability since residue 102 is located in an unordered region outside the folded C-terminal domain (16–18). The local conformation of PrP(90–231) in the vicinity of residue 102 may be probed by measuring the accessibility of the sole tryptophan at position 99 to fluorescence quenchers such as acrylamide (33). The acrylamide quenching curves for the wild-type protein and the P102L mutant are shown in Fig. 5. The Stern-Volmer constants derived from these curves are essentially identical (10 ± 0.2 M\(^{-1}\)), indicating for both proteins an almost complete exposure of Trp\textsuperscript{99} to water. These data argue against the possibility that the Pro to Leu substitution induces local ordering in the flexible N-terminal portion of the protein.

**DISCUSSION**

Within the framework of the protein only hypothesis, the central molecular event in the pathogenesis of prion diseases is the conversion of PrP\textsuperscript{C} into an abnormal, conformationally altered isoform, PrP\textsuperscript{res} (1–4, 19). Recent experiments with the recombinant prion protein indicate that PrP\textsuperscript{C} is characterized...
since the PrPC to PrPres conversion likely occurs through par-
dendogenously PrP C, the conversion process in hereditary spon-
structure of PrPC (4, 21, 22). Such a hypothesis seems logical,
primary role of these mutations is to destabilize the native
basis of the disease. It has been widely postulated that the
tations should provide important clues regarding the molecular
Therefore, studies with PrP variants containing familial mu-

FIG. 4. Thermal denaturation of huPrP(90–231) variants at pH
7.2. Circles (solid line), wild-type protein; triangles (dashed line),
E200K mutant. The denaturation curve for the P102L mutant was
indistinguishable from that for the wild-type protein and is not shown
for clarity of the figure. Best fits are represented by lines and the
symbols represent every 20th experimental point.

FIG. 5. Stern-Volmer plots for acrylamide quenching of the
fluorescence of the wild type (open squares) and P102L (filled
squares) variants of huPrP(90–231) at pH 7.2. F0/F represents the
ratio of fluorescence in the absence and presence of acrylamide,
respectively.

by high thermodynamic stability (23, 34). As in the case of
classical amyloid-forming proteins, conversion of such a stable
structure would likely be a complex process involving destabi-
lization of PrP\textsuperscript{C} and formation of partially unfolded intermedi-
ates (20, 35–37). While the transmission of prion disorders
requires direct interaction between exogenous PrP\textsuperscript{Sc} and en-
dogenous PrP\textsuperscript{C}, the conversion process in hereditary spon-
iform encephalopathies appears to occur spontaneously (3).
Therefore, studies with PrP variants containing familial mu-
tations should provide important clues regarding the molecular
basis of the disease. It has been widely postulated that the
primary role of these mutations is to destabilize the native
structure of PrP\textsuperscript{C} (4, 21, 22). Such a hypothesis seems logical,
since the PrP\textsuperscript{C} to PrP\textsuperscript{Sc} conversion likely occurs through par-
tially unfolded intermediate(s) that would be promoted upon
destabilization of PrP\textsuperscript{C} (20). The general mechanism of patho-
genesis based on the concept of mutation-induced protein de-
estabilization has been relatively well documented for some
other amyloid-related diseases (35, 36, 38). However, the effect
of familial mutations on the structure and thermodynamic stability of the prion protein has yet to be tested experimentally.

To study the effect of pathogenic mutations on the thermo-
dynamic stability of prion protein, we used the recombinant
huPrP(90–231). Although the recombinant protein lacks glyco-
sylation and the glycosylphosphatidylinositol anchor, its sec-
ondary and tertiary structure appears to be very similar to that
of PrP\textsuperscript{C} isolated from the brain (39). This justifies the use of the recombinant protein as a structural model of PrP\textsuperscript{C}. However, the inherent limitation of the present study is that the thermo-
dynamic data for PrP mutants has been inferred from \textit{in vitro}
experiments performed on the unfolding pathway and not dur-
ing the \textit{in vivo} conversion reaction. While this approach affords
reliable information about the mutation-dependent stability of
PrP\textsuperscript{C}, it provides no insight into the effect of mutations on the
thermodynamic properties of the PrP\textsuperscript{Sc} species.

The huPrP(90–231) variants tested in this study correspond
to three major phenotypes of hereditary human spongiform
encephalopathies (3). While the first of these mutations has no
detectable effect on the thermodynamic stability of huPrP(90–
231), the E200K substitution appears to result in a very small
destabilization of the protein. However, it is unlikely that such
a slight destabilizing effect alone could account for the patho-
genicity associated with the E200K mutation. Furthermore, no
significant changes due to the P102L and E200K mutations
could be detected in the PrP unfolding pathway at acidic pH. As
discussed previously, acidic conditions are conducive to the
formation of a potentially “prionogenic” folding intermediate
(23). Aggregation precluded thermodynamic characterization
of the D178N variant. However, the finding that in the bacte-
rial system this mutant cannot be expressed in a soluble form
suggests that it has different physicochemical properties, pos-
sibly reflecting structural destabilization.

The present findings may be rationalized based on previous
NMR structural studies with the recombinant PrP (15, 16, 18).
The NMR data indicate a strong potential for salt bridge
between the carboxyl group of Asp\textsuperscript{178} and the guanidinium group
of neighboring Arg\textsuperscript{164}. The D178N mutation would abolish this
salt bridge, resulting in destabilization of the protein. Furth-
ernore, Asp\textsuperscript{178} is in close proximity to the disulfide bridge. A
mutation in this region may potentially interfere with the S–S
bridge formation, leading to aggregation of the protein. A
decrease in protein stability may also be predicted for a few of
the other familial mutations. This effect should be particularly
strong for the F198S variant, since the buried Phe residue
occupies a large hydrophobic space that would not be preserved
upon substitution with the small polar Ser. However, major
destabilization of PrP structure does not appear to be a com-
mon feature of all mutations associated with hereditary prion
diseases. Residue 102 is located outside the folded C-terminal
domain of PrP. Therefore, it is not all that surprising that the
destabilizing effect was not observed for the P102L variant.
Based on the results obtained for this variant, it may be pre-
dicted that all other mutations within the flexible N-terminal
region (i.e. P105L, A117V, additional octapeptide repeat units)
will have a negligible effect on the stability of PrP\textsuperscript{C}. Gly\textsuperscript{200} is
located on the protein surface and thus should have a relatively
small contribution to stability. The very small destabilization
found for the E200K mutant may be explained by unfavorable
electrostatic interactions between Lys\textsuperscript{200} and nearby Lys\textsuperscript{204}.

In conclusion, our data indicate that the pathogenic muta-
tions do not exert a uniform effect on the thermodynamic sta-
bility of the human prion protein, and consequently, not all
hereditary forms of the disease may be rationalized through a
common mechanism based on the mutation-induced decrease
in global stability of PrP\textsuperscript{C}. Clearly, the conversion models other
than the one involving protein destabilization should be con-
sidered to explain how familial mutations affect the conversion
of prion protein. It is also possible that some of the mutations
may facilitate conformational transition(s) in prion protein by
inducing abnormal interactions of PrP$^C$ with auxiliary proteins/chaperones (40, 41) or cellular membranes (42) or by interfering with cellular targeting of the protein.

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