Folding of Prion Protein to Its Native \( \alpha \)-Helical Conformation Is under Kinetic Control*

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The recombinant mouse prion protein (MoPrP) can be folded either to a monomeric \( \alpha \)-helical or oligomeric \( \beta \)-sheet-rich isoform. By using circular dichroism spectroscopy and size-exclusion chromatography, we show that the \( \beta \)-rich isoform of MoPrP is thermodynamically more stable than the native \( \alpha \)-helical isoform. The conformational transition from the \( \alpha \)-helical to \( \beta \)-rich isoform is separated by a large energetic barrier that is associated with unfolding and with a higher order kinetic process related to oligomerization. Under partially denaturing acidic conditions, MoPrP avoids the kinetic trap posed by the \( \alpha \)-helical isoform and folds directly to the thermodynamically more stable \( \beta \)-rich isoform. Our data demonstrate that the folding of the prion protein to its native \( \alpha \)-helical monomeric conformation is under kinetic control.

Although protein folding is commonly thought to be controlled by thermodynamic preferences, it has been understood by many, including Anfinsen and others (1,2), that kinetic issues can alter the folding landscape. Whereas most small globular proteins will refold spontaneously in vitro to a native conformation, in vivo folding often exploits auxiliary molecules and defined subcellular compartments to avoid the deposit of misfolded forms (3). Increasingly, a role for protein misfolding in a variety of neurodegenerative diseases has emerged. A common thread joining prion-based diseases and Alzheimer’s disease, and possibly Parkinson’s disease and frontotemporal dementia, is the conversion of a normal, cellular, monomeric isoform of a protein into a \( \beta \)-sheet-rich, polymeric form (4–6). When the deposited polymeric form is sufficiently ordered to bind Congo red and exhibit birefringence to polarized light, the pathologic term amyloid is used to cluster these and other conditions can also be refolded into \( \beta \)-rich, amyloid forms under conditions that destabilize the native state. So far, these proteins have not been associated with human deposition diseases. This finding has led to the suggestion that the ability to adopt alternative \( \beta \)-rich folds capable of forming amyloid is not a unique property of specific proteins associated with conformational diseases but reflects a general property of polypeptide chains (13). The interplay between protein concentration and the conformational preferences of the monomeric chain in driving the transition to a \( \beta \)-rich multimeric isoform remains to be more fully explored.

Glockshuber and colleagues (14) have shown that a fragment of the mouse prion protein folds very rapidly into the \( \alpha \)-helix-rich conformation with a half-life of 170 \( \mu \)s as measured at 4 °C. Here, we report that a \( \beta \)-sheet-rich conformation of the mouse prion protein (MoPrP) is thermodynamically more stable than its native \( \alpha \)-helix-rich conformation. The conformational transition from the \( \alpha \)-helical to a \( \beta \)-sheet-rich isoform is controlled by a large energetic barrier that is associated with partial unfolding and oligomerization of an intermediate state. Under partially denaturing conditions, it is possible to avoid the kinetic trap that leads to the normal cellular isoform, PrP\( ^C \), and fold the prion protein directly to a thermodynamically more stable, non-native \( \beta \)-isoform. Our data demonstrate that folding the prion protein to its native \( \alpha \)-conformation is under kinetic, not thermodynamic, control.

**EXPERIMENTAL PROCEDURES**

Protein Preparation—The expression and purification of recombinant MoPrP 89–231 was performed as described by Mehlhorn et al. (15).

Circular Dichroism—CD spectra were recorded with a J-720 CD spectrometer (Jasco, Easton, MD) scanning at 20 nm/min, with a bandwidth of 1 nm and data spacing of 0.5 nm using a 0.1-cm cuvette. Each spectrum represents the accumulation of three individual scans after subtracting the background spectra. To monitor the refolding curves, MoPrP was diluted from 10 \( \mu \)M to various concentrations of urea in 20 mM sodium acetate in the absence or in the presence of 0.2 \( \mu \)M NaCl, pH 3.6, and then incubated at room temperature for different periods of time. No change in pH value was detected during the time course of incubation. To monitor the kinetic trace of the conformational transition, MoPrP was rapidly mixed with 10 \( \mu \)M urea in a 1:1 volume ratio, whereas to monitor the kinetics of refolding to the \( \beta \)-MoPrP, MoPrP unfolded in 10 \( \mu \)M urea was mixed with buffer, again at a 1:1 volume ratio. All kinetic experiments were carried out in 20 mM sodium acetate and 0.2 \( \mu \)M NaCl, pH 3.6.

Analysis of the Kinetic Data—The rate constant and apparent rate order of refolding were calculated from Equation 1,

\[
1/C_n^k = 1/C_0^k + (n - 1) h t
\]

in which \( C_n \) is concentration of the monomer MoPrP at zero time, \( C_0 \) is concentration of monomer MoPrP at time \( t \), and \( n \) is apparent order of the process. \( \Delta E^\ast \) was calculated from the Arrhenius relation (1) with \( k_{\text{obs}} \), measured experimentally and determined from the equation for diffusion-controlled reaction, assuming that the reaction follows fifth-order kinetics.

Size-exclusion Chromatography—All separations were performed at 23 °C with a flow rate of 1 ml/min using TSK-3000 high pressure liquid

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† The abbreviations used are: MoPrP, mouse prion protein; PrPrP, prion protein; PrP\( ^C \), cellular isoform; PrP\( ^S \), scrapie isoform; SEC, size-exclusion chromatography.
chromatography gel filtration column (300 mm × 7.80 mm) equilibrated in 20 mM sodium acetate, 0.2 mM NaCl, pH 3.6, and the corresponding concentration of urea.

*Thioglycollate T Assay*—To follow the kinetics of amyloid formation, 0.64 mg/mL MoPrP was incubated in 20 mM sodium acetate and 0.2 mM NaCl, pH 5.5, constantly shaken at 36 °C. In the time course of incubation, aliquots of MoPrP were diluted 8 times by phosphate-buffered saline, pH 7.0, and the fluorescence was measured using a LS50B fluorimeter (PerkinElmer Life Sciences) at 482 nm (excitation at 450 nm, emission slit is 5 nm, emission slit is 10 nm, 0.4-cm rectangular cuvettes) with 5 μM thiolavin T.

*Congo Red Binding*—Congo red (Sigma) was dissolved in 5 mM potassium phosphate, 150 mM NaCl, filtered 5 times with a 0.22-mm filter (Millipore, Bedford, MA), and adjusted to 0.2 mM. The difference spectra of original MoPrP and MoPrP at 10 M concentration is performed in a high salt buffer (0.2 mM NaCl, 20 mM sodium acetate), the refolding curve under- going a gradual time-dependent transformation from a single cooperative transition between the α-isofrom and the unfolded state (Fig. 1a). When α-MoPrP is unfolded in 10 M urea and then refolded by diluting the urea concentration, its refolding curve expresses hysteresis, a phenomenon indicative of a non-two-state process (Fig. 1a). Both the unfolding and refolding limbs of the curve remain stable for at least 5 weeks when MoPrP is kept in a low salt buffer (20 mM sodium acetate). However, when refolding of MoPrP at 10 μM concentration is performed in a high salt buffer (0.2 mM NaCl, 20 mM sodium acetate), the refolding curve undergoes a gradual time-dependent transformation from a single cooperative transition to a transition with local intermediates (Fig. 1b). If a similar experiment is performed at 30 μM MoPrP, the migration of refolding curve occurs more rapidly (Fig. 1c).

Unfolded MoPrP folds first to the α-helical form upon dilution from 10 μM urea (Fig. 1d). During incubation for 5 weeks in the high salt buffer, it undergoes a slow conformational transition to the β-rich form as illustrated by the change in the overall CD spectra, as well as by reduction of the CD signal at 222 nm (Fig. 1, b and d). The conformational transition from the α-helical to a β-sheet-rich isofrom is accompanied by oligomerization as judged by size-exclusion chromatography (SEC) (Fig. 1e). Immediately after dilution from 10 μM urea, a new peak corresponding to an oligomer appears, in addition to the peak that represents a monomer. During the conformational transition, the population of monomer decreases whereas the fraction of oligomer grows. Although the square variance analysis of the oligomer peak indicates that there is certain heterogeneity of the oligomer species, electrospray mass spectrometry suggests that an octamer is the dominant multimeric assembly (data not shown).

The unfolding and refolding behavior of MoPrP demonstrates hysteresis, a time-dependent transformation of the single transition curve into a double transition curve, and a concentration-dependence for this process. These observations challenge the application of either of the two possible classical three-state models used previously to estimate the thermodynamic parameters for PrP unfolding (16, 17). In contrast, a model with two independent transitions, one between the α-isofrom and unfolded and the other between the β-isofrom and unfolded, can be used to fit the data. We have observed that the refolding to the α-isofrom is much faster than the refolding to a β-isofrom, whereas the time-dependent accumulation of a β-isofrom indicates that it is thermodynamically more stable than the α-isofrom. Thus, MoPrP diluted out of urea folds predominantly to the α-isofrom with little β-isofrom present. The presence of a β-isofrom would account for the hysteresis between the unfolding and the refolding curves (Fig. 1b). With time, the refolding curve transforms from an apparent single transition to the double transition, demonstrating equilibration of the α- and the β-isofroms.

Direct comparison of the thermodynamic stability of the α- and the β-isofroms illustrate that the α-isofrom is not the lowest energy state. First, we estimated the thermodynamic parameters for the α-isofrom using the urea-induced unfolding curve and applying a classical two-state model (see Fig. 1a and Table I (18). To evaluate the thermodynamic stability of the β-isofrom, two parameters, the molar ellipticity at 222 nm and the fraction of the oligomer, were monitored in parallel as a

![Fig. 1. Urea-induced unfolding and refolding transitions of MoPrP monitored by CD at pH 3.6.](image-url)

FIG. 1. **Urea-induced unfolding and refolding transitions of MoPrP monitored by CD at pH 3.6.** a, unfolding (squares) and refolding (circles) curves monitored upon incubation of 10 μM MoPrP for 10 min (filled circles) and for 5 weeks (open symbols) at various concentrations of urea. Data were analyzed according to a two-state transition model; the result of the fitting is represented by solid curves (18). b, refolding curves measured upon incubation of 10 μM MoPrP for 10 min (filled circles), 72 h (open circles), 1 week (filled triangles), and 5 weeks (open triangles) after dilution at a particular concentration of urea and 0.2 mM NaCl. The solid line represents the unfolding curve from panel a. c, refolding curves measured upon incubation of 30 μM MoPrP for 10 min (filled circles), 72 h (open circles), 1 week (filled triangles), and 5 weeks (open triangles) after dilution at a particular concentration of urea and 0.2 mM NaCl. The solid line represents the unfolding curve from panel a. d, far-UV CD spectra of original α-MoPrP (solid line), recorded immediately after dilution from 10 μM urea (long-dashed line) and after a 5-week incubation following dilution (short-dashed line). e, SEC profile of original α-MoPrP (1) and profiles obtained upon incubation for 10 min (2), 72 h (3), 1 week (4), and 5 weeks (5) after dilution from 10 to 1 μM urea.
function of urea concentration after re-equilibration of MoPrP for 5 weeks. Despite the fact that a small fraction of MoPrP remains trapped in the α-helical conformation even after 5 weeks, we have exploited the fraction of the β-oligomer as directly measured by SEC to analyze the “unfolded ↔ β-isofrom” equilibrium using the two-state model (Fig. 2a). The unfolding curve measured by CD requires deconvolution, because it is composed of signals from the β-isofrom, the unfolded state, and the α-isofrom. Using the population of the monomer measured by SEC as a function of urea concentration and the thermodynamic parameters estimated previously for the “α-isofrom ↔ unfolded” equilibrium, we calculated the contribution of the α-isofrom to the CD curve (Fig. 2b). When this contribution is subtracted from the original curve, a curve reflecting the unfolded ↔ β-isofrom transition results. As shown in Fig. 2c, the transition curves for the β-isofrom are superimposable, with ΔG, m, and C1/2 determined from the two techniques equal within the uncertainty of the experiment (Table I). Both ΔG and C1/2 demonstrate that the β-isofrom is thermodynamically more stable than the α-isofrom (see Fig. 2c and Table I). Because both isofroms can be refolded directly from the unfolded state, we have used the unfolded state as a reference in the free energy diagram (Fig. 2d).

Although the β-isofrom is thermodynamically more stable than the α-isofrom, it might be not a true global energy minimum state, because the β-isofrom can undergo an additional time-dependent transition to a polymeric amyloid form. Incubation of β-MoPrP at 37 °C and constant shaking lead to the formation of higher molecular weight aggregates that possess amyloid properties. The process of amyloid formation monitored by thioflavin T binding displays an apparent latent period and then an exponential accumulation of the aggregate (Fig. 3a). In addition to thioflavin T, the amyloid of MoPrP binds Congo red in a specific manner as judged by birefringence of polarized light and typical red shift of absorbance spectra (Fig. 3b). Aggregated MoPrP forms numerous twisted fibrilar filaments as seen by electron microscopy (Fig. 3c).

Why is the thermodynamically more stable β-isofrom not accessible during folding under native conditions? Previously, it has been shown that the folding of PrP to the α-isofrom is an extremely fast, first-order process (14). Folding to the β-isofrom is slower by several orders of magnitude and is concentration-dependent. To prevent the conformational conversion, the α-isofrom has to be separated by a large energetic barrier from the β-isofrom. Although the free energy diagram does not provide a view of the actual kinetic pathway for the conformational transition, several important observations can be made concerning the origin of the energetic barrier. First, the α-isofrom has to unfold substantially on route to the β-isofrom. As we have seen before, the α-isofrom converts very slowly to the β-isofrom at pH 3.6 in the absence of urea (Fig. 1b). This process can be accelerated by shifting the α-isofrom ↔ unfolded equilibrium toward the unfolded state. After jumping the urea concentration from 0 to 5 M, we observed a very fast loss of secondary structure by the α-monomer within the dead time of manual mixing, followed by an accumulation of a β-sheet-rich conformation (Fig. 4a). This result illustrates that a substantial portion of the energetic barrier requires partial unfolding of the α-isofrom. The connection between the structural complexity of the pretransition state and the energetic barrier is demonstrated by previous observations.

### Table I

| Isoform | ΔG (kcal/mol) | m (kcal/mol μ) | C1/2 (M) |
|---------|---------------|---------------|----------|
| α-PrP   | -3.95 ± 0.12  | 1.18 ± 0.03   | 3.3      |
| β-PrP by SEC | -6.07 ± 0.12  | 0.88 ± 0.02   | 6.9      |
| β-PrP by CD/SEC | -6.19 ± 0.55  | 0.91 ± 0.11   | 6.8      |

### Fig. 2

Estimated thermodynamic stability of β-MoPrP. MoPrP unfolded at 10 M urea was diluted to various concentrations of urea at pH 3.6 in the presence of 0.2 M NaCl and incubated for 5 weeks at 23 °C before SEC and CD measurements. α, examples of SEC profiles monitored at different concentrations of urea, from bottom to top: 1, 2, 3, 3.5, 4, 5, 6, 7, 8, and 9 M. To apply a two-state model, the population of the β-oligomer versus urea concentration was normalized relative to the amount of the β-oligomer observed with 1 M urea taken at 100%. b, CD measurements of the original refolding curve (filled circles) and the transition curve after subtracting the contribution of the α-isofrom (open circles). The solid line represents the result of the fitting to the two-state model. c, normalized transition of the α-isofrom (filled circles) and the β-isofrom (open circles) as determined by SEC (solid line) and by CD (dashed line) against varying concentrations of urea. d, free energy diagram for the α- and the β-isofrom of MoPrP determined at pH 3.6. U represents the unfolded state.

### Fig. 3

β-MoPrP assembles into amyloid fibrils. a, the kinetics of assembly of 40 μM MoPrP monitored by thioflavin T binding. b, difference spectra obtained at 1.7 (solid line), 3.4 (dotted line), 5.1 (dashed line), and 6.8 μM (dotted-dashed line) Congo red in the presence of 1.5 μM MoPrP taken after 160 h. c, electron micrograph of fibrils negatively stained with ammonium molybdate.
Kinetic Control of Prion Protein Folding

that conversion of PrP-derived peptides with low structural complexity into β-rich isoforms occurs spontaneously and does not require partially denaturing conditions (19–21). Whether the transition state on the way from the α- to the β-isofrom is predominantly unfolded under native conditions or whether it has residual β-sheet or α-helical structure remains to be established. A significant contribution to the energetic barrier seems to be associated with the process of oligomerization. As shown on Fig. 4b, the accumulation of a β-rich conformer is accompanied by oligomerization. The fact that both kinetic curves are superimpose illustrates that the two processes are coupled (Fig. 4a). MoPrP can be refolded directly to the β-isofrom if the unfolded protein is diluted first to 5 mM urea (Fig. 4b). When dialyzed out of urea and salt, β-MoPrP is stable for months at room temperature with no detectable conversion to the α-isofrom. Analysis of the kinetic traces indicates that the process of folding to the β-isofrom represents a single transition with apparent reaction order of 5, regardless of whether the refolding is initiated by dilution of urea from 10 to 5 mM, a jump of the urea concentration from 0 to 5 mM, or if the conformational transition occurs in the absence of urea. Such a high order of reaction suggests that the conformational transition will depend upon the concentration of the transition state.

To estimate the energy of activation (ΔG*) of the conformational transition, the Arrhenius relation,

\[ k_{obs} = k_0 \exp(-\Delta G^*/RT) \quad \text{(Eq. 2)} \]

can be used, in which \( k_{obs} \) is the constant rate of the conformational transition measured experimentally, and \( k_0 \) is the rate of the process under diffusion control. Under experimental conditions employed (pH 3.6 and 10 μM MoPrP), we found that the α-isofrom is separated from the β-isofrom by an energy barrier of 20 kcal/mol (Fig. 4c). The energetic barrier is predicted to be much higher under physiological conditions because of the lower concentration of PrP and the higher thermodynamic stability of the α-isofrom at pH 5–7 (Fig. 4d). For wild-type MoPrP, the calculated energy barrier of 35–45 kcal/mol is sufficient to prevent the process of conformational transition over the functional lifetime of the protein. Hence, a large energetic barrier prevents the conversion of the α-isofrom to the thermodynamically more stable β-isofrom. From the kinetic perspective, the process of conformational transition can be facilitated by the reduction of the energetic barrier (22). Thus, single point mutations associated with inherited forms of prion diseases might reduce the energetic barrier by stabilizing the transition state. Additionally, if PrPSc provides a template for the conversion of PrP to PrPSc by binding and stabilizing the transition state, this would also speed up the conformational conversion.

Our results clearly indicate that the folding of native PrPC is under kinetic control. The observations that many proteins are able to adopt alternative amyloid-like folds require us to revisit the role of kinetic traps in protein folding (8–12). If a β-rich amyloid competent structure is an intrinsic preference especially at a high protein concentration, then compartmentalization of partially folded intermediates and proteins that mediate unfolding and clearance of misfolded proteins play critical roles in cellular health. In addition, side-chain patterns that favor the formation of amyloid, such as alternating polar and nonpolar amino acid residues, will be avoided (23). Despite these strategies, some proteins, including PrP, Aβ, α-synuclein, parkin and tau, find a route to a β-rich, multimeric structure with unfortunate consequences.

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