Species-specific Differences in the Intermediate States of Human and Syrian Hamster Prion Protein Detected by High Pressure NMR Spectroscopy*

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Human (huPrP) and Syrian hamster (ShaPrP) prion proteins have barriers for mutual infectivity, although they fold into almost an identical structure. The pressure responses of huPrP and ShaPrP characterized by high pressure NMR spectroscopy show differences in their excited states, as monitored by pressure-induced chemical shifts and intensity changes of individual residues in the $^1$H/$^1$H HSQC spectra. Both proteins fluctuate rapidly between two well folded (native) conformations N1 and N2 and less frequently between N and the excited states I1 and I2 with local disorder that may present structural intermediates on the way to PrP$^{Sc}$. These four structural states can be observed in the hamster and human PrP. At ambient pressure, less than 5 molecules of 10,000 are in the intermediate state I2. From the structural point of view, the different states are mutually different, particularly in positions strategically important for generating species barriers for infection. The results point to the notion that excited state conformers are important for infection and that their structural differences may crucially determine species barriers for infection.

Transmissible spongiform encephalopathies are associated with the formation of an oligomeric conformational scrapie isomer, PrP$^{Sc}$, of the host-encoded monomeric prion protein PrP$^{C}$ (1). They are infectious fatal disorders of the central nervous system, which include Creutzfeldt-Jakob disease in humans, bovine spongiform encephalopathy in cattle, scrapie in sheep, and chronic wasting disease in deer and elk (2). According to the “protein only” hypothesis, PrP$^{Sc}$ is the sole component of transmissible prions (3). The formation of PrP$^{Sc}$ involves a conformational switch by which PrP$^{C}$ is transformed into the PrP$^{Sc}$ conformation with PrP$^{Sc}$ as template (4). A puzzle of this hypothesis is the existence of a wide variety of distinct so-called prion strains with differing infectivity and related to them a species barrier (i.e. the degree of the low interspecies infectivity of a prion strain) (5). Primarily, the differences in PrP$^{Sc}$ amino acid sequence related to heterology of the PrP genotype may account for the formation of distinct prion strains and for the species barrier (6). For this reason, hamster prions are usually not infectious to wild-type mice, and there is a moderate species barrier for transmission of mouse prions to hamster. However, formation of distinct prion strains is known even when the strains have identical primary structure and the hosts are genetically identical (7). The species barrier can be defined as the inability of the host to replicate the conformation of the infectious isomer and is broken only by altering the original conformation and generating a conformationally distinct isoform (8). Vanik et al. (9) showed that sequence-based barriers that prevent cross-seeding between prion proteins from different species can be bypassed in vitro by a template-induced adaptation process, leading to the emergence of new strains of prion fibrils.

Basic three-dimensional structures of the cellular prion proteins from different species are known, which include hamster (10, 11), human (12–14), and bovine (15). The amino acid sequences of all mammalian prion proteins are rather similar (e.g. see Fig. 1), and global folds are well conserved. This indicates that the basic folded structures themselves cannot tell the molecular mechanism for the species barrier for infection. On the other hand, only a couple of sequence variations may change the infectivity (9, 16). This suggests that the structural basis for the species barrier could be very subtle and local. Our idea is then that we must seek for the structural basis in a site-specific manner with NMR spectroscopy, not in the stable folded state but rather in an unstable and more reactive excited state.

To this end, NMR with pressure perturbation is one of the best available methods of study. Application of high hydrostatic pressure will increase the population of an excited state conformer through the difference in their partial molar volumes to the level of spectroscopic detection and may allow the determination of their structures at least qualitatively (17). Recently, we have successfully applied the technique to prion proteins from Homo sapiens (18) and Syrian hamster (19) that are mutually not infectious to detect the excited state conformers. In the present work, we have analyzed the two high pressure NMR data sets from the two proteins in an identical manner to find the similarity and differences in their excited state conformers.
MATERIALS AND METHODS

Protein Expression and Sample Preparation—Recombinant human prion protein huPrP-(121–230) (residues 121–230) was prepared as described previously (12), and recombinant Syrian hamster prion protein ShaPrP-(90–231) (residues 90–231) was prepared as described by James et al. (10). As a cloning artifact huPrP-(121–230) contains two additional N-terminal amino acids, Gly119 and Ser120. For the NMR experiments, a 1.2 mM solution of 15N-enriched huPrP-(121–230) in 10 mM sodium acetate buffer, pH 4.8, was used. The measurements were performed in 2H2O with 5% 2H2O added. The samples contained 0.1 mM 2,2-dimethyl-2-silapentane-5-sulfonate as an internal reference. 15N-enriched ShaPrP-(90–231) in 20 mM sodium acetate buffer, pH 5.2, in 1H2O with 5% 2H2O was measured with trace amounts of sodium 3-(trimethylsilyl [2,2,3,3,-2H]tetradecuteropropionate added as a chemical shift reference.

High Pressure NMR Measurements—Online high pressure NMR systems were used in a commercial 5 mm NMR probe from Bruker. To apply high pressure on huPrP-(121–230), we used an on-line variable pressure cell system (20, 21) with a sapphire capillary of 1.72-mm inner and 3.14-mm outer diameter (22, 23). One-dimensional 1H and two-dimensional 1H-15N TROSY spectra were acquired at hydrostatic pressures of 3, 50, 100, 125, 150, 175, 200, and 250 MPa and at 303 K. HSQC spectra of ShaPrP-(90–231) were measured in a quartz tube with 1-mm inner and 3-mm outer diameter (24) at pressures of 50, 100, 125, 150, 175, 200, and 250 MPa and at 303 K.

Analysis of the Chemical Shifts—The pressure dependence of the chemical shift values of the amide protons and nitrogens in the HSQC-TROSY spectra of the two proteins was fitted in two different ways, using a second order Taylor expansion (Equation 1) or a two-site fast exchange model (Equation 2).

\[
\delta(p, T_0) = \delta_0(p, T_0) + B_1(p, T_0)(p - p_0) + B_2(p, T_0)(p - p_0)^2
\]  
(Eq. 1)

\[
\delta = \frac{1}{1 + K^1} \delta_1 + \frac{K}{1 + K^2} \delta_2 = \frac{\delta_1 + \delta_2 \exp\left(\frac{\Delta G}{RT}\right)}{1 + \exp\left(\frac{\Delta G}{RT}\right)}
\]  
(Eq. 2)

\(\delta_0\) is the chemical shift at ambient pressure \(p_0\) at temperature \(T_0\). The first order pressure coefficient \(B_1\) and the second order pressure coefficient \(B_2\) of each amide proton were corrected by subtracting the pressure coefficients of the standard peptide (Gly-Gly-X-Ala) of the corresponding amino acid as published by Arnold et al. (23, 25). The corrected coefficients are referred to as \(B_1^1\) and \(B_2^2\).

If there is an equilibrium between two conformations of the protein that is in fast exchange on the time scale of the NMR experiment, the observed chemical shifts are the weighted average of the chemical shifts \(\delta_1\) and \(\delta_2\) of the two states. With an equilibrium constant for the conformational exchange \(K\), the chemical shift \(\delta\) is given by Equation 2.

The free energy at ambient pressure \(\Delta G_0\) can be determined by fitting the data to Equation 4.

\[
\Delta G = \Delta G_0 + \Delta V(p - p_0) + \frac{1}{2} \Delta \kappa (p - p_0)^2
\]  
(Eq. 4)

Here, \(\Delta V\) is the partial molar volume, and \(\Delta \kappa\) is the compressibility. For our calculations of the \(\Delta G_0\) values, we neglected the quadratic term in Equation 4.

Analysis of the Signal Volumes—If there is a conformational exchange that is slow relative to the time scale of the NMR experiment, the signal volume of one state is proportional to its population \(N_i\). Assuming a two-state transition for the change of the conformational equilibrium with increased pressure, the equilibrium constant \(K\) can be calculated from the relative cross-peak intensities of the TROSY spectra by Equation 5 (19).

\[
K = \frac{I(p)}{I_0} = \frac{1}{1 + \exp\left(-\frac{\Delta G}{RT}\right)}
\]  
(Eq. 5)

\(I(p)\) is the relative cross-peak intensity at pressure \(p\), and \(I_0\) is the intensity when 100% of the protein is in the native state. With this, one obtains the free energy \(\Delta G\) associated with the pressure-induced conformational change by Equation 6.

\[
I(p) = \frac{I_0}{1 + \exp\left(-\frac{\Delta G}{RT}\right)}
\]  
(Eq. 6)

The free energy at ambient pressure \(\Delta G_0\) is defined by Equation 4 and can be obtained from a three-parameter fit with \(I_0\), \(\Delta V_0\), and \(\Delta G_0\), neglecting the second order term in \(p\).

Analysis of Pressure-dependent Shift Correlations—When the chemical shift changes in two samples A and B (e.g. the same spin in two constructs of a protein or the hydrogen and nitrogen atoms of an amide group) are governed by the same physical process that can be approximated by a two-site exchange, then the chemical shift changes can be described by the same free energy \(\Delta G\). In this case, the pressure-dependent chemical shift changes \(\Delta \delta^A\) and \(\Delta \delta^B\),

\[
\Delta \delta^A = \delta^A(p) - \delta^A(p_0) = (\delta^A_2 - \delta^A_1)f(p)
\]  
(Eq. 7)

\[
\Delta \delta^B = \delta^B(p) - \delta^B(p_0) = (\delta^B_2 - \delta^B_1)f(p)
\]  
(Eq. 8)

follow the linear relation,

\[
\Delta \delta = \frac{(\delta^A_2 - \delta^A_1)}{(\delta^B_2 - \delta^B_1)} \Delta \delta^B
\]  
(Eq. 9)

RESULTS

General Pressure Effects on the Folded Core of Human and Syrian Hamster Prion Protein—Human and Syrian hamster PrP show a species-specific sequence polymorphism that extends
Pressure-induced chemical shifts are sensitive measures of conformational fluctuation of a protein, which takes place within the folded manifold of the protein. In general, the pressure response of proteins is not isotropic, and the chemical shift changes induced by the local and global conformational changes lead to a nonlinear dependence of the chemical shifts on pressure. The pressure dependence was described by the zero-order coefficient $\delta_0 (p, T_0)$, the first order coefficient $B_1$ and the second order coefficient $B_2$ of a Taylor expansion around the pressure $p_0$ and the temperature $T_0$ (see Equation 1) (24).

When the pressure effects are assumed to be composed of two contributions, an unspecific effect as observed in random coil peptides and a specific, structure-dependent effect, then the influence of these effects on the Taylor coefficients can be assumed as additive as long as the two events are not coupled or are only weakly coupled (see “Materials and Methods”). In this case, the corrected, conformation-dependent pressure (Taylor) coefficients $B_1^*\Delta S$ and $B_2^*\Delta S$ can be obtained by subtracting the corresponding pressure coefficients from random coil peptides. Random coil pressure coefficients are available for the $^1$H shifts from a study of the model Gly-Gly-X-Ala peptides (25) but not for the $^{15}$N shifts.

The first order conformation-dependent pressure coefficients $B_1^*(H)$ for the $^1$H$^N$ shifts and of the uncorrected pressure coefficient $B_1^*(N)$ of the $^{15}$N$^N$ chemical shift as a function of the primary and the secondary structure are displayed in Fig. 2 for the structured core of huPrP and ShaPrP. Analogously, the second order coefficients $B_2^*(H)$ and $B_2^*(N)$ are plotted as a function of the sequence position in Fig. 3.

The mean values of the absolute first order pressure coefficients $|B_1^*(H)|$ and $|B_1^*(N)|$ of the human protein are 0.54 and 3.35 ppm/GPa. The corresponding values for the Syrian hamster protein (only residues 121–230) are quite similar, at 0.58 and 3.03 ppm/GPa. In the case of the second order coefficients $|B_2^*(H)|$ and $|B_2^*(N)|$, there are significantly larger differences in the mean values: 1.69 and 7.29 ppm/GPa$^2$ (human) and 1.19 and 6.15 ppm/GPa$^2$ (hamster).

The same is observed for the correlation coefficients of $B_1^*(H)$, $B_1^*(N)$, $B_2^*(H)$, and $B_2^*(N)$ of the two proteins. The correlation coefficients are 0.67 ($B_1^*(H)$), 0.52 ($B_1^*(N)$), 0.18 ($B_2^*(H)$), and 0.16 ($B_2^*(N)$), and the corresponding slopes are 0.70, 0.52, 0.13, and 0.14. The rather low correlations, especially for the second order coefficients, are not only caused by the difference of the two proteins. For residues with small pressure-induced shifts, the second order Taylor coefficients cannot be determined from the data with high precision; their relatively large errors remove possibly existing global correlations almost completely. Calculating the correlation coefficients for huPrP and ShaPrP directly from the chemical shifts (see Equation 9), leads to much higher values. The mean value of the correlation coefficients of each residue of huPrP to its corresponding residue of ShaPrP is 0.82 in the case of HN and 0.86 for the NH.

However, distinct local differences and interesting trends of shifts are found in both proteins.

Pressure-induced Shifts and First Order Pressure Coefficient—

The first order coefficients $B_1^*(H)$ for the $^1$H$^N$ shifts (Fig. 2a) are plotted as deviations from corresponding shifts in model peptides. The positive coefficient generally means that the hydrogen bond is less compressed than in the model peptide, whereas the negative coefficient generally means that the hydrogen bond is compressed more than that in the model peptide. In the used temperature range, the contribution is very small and (as we showed earlier) almost all resonances. In addition, in TROSY-HSQC spectra, the residue-specific pattern of the pressure response is not within the limits of error. More importantly, we showed that the increased pressure leads to a decrease of the signal volumes of a number of resonances. Some signals disappear completely, whereas others show no significant volume changes (18, 19).

Increasing pressure results in chemical shift changes of almost all resonances. In addition, in TROSY-HSQC spectra, the increased pressure leads to a decrease of the signal volumes of a number of resonances. Some signals disappear completely from the spectra, whereas others show no significant volume changes (18, 19).

Pressure-induced chemical shifts are sensitive measures of conformational fluctuation of a protein, which takes place within the folded core of the protein (Fig. 1). One-dimensional $^1$H and two-dimensional $^1$H-$^{15}$N TROSY NMR spectra of $^{15}$N-enriched human prion protein, huPrP-(121–230), were recorded at pH 4.8 (acetate buffer) at various pressures under solution conditions identical to those used for the structure determination (12). At 293 K, hydrostatic pressures in steps of 0.1, 50, 100, 125, 150, 175, and 200 MPa were applied. The data of ShaPrP-(90–131) recorded at 303 K and published earlier (19) were reevaluated in an identical manner to those from the human protein. Slightly different experimental temperatures were selected for the two proteins, since optimal (most complete) NMR spectra were obtained at 303 K for hamster and 293 K for human protein and since the two NMR structures were solved at these temperatures. $\Delta G$ is per se temperature-dependent by its $T\Delta S$ contribution. However, in the used temperature range, the contribution is very small and (as we showed earlier) within the limits of error. More importantly, we showed that the residue-specific pattern of the pressure response is not influenced significantly by a temperature change from 293 to 303 K (18).

FIGURE 1. Sequence comparison of the core domain of human and Syrian hamster prion protein. Differences in the sequence are highlighted.
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Graph a

Graph b
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Assuming a two-site model, the pressure-induced changes of signal volumes and thus of the corresponding populations can be used to calculate a molar free energy difference ($\Delta G_p$) for every single amino acid residue in the polypeptide chain. The distribution of the $\Delta G_p$ values of the individual residues indicates for both species the existence of (at least) two different independent conformational exchange processes and therefore the existence of two pressure-stabilized intermediate states, I$_1$ (intermediate $\Delta G_p$) and I$_2$ (high $\Delta G_p$) (Fig. 6, a and b, and Table 1).

The corresponding energy distributions for the transitions to the intermediate states are shifted to somewhat higher values for the hamster PrP (Fig. 6) (i.e. higher energies are necessary to shift the equilibrium to the transition state for ShaPrP). Correspondingly, about 0.8 and 0.01% of hamster PrP occur in states I$_1$ and I$_2$ at ambient pressure, respectively. For the human PrP, slightly higher values of 1.1 and 0.05% are obtained at ambient pressure.

**DISCUSSION**

Common Features of the Two Prion Proteins—In the part of the protein studied in this paper (amino acids 121–230) and corresponding essentially to the folded core of the hamster and human prion protein, only 14 amino acids differ. In addition, the human protein contains two C-terminal deletions and one insertion compared with the Syrian hamster protein (Fig. 1). These sequence variations influence the conformation of the folded core and the pressure response of the protein. However, the observed effects may vary in their extent considerably. They are also connected to the species barrier, but again all sequence variations are not equally important (29).

In general, two different pressure responses are to be expected: 1) a small structural change that depends on the local compressibility of the protein and 2) a shift of the conformational equilibrium between different structural states. Both processes usually cause chemical shift changes that can be described by the first and second order pressure coefficients. The general correlation analysis shows that the overall pressure response measured by Equation 9 in the two proteins is similar (correlation coefficients of 0.82 and 0.86, respectively). Such a result is expected, since the general folds of the two prion proteins are similar.

Pressure response 1 mainly encompasses a shortening of amide hydrogen bonds and due to a distortion of the structure also lengthening of hydrogen bonds at some places in the structure. This can be detected by pressure-dependent amide proton shifts and the magnitude of their corresponding first order pressure coefficients $B_1(H)$. The $1^H$NN linear pressure-induced shifts are thus a measure for the local compressibility that is usually interpreted as a measure for the size of the equilibrium fluctuations of the hydrogen bonds at 0.1 MPa (fluctuations within the folded manifold). Again, a significant correlation of the first order proton pressure coefficients (correlation coefficient 0.67) is observed, indicating that the local compressibility in larger...
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parts of the proteins is similar. The second order coefficients are thought to be indicative for more global responses and large structural changes and conformational equilibria. Here, almost no correlation is found for the proteins, suggesting that here considerable differences between the two proteins can be found.

As to be expected for the folded core (residues 121–230) of hamster and human prion protein, the majority of residues show negative $B_1^*(H)$ values, since the structures are stabilized by internal hydrogen bonds that are mainly compressed. Small negative or slightly positive values are observed for the two proteins for the residues located N-terminal to the first $\beta$-strand $\beta_1$, in the center of the loops interconnecting $\beta_1$ with helix $\alpha_1$ and strand $\beta_2$ with helix $\alpha_2$. Negative trends are observed for residues of 146–157, 181–192, and 199–221, which largely correspond to helices $\alpha_1$, $\alpha_2$, and $\alpha_3$. It is intriguing to note that these residues do not exactly match the residues reported for helices $\alpha_1$, $\alpha_2$, and $\alpha_3$ for the two proteins (10–12). This discrepancy is obvious for residues 173–179 of $\alpha_2$, suggesting that the initial part of helix $\alpha_2$ does not form stable hydrogen bonds with carbonyls. Anomalies of shifts are also found for residues of $\alpha_3$, particularly for residue 211. A characteristic pattern is observed at the C-terminal part of $\alpha_2$ and the loop interconnecting $\alpha_2$ and $\alpha_3$. The second half of helix 2 shows negative coefficients with a maximum at residue 188, and the loop shows positive values with a maximum value at residue 195; in a simple interpretation, that part would be expanded, and the loop would be compressed or would form stronger hydrogen bonds in both proteins.

Above we have discussed mainly the magnitude of the sequence-dependent pressure-induced chemical shift changes and its similarities in the two proteins. An equally important general feature is the magnitude of the Gibbs free energies that describe the pressure dependence. Here, the two proteins show a pressure response that requires the existence of at least four different conformational states that we call $N_1$, $N_2$, $I_1$, and $I_2$.

The transition between state $N_1$ and $N_2$ is fast on the NMR time scale in both proteins. The continuous shift change as function can be perfectly fitted with the corresponding model (Eq. 2). From the condition $|\Delta \omega \tau_p| \gg 1$ and typical $^1$H$^0$ shift differences of 0.2 ppm, a typical upper limit of the exchange correlation time would be 1.3 ms. The $I_1$ to $I_2$ transition is characterized by a loss of cross-peak intensity. There are two possible explanations for this, namely increased loss of intensity during the INEPT periods by increased average $T_2$ relaxation and/or a two-site exchange that is slow on the NMR time scale and for which the second signal is too weak to be observable (30). The increased transverse relaxation rate can be due to an exchange broadening or an increased effective molecular size by protein aggregation. As pointed out before, a two-site exchange that is slow on the NMR time scale is the most probable explanation (18, 19). The disappearance of the second signal in the slow exchange regime is probably mainly due to a line broadening by a local disorder in the intermediate state, since the proton NMR lines of most aliphatic protons are still visible at 200 MPa (18). The observed line broadening of these lines would be consistent with the formation of a dimer but could explain the observed disappearance of the HSQC signals only partly.

The existence of similar conformational states in the two proteins is an important finding that may apply to other prion proteins.

**FIGURE 4. Variation of pressure coefficients between hamster and human prion protein.** NMR structures of huPrP (a) and ShaPrP (b) with molecular cavities added (taken from Protein Data Bank codes 1QM2 and 1B10). Cavities were calculated with a probe radius of 0.12 nm. The amino acid substitutions between the species are marked in the structures. c, residues are depicted in orange and red, where $|B_1^*(H, hu) - B_1^*(H, sha)|$, $|B_1^*(N, hu) - B_1^*(N, sha)|$, or $|B_1^*(H, hu) - B_1^*(H, sha)|$ is larger than $\sigma_1$ and $2\sigma_1$, respectively. $\sigma_1$ is the S.D. relative to 0. Models were prepared using MOLMOL (31).

**FIGURE 3. Second order pressure coefficients of the $^1$H$^0$ and of the $^{15}$N$^0$ chemical shifts taken from the TROSY/HFQC spectra of human and hamster PrP.** Only values for the folded core (residues 120–230) are given. The conformation-dependent pressure coefficients $B_0^*(H)$ (a) for the $^1$H$^0$ shifts and the uncorrected pressure coefficient $B_0(N)$ (b) for the $^{15}$N$^0$ chemical shifts are plotted as a function of the sequence position. Black bars, human protein; white bars, hamster protein. In addition, the secondary structure of the human prion protein is symbolized by arrows (β-strands) and lanyards (α-helices). Note that huPrP(121–230) contains two additional N-terminal amino acids, Gly$^{119}$ and Ser$^{120}$, as cloning artifacts.
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proteins. It cannot be derived directly from the similarities of the reported structures that are an average over the different states at ambient pressure. However, as we discuss later, also important differences are evident when the localization and magnitude of the free energy changes are taken into account.

Differences in Pressure Response in huPrP and ShaPrP—Although there are clear general similarities in the pressure response of the proteins from human and Syrian hamster, significant differences between the two proteins can be identified (Fig. 4). Such differences can be seen in the β-pleated sheet β1, in loop L2 connecting β1 with helix α1, and in the helix α1. Loop L2 contains at positions 138, 139, 143, and 145 and helix α1 at position 155 species-specific sequence differences. Some of these sequence differences are known to be main determinants of the species barrier between humans and hamsters. Loop L3 and β-strand β2 show significant differences in the pressure response of the two proteins. In loop L4, only one residue (S170N) shows differences in the pressure response. This residue is flanked by two sequence polymorphisms at positions 166 and 168 (M166V and E168Q). Helix α2 shows differences in its second half but does not contain sequence polymorphisms in this part of the structure. The long interconnecting loop L5 reacts similarly to pressure in the proteins ShaPrP and huPrP. Remarkedly, β1, β2, and L3 react differently in the two proteins, although they do not contain species-specific sequence differences. Helix α3 contains five sequence variations in the two species. This causes a different pressure response of a significant part of the helix.

The observed differences in pressure response of the folded core of the prion protein are not caused by the different lengths of the hamster and human protein. We showed earlier that only weak interaction with the unfolded part of huPrP-(20–130) exists. Small changes of the pressure-induced shifts of Glu168, His187, Thr192, Glu207, Glu211, and Tyr226 can be observed when the unfolded part is present in the protein. However, these changes induced by the interaction with the N terminus do not influence our analysis of the differences between hamster and human protein significantly.

Although the linear pressure response is mainly explained by equilibrium fluctuations within the basic folded conformer N1, the nonlinear shifts are likely to be correlated with the low lying excited state. Large second order shift coefficients are usually found close to cavities in the protein. Indeed, they are found in huPrP close to the species-specific sequence variations I138M and I139M in loop L2 in the neighborhood of a large cavity but not in the hamster protein, where only small cavities close to that position are found (Fig. 4).

The intermediate states I1 and I2 not only differ in their populations at ambient pressure but also show significant differences between the two species in the structural elements that show strong responses. Fig. 6, c and d, shows the residues mainly influenced by the transition to intermediate state I1.
In the two proteins, helix $\alpha3$ is influenced by the transition to $I_1$, and helix $\alpha1$ is influenced by the transition to $I_2$. In the two proteins, the short $\beta$-pleated sheet shows effects in the first and the second transition. Clear differences are seen in helix $\alpha2$ and loop L5. In the hamster protein, strong structural effects are coupled with transition $I_1$, whereas only a few residues of helix $\alpha2$ show effects in huPrP, and no effects are seen in L5 connecting helix $\alpha2$ with helix $\alpha3$. An interesting difference is visible in loop L2, where a cluster of residues around residues Ile$^{138}$ and Ile$^{139}$ is involved in the $I_1$ transition in huPrP but not in ShaPrP.

The regions that show the largest volume effects in the $I_1$ and $I_2$ transitions in the two proteins tend to show also the largest effects in the $N_1$-$N_2$ transitions. In line with this observation, regions in the human and hamster protein that behave differently in the $N_1$-$N_2$ transitions behave also differently in the transition to the intermediate states. This means that the two processes in the PrPs are structurally coupled.

Conclusions—High pressure NMR was able to detect in both prion proteins different conformers that coexist in solution. As any protein, PrP also exists in a multitude of structural substates in solution, but on the basis of the corresponding distribution of the Gibbs free energies, they can be grouped into four main states. The two native states, $N_1$ and $N_2$, dominate at ambient pressure; however, the two intermediate states $I_1$ and $I_2$ are also present at low concentrations. About five molecules of 10,000 are in state $I_2$ in a solution of human prion protein, even less in the hamster protein. Intermediate state $I_1$ with its low abundance could be a candidate representing a structural intermediate on the pathway from PrP$^{Sc}$ to PrP$^{Sc}$. Part of the structure in the excited states $I_1$ and $I_2$ is probably locally disordered, and a dimer formation in state $I_2$ would be in line with the experimental data (see above).

The amino acid sequences of human and hamster prion protein are highly conserved; the few amino acids that are different must be responsible for the species barrier. In fact, probably
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only a subset of these residues represents the main determinant of the species barrier (24). In the folded part of the Syrian hamster and human protein about 87% of all amino acids are identical. As a consequence, the general fold of the two proteins is very similar (Fig. 4). However, this difference is sufficient to establish a species barrier for the transmission of prion diseases between these species as indicated by cross-seeding experiments (9, 16). Especially, the amino acid alterations I138M and I139M are responsible for the fact that huPrP does not seed ShaPrP fibrils and vice versa (9). High pressure NMR spectroscopy tests the physical consequences of the sequence variations (local compressibility) as well as pressure-dependent shifts of conformational equilibria.

In fact, there are regions in the protein where the species-specific sequence differences have almost no effect on the pressure response of the chemical shifts and other regions where clear differences can be observed. The most prominent example for a differential pressure response is located around loop L2 and helix α1 (Fig. 4C), where also amino acid exchanges between huPrP and ShaPrP occur. I138M, I139M, H140H, and F141F form a cluster of residues with high differences in their pressure coefficients. It is also a region where a strong exchange broadening in the human protein is observed during the transition to the intermediate state.

In summary, the data would fit to the hypothesis that the species barrier is mainly related to structural differences in the excited states of the proteins, not to those in the ground state dominated by the native structural states N1 and N2. The high pressure data show that such states exist at ambient pressures in solution with very low populations and can be thermodynamically characterized (Table 1). They are less abundant in the hamster protein. An intriguing suggestion would be that the experimentally detected states I1 and I2 represent critical structural states during the structural rearrangement during fibril formation. However, this hypothesis cannot be proved on the basis of the experimental data presented here. In line with this hypothesis, the two proteins show distinct differences in their pressure response, indicating also structural differences of their intermediate states in a region critical for the species barrier in fibril formation and the PrP<sup>C</sup> → PrP<sup>Sc</sup> transition.

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