Folding Intermediates of the Prion Protein Stabilized by Hydrostatic Pressure and Low Temperature*

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Prion diseases are associated with conformational conversion of the cellular prion protein, PrP<sup>C</sup>, into a misfolded form, PrP<sup>Sc</sup>. We have investigated the equilibrium unfolding of the structured domain of recombinant murine prion protein, comprising residues 121–231 (mPrP-(121–231)). The equilibrium unfolding of mPrP-(121–231) by urea monitored by intrinsic fluorescence and circular dichroism (CD) spectroscopies indicated a two-state transition, without detectable folding intermediates. The fluorescent probe 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (bis-ANS) binds to native mPrP-(121–231), indicating exposure of hydrophobic domains on the protein surface. Increasing concentrations of urea (up to 4 M) caused the release of bound bis-ANS, whereas changes in intrinsic fluorescence and CD of mPrP took place only above 4 M urea. This indicates the existence of a partially unfolded conformation of mPrP<sub>C</sub>, characterized by loss of bis-ANS binding and preservation of the overall structure of the protein, stabilized at low concentrations of urea. Hydrostatic pressure and low temperatures were also used to stabilize partially folded intermediates that are not detectable in the presence of chemical denaturants. Compression of mPrP to 3.5 kbar at 25 °C and pH 7 caused a slight decrease in intrinsic fluorescence emission and an 8-fold increase in bis-ANS fluorescence. Lowering the temperature to −9 °C under pressure reversed the decrease in intrinsic fluorescence and caused a marked (−40-fold) increase in bis-ANS fluorescence. The increase in bis-ANS fluorescence at low temperatures was similar to that observed for mPrP at 1 atm at pH 4. These results suggest that pressure-assisted cold denaturation of mPrP stabilizes a partially folded intermediate that is qualitatively similar to the state obtained at acidic pH. Compression of mPrP in the presence of a subdenaturating concentration of urea stabilized another partially folded intermediate, and cold denaturation under these conditions led to complete unfolding of the protein. Possible implications of the existence of such partially folded intermediates in the folding of the prion protein and in the conversion to the PrP<sup>Sc</sup> conformer are discussed.

Mammalian prion diseases, also known as transmissible spongiform encephalopathies, are a group of fatal neurodegenerative disorders that include scrapie in sheep and goat, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt-Jakob disease in humans. Creutzfeldt-Jakob disease generally presents itself as a form of progressive dementia, whereas scrapie and BSE are generally manifest as ataxic illnesses (1). Prion diseases can arise sporadically or can have an infectious or genetic etiology (2). Neuropathologically, these disorders are characterized by neuronal loss and astrogliosis and sometimes by spongiform degeneration of the brain and deposition of amyloid plaques (2). Several lines of evidence indicate that PrP<sup>Sc</sup>, a misfolded pathogenic isoform of the host-encoded cellular prion protein (PrP<sup>C</sup>), plays a key role in the origin of prion diseases (for reviews, see Refs. 2 and 3).

The human cellular prion protein, PrP<sup>C</sup>, is a cell surface glycoprotein normally found in neurons and in several other cell types (4, 5). Important physiological roles have been attributed to PrP<sup>C</sup>, including participation in copper uptake, protection against oxidative stress, cell adhesion, differentiation, signaling, and cell survival (6). PrP<sup>C</sup> contains a disulfide bond, is N-glycosylated, and is attached to the plasma membrane by a glycosyl phosphatidylinositol anchor at its C terminus (2). The three-dimensional NMR structures of recombinant prion proteins from mouse and hamster, PrP-(23–231), have revealed that the whole N-terminal segment comprising amino acid residues 23–120 is flexibly disordered and that only the segment containing the C-terminal 111 residues, PrP-(121–231), possesses a defined three-dimensional structure (7, 8). The latter is a self-folding domain and contains three α-helices and a two-stranded antiparallel β-sheet (9, 10).

Prion diseases may be considered as disorders resulting from abnormal protein folding. The transition between PrP<sup>C</sup> and PrP<sup>Sc</sup> occurs by a post-translational mechanism and appears to take place without any detectable covalent modification of the protein (11). One of the main characteristics distinguishing PrP<sup>C</sup> from PrP<sup>Sc</sup> is the resistance of the latter to proteolytic digestion (12, 13). In addition, their secondary, tertiary, and quaternary structures differ (14–18). PrP<sup>C</sup> is monomeric, whereas PrP<sup>Sc</sup> adopts a multimeric arrangement. Fourier transform infrared and CD spectroscopy studies indicate that PrP<sup>C</sup> is highly helical (42%), with little β-sheet structure (3%) (16). In contrast, PrP<sup>Sc</sup> contains a large amount of β-structure (43%) and less helical structure (30%). According to the current hypothesis, PrP<sup>Sc</sup> propagates by impressing its abnormal conformation on PrP<sup>C</sup>, thereby generating additional PrP<sup>Sc</sup> mole-
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cules in an autocatalytic reaction (19). This conversion reaction is thought to occur either by a template-assisted refolding mechanism in which binding of PrPSC to PrPC lowers the activation energy that separates the two states (20) or via a nucleated polymerization process in which PrPSC serves as a seed that recruits and stabilizes abnormal conformations of PrP that are in dynamic equilibrium with PrPC (21, 22).

Despite intensive research, the molecular mechanisms underlying the conformational transition between the normal and pathogenic conformers of PrP remain unknown. Partially structured folding intermediates have been implicated in the conversion of PrPSC to PrPSc (23). Partially folded intermediates are also believed to play central roles in amyloid formation by others proteins, such as transthyretin and lysozyme variants (24, 25). However, folding intermediates of the prion protein have proved very difficult to detect and characterize (26–28). In recent years, elevated hydrostatic pressure and low temperatures have been used as tools to stabilize partially folded intermediate states that are not detectable in equilibrium in the presence of chemical denaturants (29–32). Here, we characterize two partially folded equilibrium intermediates of the murine prion protein stabilized by high hydrostatic pressure and low temperature. Demonstration of the existence of these species supports the notion that partially folded intermediates present during the folding of the prion protein may have a potential involvement in the PrPSC → PrPSc conversion or in the formation of neurotoxic PrP species in vivo.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—bis-ANS was from Molecular Probes (Eugene, OR). Stock solutions were prepared in methanol, and the concentration was determined using the extinction coefficient, ε_{290} = 23,000 M⁻¹ cm⁻¹ (33). Urea was from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest analytical grade commercially available.

**Protein Expression and Purification**—The plasmids for bacterial expression of wild-type and F175W variant of murine PrP-(121–231) were generous gifts from Dr. R. Glockshuber (Eidgenössische Technische Hochschule, Switzerland). The recombinant proteins were expressed in *Escherichia coli* strain BL21(DE3) and purified as previously described (34). The concentrations of wild-type and F175W mPrP-(121–231) were determined using ε_{290} = 19,890 M⁻¹ cm⁻¹ and ε_{280} = 28,800 M⁻¹ cm⁻¹ (26, 34), respectively.

**Fluorescence Measurements**—Unless otherwise noted, fluorescence emission spectra were measured at 25 °C on an ISS PC1 spectrofluorometer (ISS Inc., Champaign, IL). For intrinsic fluorescence measurements, excitation was at 280 nm and emission spectra were recorded from 300 to 420 nm using a WG 320 filter on the emission channel. bis-ANS fluorescence was excited at 365 nm, and emission spectra were recorded from 420 to 600 nm. Bandpasses of 8 and 16 nm were used for excitation and emission, respectively. Fluorescence measurements under pressure were performed using a pressure cell equipped with sapphire optical windows, similar to that originally described by Paladini and Weber (35). The temperature was controlled by connecting the thermostatted pressure cell to a circulating bath. During compression or cooling, the samples were allowed to equilibrate for 10 min at each condition prior to acquisition of the emission spectra. In low temperature experiments, the windows of the pressure bomb were flushed with nitrogen to prevent condensation. All experiments (unless otherwise indicated) were carried out in 50 mM sodium phosphate, pH 7.0, with 2.0 μM mPrP-(121–231). The concentration of bis-ANS was 8.9 μM.

**Thermodynamic Parameters of Unfolding of mPrP-(121–231) → PrPSc (the free energy of unfolding in the absence of denaturant)** and the m value, which measures the steepness of the dependence of the unfolding transition on the concentration of denaturant, were obtained from urea unfolding data using a two-state model for monomer unfolding. The dimensionless equilibrium unfolding constant at atmospheric pressure (K_p) and the molar volume change of folding (ΔV) can be calculated from the following thermodynamic relation (31),

\[
K_p = K_u \exp(\rho \Delta V/RT) \tag{1}
\]

where K_u is the unfolding constant at pressure p, and R and T have their usual meanings. The equation can be rewritten by introducing the degree of unfolding, α_p, at pressure p,

\[
\ln(\alpha_p/(1 - \alpha_p)) = \ln K_u + p/\Delta V/RT \tag{2}
\]

where ln(α_p/(1 − α_p)) equals ln K_u for the unfolding of a monomer. Thus, a plot of ln(α_p/(1 − α_p)) versus pressure yields the molar volume change of folding (ΔV) from the slope and K_u from the intercept on the ordinate.

At constant pressure, the temperature dependence of the equilibrium constant for a two-state unfolding transition is described by the van't Hoff equation,

\[
\Delta G/T = (1/T)\Delta H - \Delta S = -R \ln K_T \tag{3}
\]

where K_T is the equilibrium constant for denaturation at temperature T, and ΔG is the corresponding Gibbs free-energy change. From a plot of ΔG/T versus the inverse temperature, the changes in enthalpy (ΔH) and entropy (ΔS) of unfolding can be extracted.

**RESULTS**

In the present study, the equilibrium unfolding of the recombinant murine prion protein, mPrP-(121–231), was investigated using chemical and physical denaturants. mPrP-(121–231) corresponds to the structured 111-residue domain of the cellular prion protein, and its structure is the same as that in the context of the full-length protein (7, 10). Wild-type mPrP-(121–231) does not exhibit a change in intrinsic fluorescence upon unfolding, probably because of the solvent-exposed location of the sole tryptophan residue (Trp-145) at the N terminus of α-helix 1 (26). In the structure of wild-type mPrP-(121–231), Phe-175 is located in α-helix 2, with its side chain participating in the hydrophobic core of the protein (26). Replacement of this Phe residue by a Trp residue allowed creation of a folding-sensitive fluorescent variant of mPrP-(121–231), known as variant F175W (26). In contrast to the wild-type protein, the variant exhibits a 2.5-fold increase in intrinsic fluorescence upon unfolding (26). In addition, the urea-induced unfolding of mPrP-(121–231) monitored by tryptophan fluorescence and circular dichroism has been shown to be a two-state transition, without detectable folding intermediates (26).
Fig. 1 shows the equilibrium unfolding of F175W mPrP-(121–231) by urea monitored by both intrinsic and bis-ANS fluorescence intensities. In accord with a previous report (26), unfolding of mPrP-(121–231) took place between 4 and 8 M urea, with a 2.5-fold increase in intrinsic fluorescence emission and a transition mid-point at ~5.5 M urea. The changes in intrinsic fluorescence were paralleled by changes in CD, as monitored by the loss of ellipticity at 222 nm (data not shown). The structural changes induced by urea in mPrP-(121–231) were also monitored by bis-ANS fluorescence. Bis-ANS is an environment-sensitive fluorescent dye that normally binds to exposed hydrophobic patches on the protein surface in the native state. A sharp decrease in bis-ANS fluorescence, whereas changes in intrinsic fluorescence emission of mPrP caused by pressure alone. Indeed, the fluorescence intensity of the sample highly depends on the presence of hydrophobic surfaces in partially folded intermediates more tightly than to both the native and random coil states of proteins (37). Binding to exposed hydrophobic domains in proteins brings about a large increase in bis-ANS fluorescence emission and a blue shift of the emission maximum (31, 32, 38, 39). Titration of the native prion protein (2 μM) with bis-ANS (0–12 μM) showed saturation of bis-ANS binding sites at 4 μM bis-ANS, suggesting that there are two bis-ANS binding sites/molecule of prion protein (data not shown). Interestingly, maximal binding of bis-ANS to F175W mPrP-(121–231) occurred in the absence of urea (Fig. 1), indicating the exposure of hydrophobic patches on the protein surface in the native state. A similar result was obtained when binding of bis-ANS to wild-type mPrP was investigated (data not shown). Surprisingly, increasing concentrations of urea (between 1 and 4 M) caused a sharp decrease in bis-ANS fluorescence, whereas changes in intrinsic fluorescence emission (Fig. 1) and in CD (data not shown) only took place above 4 M urea.

It is noteworthy that the fluorescence emission of bis-ANS in the presence of prion protein, at pH 7.0 and in the absence of urea, is 3- to 4-fold higher than the fluorescence of the free dye (Figs. 1 and 3, insets). In addition, the fluorescence of free bis-ANS hardly changes as a function of increasing urea concentrations (Fig. 1, inset), indicating that the decrease in bis-ANS fluorescence observed in Fig. 1 is not due to a direct effect of urea on the fluorescence intensity of the free dye. Importantly, previous studies have shown that the decrease in bis-ANS fluorescence at high urea concentrations is not due to competitive inhibition of bis-ANS-protein interactions (40). These results suggest that conformational changes causing disorganization of bis-ANS binding sites on the prion protein surface take place at lower concentrations of urea than those needed to bring about changes in the intrinsic fluorescence and secondary structure of mPrP. These observations challenge the notion that the transition between native and unfolded mPrP-(121–231), at atmospheric pressure and 25 °C, is a true two-state transition.

In recent years, hydrostatic pressure and low temperatures have been used to stabilize partially folded intermediate states of proteins that are not detectable in the presence of chemical denaturants (for recent examples, see Refs. 29–32). Fig. 2 shows the pressure-assisted cold denaturation of F175W mPrP-(121–231). Compression of mPrP to 3.5 kbar at 25 °C and pH 7.0 caused a 40% decrease in intrinsic fluorescence emission (Fig. 2A). A similar result was obtained when wild-type mPrP (in which there is a single Trp residue at position 145) was compressed to 3.5 kbar under the same conditions (data not shown), indicating that the change in intrinsic fluorescence emission observed upon compression of mPrP reflects the effect of pressure on the fluorescence intensity of Trp-145. An 8-fold increase in bis-ANS fluorescence was observed upon compression of mPrP to 3.5 kbar at 25 °C and pH 7.0 (Fig. 2C), suggesting increased exposure of hydrophobic domains on the protein surface.

To further investigate the existence of folding intermediates of F175W mPrP-(121–231), we carried out cold denaturation experiments under pressure. The freezing point of water is significantly decreased under pressure (41), allowing analysis of aqueous samples at sub-zero temperatures without the need for addition of cryosolvent additives. Fig. 2B shows that cooling the sample to ~9 °C at 3.5 kbar of pressure reversed the decrease in intrinsic fluorescence emission of mPrP caused by pressure alone. Indeed, the fluorescence intensity of the sample
at 1 atm and 25 °C was quite similar to that observed at −9 °C and 3.5 kbar. This suggests that the combined pressure/cold treatment did not lead to significant unfolding of mPrP. Interestingly, however, a marked (−40-fold) increase in bis-ANS fluorescence was observed under the same conditions (Fig. 2). Importantly, upon return of the sample to 25 °C and decompression, complete recoveries of both intrinsic and bis-ANS fluorescence emission were obtained, indicating reversible refolding of both intrinsic and bis-ANS fluorescence emission of the pressure-denatured state seems to correspond to a stable partially unfolded intermediate of mPrP. Interestingly, this partially unfolded intermediate does not bind bis-ANS (data not shown), suggesting disorganization of bis-ANS binding domains on the protein. Fig. 3 shows that lowering the temperature to −9 °C under pressure led to an additional increase in intrinsic fluorescence emission, reaching a plateau approaching an overall 2.5-fold increase in fluorescence. Upon return of the sample to 25 °C and decompression, the fluorescence intensity completely recovered to that observed at atmospheric pressure, indicating reversible refolding of mPrP-(121–231) (Fig. 5, open symbols). Comparison of the results presented in Figs. 2 and 5 indicates that the spectral red-shift and the increase in fluorescence intensity observed in the pressure-assisted cold denaturation of mPrP are a consequence of a conformational change that is only observed in the presence of 4 M urea (Fig. 5), but not in the absence of denaturant (Fig. 2), and are not due to direct effects of pressure and temperature on the fluorescence emission of the tryptophan residues of the protein.

Fig. 6 shows intrinsic fluorescence emission spectra of F175W mPrP-(121–231) in the native state and in the presence of 4 M urea. The spectrum of the pressure-denatured state exhibited a red-shift (~4 nm) fluorescence emission and a 1.3-fold increase in intrinsic fluorescence at 360 nm compared with native mPrP-(121–231). On the other hand, the fluorescence spectra of the cold-denatured state and of the completely unfolded protein (i.e., in the presence of 8 M urea) were very similar and exhibited ~2.5-fold fluorescence increase in addition to the red shift of the emission.

The thermodynamic parameters obtained for the transitions to different (partially) denatured states of F175W mPrP-(121–231) are shown in Table I. It is interesting to note that the sum
of the individual free-energy changes corresponding to the transitions to the urea-denatured and pressure-denatured states of F175W mPrP-(121–231) is in excellent agreement with \( \Delta G_{\text{un}} \), the overall free-energy change obtained from full unfolding of F175W mPrP-(121–231) at high concentrations of urea, adding support to the notion that folding intermediates of mPrP-(121–231) are stabilized under these experimental conditions. This allowed us to construct a free-energy diagram that describes the equilibrium (un)folding transitions of mPrP (Fig. 7).

**DISCUSSION**

The key event in the pathogenic process of transmissible spongiform encephalopathies is the transition between the normal conformation of the cellular prion protein, PrP\(^c\), and its misfolded conformation, PrP\(^sc\). The molecular mechanisms underlying the conformational transition between the normal and pathogenic conformers of the protein remain largely unknown. Although folding intermediates of the prion protein have proved very difficult to detect and characterize (26–28), a recent study presented evidence for a kinetic intermediate during the folding of the human prion protein (42). We have now characterized partially folded equilibrium intermediates of the murine prion protein stabilized by high hydrostatic pressure and low temperature.

In a previous report, the urea-induced unfolding of F175W mPrP-(121–231) monitored by intrinsic fluorescence and circular dichroism indicated a two-state transition, without detectable folding intermediates (26). However, when the unfolding transition was monitored by bis-ANS fluorescence (Fig. 1), the mid-point of the curve was very different from that reported by tryptophan fluorescence. This type of behavior is characteristic of the existence of at least one folding intermediate (i.e. a non-two state transition (43)) and indicates a major disorganization of bis-ANS binding sites on the protein surface at lower concentrations of urea than those needed to bring about changes in intrinsic fluorescence emission and in secondary structure of mPrP (reported by CD).

Interestingly, the appearance of partially folded intermediate states is usually accompanied by an increase in bis-ANS fluorescence, whereas the folding intermediate of mPrP stabilized at 4 M urea is characterized by decreased bis-ANS binding. Previous examples of folding intermediates that bind less bis-ANS than the corresponding native states of the proteins include troponins I and T (39) and Arc repressor (44). In addition, synchrotron radiation small angle x-ray scattering measurements on the prion protein in the presence of 4.0 M urea showed that the protein retains a compact but somewhat expanded shape compared with native state at pH 7.0,\(^2\) which corroborates the existence of a prion protein folding intermediate under our experimental conditions.

It is generally assumed that pressures below 5 kbar do not significantly disturb the secondary or tertiary structures of monomeric proteins (45). Hydrogen bonds, the stabilizing elements of \( \alpha \)-helices and \( \beta \)-sheets, are permanent dipoles and relatively insensitive to pressure changes. However, hydrostatic pressure weakens hydrophobic interactions by favoring water penetration into the hydrophobic cores of proteins. mPrP-(121–231) has 20 amino acid residues forming a tightly packed hydrophobic core (46). The \(-8\)-fold increase in bis-ANS fluorescence observed when mPrP was compressed to 3.5 kbar at 25 °C and pH 7.0 (Fig. 2C) supports the notion that additional hydrophobic domains become exposed in mPrP under these conditions. A very recent study reported a similar result with hamster recombinant PrP\(^sc\)-(90–231) compressed to 4.0 kbar (47). In addition, the combination of high hydrostatic pressure and low temperatures allowed characterization of a mPrP folding intermediate exhibiting very strong bis-ANS flu-
Table I
Thermodynamic parameters of different (partially) denatured states of F175W mPrP(121–231)

|                     | Urea denaturation<sup>a</sup> | Pressure denaturation<sup>a</sup> | Cold denaturation<sup>a</sup> |
|---------------------|-------------------------------|---------------------------------|--------------------------------|
| $\Delta G_{\text{unf}}$ | 5.1 ± 0.2 kcal/mol            | 1.1 ± 0.1 kcal/mol              |                                |
| $m_{\text{ov}}$      | 0.9 ± 0.1 kcal/mol-M          |                                  |                                |
| $\Delta G_{\text{int}}$ | 2.2 ± 0.1 kcal/mol            |                                  |                                |
| $m_{\text{ov}}$      | 0.6 ± 0.05 kcal/mol-M         |                                  |                                |

<sup>a</sup>$\Delta G_{\text{unf}}$, the overall free-energy change of unfolding of mPrP-(121–231), was obtained from the intrinsic fluorescence data shown in Fig. 1, assuming a two-state transition from the native to the fully unfolded states and using the linear extrapolation method (36). $\Delta G_{\text{int}}$, the free-energy change corresponding to the transition from the native state to the partially folded intermediate state stabilized by urea, was calculated from the bis-ANS fluorescence data shown in Fig. 1, using the linear extrapolation method.

<sup>b</sup>$\Delta G_{\text{unf}}$, the free-energy change corresponding to the transition from the intermediate stabilized at atmospheric pressure in the presence of 4 M urea to the partially folded pressure-denatured state, was calculated as described under “Experimental Procedures” from the data shown in Fig. 5A.

<sup>c</sup>$\Delta G_{\text{unf}}$, the free-energy change corresponding to the transition from the pressure-denatured intermediate state to the fully unfolded state, was calculated as described under “Experimental Procedures” from the data shown in Fig. 5B.

Fig. 7. Free-energy diagram for the (un)folding transitions of mPrP-(121–231).

The effect of pressure on protein structure depends on changes in volume and compressibility that accompany protein conformational transitions, in particular partial or complete unfolding. Volume changes can be interpreted in terms of the balance of forces responsible for protein stability. It is widely accepted that exposure of polar and charged groups leads to a decrease in volume caused by electrostriction of water around the unpaired charged residues (50). The denaturation of monomeric proteins is often accompanied by similar effects, resulting in stronger hydration. It is interesting to compare the volume change measured for the unfolding of mPrP-(121–231) with the volume changes reported for pressure denaturation of other proteins of comparable molecular weight (50). Lysozyme ($M_r$ 14,400), for example, shows a specific volume change of $-1.4 \mu l/g$ upon unfolding by pressure (50). Therefore, the relatively large specific volume change of 2.9 $\mu l/g$ observed for the folding transition of the I$_2$ intermediate to I$_1$ of mPrP-(121–231) (Table I) occurs most likely with significant burial of polar side-chain groups.

The $m$ values obtained from equilibrium unfolding studies using chemical denaturants have been suggested to reflect the static pressure and low temperature may be qualitatively similar to the conformation adopted by mPrP-(121–231) at 1 atm at pH 4.0. Together, these data indicate the intrinsic propensity of mPrP to adopt partially folded conformations and suggest that factors other than acidic pH may induce the formation of a PrP folding intermediate in vivo. Under specific conditions, this conformer might be the first of several structural rearrangements required for the conversion of PrPC to PrPSc or to another potentially neurotoxic species. The structural plasticity of the prion protein implied by its propensity to populate partially folded states may also be related to the capacity to interact with multiple, distinct ligands at the cell surface and to carry out the diverse biological functions that have been proposed for this protein (6).

The pressure-induced formation of partially structured intermediate states represents an interesting phenomenon. In some cases, pressure apparently allows the detection of metastable states that are not readily trapped by other methods (29–32) but may correspond to equilibrium intermediates formed during protein folding. Interestingly, pressure denaturation of F175W mPrP-(121–231) in the presence of 4 M urea allowed the stabilization of another partially folded intermediate (Fig. 5A). Cooling this intermediate to $-9^\circ C$ led to complete unfolding (Fig. 5B). These results suggest that one possible (un)folding transition of mPrP-(121–231) can be summarized by the following reaction, $N \leftrightarrow I_1 \leftrightarrow I_2 \leftrightarrow U$, where N and U are the native and the unfolded states, respectively, and I$_1$ and I$_2$ represent the two partially (un)folded intermediates revealed by urea-induced unfolding and high pressure experiments, respectively (see Fig. 7).

The association of high pressure and low temperatures is considered to drive proteins to a lower entropic state, favoring the interaction of nonpolar amino acid residues with water (48). Therefore, lowering the temperature under pressure often induces the exposure of hydrophobic side chains to the solvent, leading to protein conformational changes or even subunit dissociation and cold denaturation (31, 32). Thus, the destabilization of mPrP at low temperature indicates a significant contribution of hydrophobic interactions to the folding of this protein. For several proteins, folding and subunit association have been found to be entropy-driven processes, involving the displacement of solvent molecules and the burial of previously solvent-exposed nonpolar side chains (31, 32). The transition from the unfolded state to the I$_2$ intermediate of mPrP-(121–231) was found to be an entropy-driven transition, with $T\Delta S = +21.9 \text{ kcal/mol at } 25^\circ C$ and a van’t Hoff enthalpy of $\Delta H = +20.8 \text{ kcal/mol}$, resulting in $-1.1 \text{ kcal/mol}$ conformational stability (Table I). The entropy-driven nature of the U $\leftrightarrow$ I$_2$ transition suggests that a hydrophobic collapse may be involved at this stage of folding of mPrP-(121–231).

The conformational properties of recombinant prion proteins are strongly pH-dependent. Acidic pH induces a dramatic increase in the exposure of hydrophobic patches on the surface of huPrP-(90–231), without affecting the overall secondary structure of the protein (49). Interestingly, the marked increase in bis-ANS fluorescence observed in the pressure-assisted cold-denatured state at pH 7.0 (Fig. 3) is similar to that observed for mPrP-(121–231) at 1 atm at pH 4.0 (Fig. 4). This suggests that the partially folded conformation of mPrP stabilized by hydrodynamic pressure and low temperature may be qualitatively similar to the conformation adopted by mPrP-(121–231) at 1 atm at pH 4.0. Together, these data indicate the intrinsic propensity of mPrP to adopt partially folded conformations and suggest that factors other than acidic pH may induce the formation of a PrP folding intermediate in vivo. Under specific conditions, this conformer might be the first of several structural rearrangements required for the conversion of PrPC to PrPSc or to another potentially neurotoxic species. The structural plasticity of the prion protein implied by its propensity to populate partially folded states may also be related to the capacity to interact with multiple, distinct ligands at the cell surface and to carry out the diverse biological functions that have been proposed for this protein (6).
change in accessible surface area (ΔASA) upon folding of small single-domain proteins (51). Interestingly, the overall m value found for mPrP-(121–231) obtained from equilibrium urea-folding experiments (Table I) was lower than those reported for proteins of comparable sizes (51). The lower overall m value found for mPrP-(121–231) suggests deviation from a two-state unfolding mechanism (52), which is in agreement with our results.

A previous study reported that in the presence of relatively low concentrations of guanidine hydrochloride under acidic conditions hPrP-(90–231) undergoes a major transition from an α-helical conformation to a β-sheet structure (49). A similar transition was also reported for mouse PrP-(121–231) upon incubation at acidic pH in the presence of urea (53). These results suggested that the β-sheet-rich conformer of hPrP-(90–231) in guanidine hydrochloride (or mouse PrP-(121–231) in urea) represents a stable monomeric (90)

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REFERENCES

1. Wells, G. A. H., Scott, A. C., Johnson, C. T., Gunning, R. F., Hancock, R. D., Jeffrey, M., Dawson, M., and Bradley, R. (1987) Vet. Rec. 121, 419–420
2. Prusiner, S. B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13363–13368
3. Collinge, J. (2001) Annu. Rev. Neurosci. 24, 519–550
4. Kretzschmar, H. A., Prusiner, S. B., Bowman, L. E., and DeArmond, S. J. (1996) Am. J. Pathol. 122, 1–5
5. Manson, J., West, J. D., Thomson, V., McBride, P., Kaufman, M. H., and Hope, J. (1992) Development 115, 117–122
6. Matinas, V. R., Linden, R., Prade, M. A. M., Wals, R., Sakamoto, A. C., Izquierdo, I., and Brentani, R. R. (2002) FEBS Lett. 512, 25–28
7. Riek, H., Hornemann, S., Wider, G., Glockshuber, R., and Wuthrich, K. (1997) FEBS Lett. 413, 262–268
8. Donne, D. G., Viles, J. H., Geth, D., Mohlorn, 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
9. Hornemann, S. and Glockshuber, R. (1996) J. Mol. Biol. 261, 614–618
10. Riek, H., Hornemann, S., Wider, G., Billette, M., Glockshuber, R., and Wuthrich, K. (1996) Nature 382, 180–182
11. Stahl, N., Baldwin, M. A., Teplow, D. B., Hood, L., Gibson, B. W., Burlingame, A. L., and Prusiner, S. B. (1993) Biochemistry 32, 1991–2002