Differential stability of the bovine prion protein upon urea unfolding

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Abstract: Prion diseases, or transmissible spongiform encephalopathies, are a group of infectious neurological diseases associated with the structural conversion of an endogenous protein (PrP) in the central nervous system. There are two major forms of this protein: the native and noninfectious cellular form, PrP^C; and the misfolded, infectious, and proteinase K-resistant form, PrP^{Sc}. The C-terminal domain of PrP^C is mainly α -helical in structure, whereas PrP^{Sc} is known to aggregate into an assembly of β -sheets, forming amyloid fibrils. To identify the regions of PrP^C potentially involved in the initial steps of the conversion to the infectious conformation, we have used high-resolution NMR spectroscopy to characterize the stability and structure of bovine recombinant PrP^C (residues 121 to 230) during unfolding with the denaturant urea. Analysis of the 800 MHz ¹H NMR spectra reveals region-specific information about the structural changes occurring upon unfolding. Our data suggest that the dissociation of the native β -sheet of PrP^C is a primary step in the urea-induced unfolding process, while strong hydrophobic interactions between helices α 1 and α 3, and between α 2 and α 3, stabilize these regions even at very high concentrations of urea.

Keywords: NMR; PrP; stability; urea unfolding; structural conversion

Introduction

Prion diseases, or transmissible spongiform encephalopathies, are a group of fatal neurological disorders affecting both humans and animals that share in com-

mon spongiform degeneration.^{1–6} Prion diseases can be sporadic, inherited, or acquired by infectious means. These disorders are commonly known as kuru, fatal familial insomnia, and Creutzfeldt-Jacob disease in humans, mad cow disease or bovine spongiform encephalopathy in cattle, chronic wasting disease in cervids, and scrapie in sheep. Independent of the species, these conditions are associated with an accumulation in the brain of an abnormally folded endogenous protein, and these proteinaceous aggregates are thought to lead to neurodegeneration.

Studies of the molecular agent responsible for scrapie revealed that the entity was extremely resistant to UV and ionizing radiation, treatments that normally inactivated nucleic acids.^{7,8} These observations lead to the generally accepted “protein-only” hypothesis suggesting that the infectious agent causing prion disease is composed for the most part, if not entirely, of endogenous protein.^{1,9} There are two major forms of prion protein: the native and noninfectious form (PrP^C), which has mainly an α -helical structure, and

Additional Supporting Information may be found in the online version of this article.

Abbreviations: bPrP, bovine prion protein; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; PrP^C, cellular, proteinase K-sensitive prion protein isoform; PrP^{Sc}, disease-associated, proteinase K-resistant prion protein isoform; TSE, transmissible spongiform encephalopathies.

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the misfolded infectious form (PrP^{Sc}), which aggregates into an assembly of β -sheets forming amyloid fibrils.^{10–12} The first NMR structure of the mouse PrP^C revealed an unstructured N-terminal region (23–121) followed by a globular domain (121–230) containing three α -helices, a small β -sheet, and a disulfide bridge between residues C179 and C214.¹³ Subsequently, more than 30 structures of the globular portion of PrP^C have been characterized using NMR and X-ray crystallography from a variety of organisms, including mammals and nonmammals. Surprisingly, only a few minor differences are observed amongst all of these structures,¹⁴ which include protein structures from organisms that seem to be immune against prion disease (e.g., rabbit). These results suggest that an understanding of prion disease and the presence of species barriers may not be fully revealed by the inspection of the structures PrP^C and/or PrP^{Sc}, but through an understanding of the mechanism by which the PrP^C protein is converted into the misfolded and infectious form. Surewicz and coworkers have recently shown that recombinant PrP^C can be converted using native-like conditions (i.e., physiologically relevant) into amyloid fibrils, consisting of proteins with parallel and in-register β -structures.^{15,16} It was shown that the conversion of recombinant prion protein into a β -sheet-rich form with similar physicochemical properties to PrP^{Sc} could be induced by acidic pH, salt, and urea.¹⁷

Over the years, many models were proposed to highlight the different characteristics of PrP^{Sc}. These include insolubility, partial proteinase K digestion, and formation of amyloid-like fibrils.² Using molecular dynamics simulation, Demarco *et al.* proposed a model for the formation of PrP^{Sc} molecules involving mainly a growth of the native β -sheet of PrP^C using the flexible N-terminal tail of the protein.¹⁸ On the other hand, based on experimental data, Cobb *et al.* suggested a parallel in-register β -structure for the amyloid fibrils using hydrogen/deuterium exchange, site-directed spin labeling coupled with electron paramagnetic resonance spectroscopy and mass spectroscopy analysis.¹⁵ One of the most popular models was presented by Govaerts *et al.*, which is based on electron microscopy data of 2D crystals that shows a left-handed β -helix trimeric structure.¹⁹ The feasibility of this model is supported by the high-resolution solid-state NMR structure of infectious material published recently for the yeast prion HET-s(218–289),²⁰ which revealed a β -solenoid with a triangular hydrophobic core that is similar to the model proposed by Govaerts *et al.*

To obtain a residue-specific structural description of the potential initial steps in the conversion process, we are using 800 MHz ¹H 1D and ¹H-¹H 2D NOESY NMR methods to characterize the differential stability of regions of the PrP^C protein in the presence of a denaturant. The use of denaturants to characterize protein unfolding is a widely used technique that has been extensively exploited to evaluate and compare

protein stabilities.²¹ According to the linear free energy model, the changes in free energy that accompany protein unfolding are linearly dependent on the concentration of denaturant.²² It has been shown that an uncharged denaturant like urea is a good choice to monitor protein unfolding compared to other denaturants,²³ and this is convenient because denaturants like guanidine hydrochloride are not ideal for NMR studies because of their ionic properties. The characterization of protein stability by NMR using urea as denaturant has been used previously to evaluate the stability of other proteins, such as the 434-repressor by Wüthrich and coworkers.²⁴ Nicholson *et al.* have also compared the global stability of Syrian hamster PrP (shPrP) and the mouse doppel protein using optical spectroscopy, and monitored the effect of urea on the amide hydrogen exchange rates using NMR.²⁵ Several other studies on the stability of the C-terminal domain of the prion protein can be found in the literature; some of them using molecular dynamics simulations,^{26,27} and biophysical techniques like Trp fluorescence, NMR and CD spectroscopies.^{12,25,28–31} The identification and characterization of the key elements favoring the prion protein transformation is an important step to obtain a more complete understanding of protein misfolding diseases.

Results

We used NMR to compare our purified bPrP^C_{121–230} with those of a previous study. Two regions of the 1D ¹H NMR spectrum of bPrP^C_{121–230} are shown in the lower traces of Figure 1(A,B). D₂O was used as a solvent to ensure a better detection of the H _{α} and aromatic protons, which would otherwise be buried under a strong water signal and overlapping amide protons, respectively. This spectrum was compared to those obtained by Wüthrich and coworkers for recombinant bPrP^C_{23–230} and bPrP^C_{23–230} isolated from healthy calf brains.³² The very close similarity between the spectra [e.g., methyl region presented in Fig. 1(A)] suggests that the proteins have a similar three-dimensional globular fold and that the D₂O did not affect the protein structure. We also acquired a 2D ¹H-¹H NOESY NMR spectrum at 800 MHz. The comparison of the cross-peaks with the chemical shifts previously deposited in the Biological Magnetic Resonance Data Bank (BMRB entry 4653) confirmed that the structure of the C-terminal domain of bPrP was properly folded and essentially identical to the previously determined structure.³³ We then performed a urea denaturation titration to evaluate the stability of the prion protein. Increasing concentrations of deuterated urea (from 0 to 10M) were added to the sample containing bPrP^C. By using deuterated urea, we would avoid reassociation of labile protons with the protein. For each step, two NMR spectra were acquired: a 1D ¹H and a 2D ¹H-¹H NOESY. The results and analysis of the two sets of experiments are presented below.

Figure 1. Titration from 0 to 10 M urea of recombinant bPrP_{121–230} followed by 1D ¹H NMR recorded at 800 MHz. (A) Stacked plot focusing on the methyl region from 0 to 0.8 ppm. I139.Hδ, I182.Hδ, and I182.Hγ₂ resonances are identified. (B) Stacked plot focusing on the aromatic region from 5.2 to 8.7 ppm. F198.Hα, Y162.Hα, Y218.Hδ, Y163.Hδ, and Y150.Hδ resonances are identified. The spectra were all referenced on the DSS signal at 0 ppm.

The 1D ¹H NMR spectra monitor the effect of urea on the chemical environment of various residues, which affects their position or chemical shift in the NMR spectrum, which in turn reflects the protein structure changes caused by the presence of the denaturant. Stacked plots of the 1D ¹H spectra acquired during the urea-induced denaturation titration for the aliphatic and aromatic regions are presented in Figure 1(A,B), respectively. For all spectra presented, the peak intensities were carefully corrected for the dilution during the urea titration from the partial specific volume of the urea added (see Materials and Methods for details). To quantify the changes in the spectra, one can utilize the chemical shifts or the peak intensity/integral of the resonances. In the presence of fast exchange (i.e., when the exchange rate is on the μs

time scale), one may use chemical shifts to monitor the structural changes since the peak intensities are likely to be constant. However, in the presence of slow conformational exchange (i.e., when the exchange rate is on the s time scale), the chemical shifts are relatively unperturbed while the peak intensities or peak areas can be used to monitor the unfolding. We have simulated the line shapes and chemical shift perturbations that would occur in the presence of fast or slow exchange (see Supporting Information Fig. S1). Using a full NMR lineshape analysis,³⁴ we have determined that the chemical exchange between the native and unfolded forms is in the NMR slow exchange limit with a lifetime on the order of ~1 s, as was previously shown by Wüthrich and coworkers for a protein of similar stability.³⁵ This justifies the use of peak integral as a measure of the fraction of the native conformation for the analysis. It also precludes a simple interpretation of the observed chemical shifts. The use of the peak integrals is a better choice than the measurement of the peak intensities for three reasons: (1) the peak integral will take into account any effect of line-broadening of the resonance, (2) it will include all frequencies of coupled protons for a given resonance, and (3) it will be less affected by the data processing method, such as drift correction, apodization, and line broadening.

We have identified the eight resonances with which to follow structural changes (I139.Hδ, I182.Hδ, I182.Hγ₂, F198.Hα, Y162.Hα, Y218.Hδ, Y163.Hδ, and Y150.Hδ). These resonances were chosen because they show no or minimal overlap with other peaks during the urea titration for a quantitative analysis. Conveniently, the eight most distinguishable resonances identified in the 1D ¹H spectrum are from residues well dispersed in different regions of PrP. By plotting the peak area of these resonances as a function of the urea concentration (Fig. 2), one can compare the relative stability of the protein as reflected by these residues. The data were fit using a sigmoidal function [see Eq. (2)] from which the two thermodynamic parameters that reflect the sensitivity of a given residue to urea can be extracted: $[D]_{1/2}$, the urea concentration required to diminish the peak area by half, and the m -value, the sensitivity of the free energy of unfolding on the urea concentration. Assuming a two-state mechanism of unfolding and a linear dependency of ΔG°_{N-U} as a function of the concentration of the denaturant, one can extrapolate to 0 M and obtain the ΔG°_{N-U} in the absence of urea. The thermodynamic data for these eight resonances are presented in Table I. The resonances were ranked according to their increasing $[D]_{1/2}$ value. The two residues most sensitive to urea are Y162.Hα and Y163.Hδ, showing the two lowest $[D]_{1/2}$ values ($5.4 \pm 0.1 M$ and $6.2 \pm 0.2 M$), while the other six resonances showed similar $[D]_{1/2}$ ranging from 7.1 to 7.7 M. The resonances Y162.Hα and Y163.Hδ also showed the two highest m -values ($4.9 \pm$

Figure 2. Peak area of the eight resonances identified in Figure 1, plotted as a function of the urea concentration: (A) I182.H δ_1 , (B) I139.H δ_1 , (C) Y218.H δ , (D) I182.H γ_2 , (E) F198.H α , (F) Y163.H δ , (G) Y162.H α , and (H) Y150.H δ . The $[D]_{1/2}$ and m -value are reported for each residue.

0.3 kJ mol⁻¹ M⁻¹ and 3.9 ± 0.3 kJ mol⁻¹ M⁻¹). Interestingly, these two residues are both located in the β -sheet of bPrP^C. This result indicates that the β -sheet region is perturbed at relatively low urea concentration compared to other residues that are located away from the β -sheet. Using these thermodynamic parameters, we have simulated the native structure populations of each residue as a function of the concentration of urea [Fig. 3(A)], and shown the location of each residue shown on the bPrP^C structure [Fig. 3(B)]. Finally, to obtain a global energy of unfolding for the C-terminal domain of bPrP^C, we have used a global fit on the individual denaturation curves to obtain the single best m -value and $[D]_{1/2}$ that fit all datasets. We obtained a m -value of 2.4 kJ mol⁻¹ M⁻¹, a $[D]_{1/2}$ of 7.2M, which lead to a global ΔG°_{N-U} of 17.3 kJ mol⁻¹ for bPrP^C. This energy of unfolding is comparable to the ΔG°_{N-U} of 4.6 kcal mol⁻¹ (~ 19.2 kJ mol⁻¹) obtained by Nicholson *et al.* for shPrP₉₀₋₂₃₀, also using urea as denaturant but CD spectroscopy to quantify the structural changes.

For several steps of the urea titration (0, 2, 5, and 8M), a 2D ¹H-¹H NOESY NMR spectrum was also acquired to monitor the presence and disappearance

Table I. Thermodynamic Properties of Different Residues of Recombinant bPrP₁₂₁₋₂₃₀ as Measured by 1D ¹H NMR

Resonance	$[D]_{1/2}$ (M)	m -Value (kJ mol ⁻¹ M ⁻¹)	ΔG°_{N-U} (kJ mol ⁻¹)
Y162.H α	5.4 ± 0.1	4.9 ± 0.3	26.7 ± 2.0
Y163.H δ	6.2 ± 0.2	3.9 ± 0.4	24.1 ± 3.5
Y150.H δ	7.1 ± 0.2	2.4 ± 0.2	16.8 ± 2.0
I182.H γ_2	7.1 ± 0.1	1.9 ± 0.7	13.7 ± 5.2
F198.H α	7.2 ± 0.1	3.0 ± 0.5	21.8 ± 4.3
I182.H δ_1	7.3 ± 0.1	2.6 ± 0.2	18.8 ± 1.9
Y218.H δ	7.6 ± 0.1	2.1 ± 0.3	15.9 ± 2.1
I139.H δ_1	7.7 ± 0.3	2.5 ± 0.1	19.1 ± 1.1

of ¹H-¹H cross-peaks as a function of the denaturant concentration (Fig. 4). The chemical shift assignment was based on deposited chemical shifts from the Wuthrich's group (BMRB 4563).³³ The presence of a cross-peak between two protons in a protein is dependent upon a number of factors, but can generally be taken as an indication that these protons are close in space (typically less than 6 Å) and show limited motions in the protein. This is made more complex if there is conformational exchange between two states

Figure 3. (A) Fraction of the native conformation of different residues of bPrP₁₂₁₋₂₃₀ as a function of the urea concentration. The dash lines correspond to the denaturation curves of resonances Y162.H α and Y163.H δ . (B) Cartoon representation of bPrP^C (PDB 1dyz). The different residues are displayed in ball-and-stick in the structure, and colored as in A).

Figure 4. Four 2D ^1H - ^1H NOESY spectra of bPrP^C acquired at 800 MHz. The spectra show the decreased number of NOE cross-peaks, as the concentration of urea is increased: (A) 0M urea, (B) 2M urea, (C) 5M urea, (D) 8M urea.

such as native and unfolded with different distances and motional properties. We have used a full relaxation matrix analysis to simulate the effect of urea denaturation on the known structure of the bovine PrP^C, and shown that the magnitude of the NOE parallels the fraction of the native conformation (see Supporting Information Fig. S2). We have used peak volumes to obtain an accurate measurement of the NOEs, since the peak intensities would not take into account changes in cross-peak lineshape or proton coupling. For each spectrum, we have measured the peak volumes as a function of the urea concentration. Only unambiguous inter-residue NOEs showing no overlap with other resonances were kept for analysis. To visualize the location of these ^1H - ^1H distances, we map the different NOEs on the three-dimensional structure of bPrP^C. Only four titration points (at 0, 2, 5, and 8M) are available for each inter-residue NOE, limiting the accuracy of individual fit to obtain thermodynamic data. To obtain the m and $[D]_{1/2}$ values that describes best the data, a global fit using Eq. (2) was performed for sets of NOEs representing each region. The a value was held constant, while the m -value and $[D]_{1/2}$ were iterated by 0.1 between 1 to 5 $\text{kJ mol}^{-1} \text{M}^{-1}$ and 1.0 and 7.0M, respectively.

We have divided the identified NOEs in three distinct regions of bPrP^C: the β 1-strand region (residues 128–133), the α 1-helix region (residues 138–157), and

the β 2-strand region (residues 160–164). The first region corresponds to inter-residual NOEs contacts between the residues of the β 1-strand, and the residues found in α 2-helix and β 2-strand (Fig. 5). These NOEs are indicative of the proper formation of the small β -sheet of bPrP^C. The global fit of the NOEs for β 1-strand region revealed a m -value of 1.5 $\text{kJ mol}^{-1} \text{M}^{-1}$ and a $[D]_{1/2}$ of 3.5M [the contour plot of error of the fit is presented in the inset of the graph shown in Fig. 5(A)]. Of special interest is the presence of multiple long-range NOEs between Y162 and L130; out of the 14 inter-residues NOEs identified for β 1-strand, five of them are between Y162 and L130, with only one of them detectable at 5M urea. These NOEs are a good probe for detecting the presence of the β -sheet, since these two protons are only close in space when the β -sheet is properly formed. The disappearance of these NOEs suggests that the β 1 and β 2-strands dissociate at a relatively low urea concentration. However, while β 1-strand dissociates from its β 2-strand partner, the β 2-strand remains strongly in contact with helices α 2 and α 3 as monitored by the second region (Fig. 6). The global fit obtained for the NOEs between β 2-strand and helices α 2 and α 3 defined this region with an m -value of 2.6 $\text{kJ mol}^{-1} \text{M}^{-1}$ and a $[D]_{1/2}$ of 5.7M [Fig. 6(A)]. The presence of these strong NOEs, many still present at 8M urea, suggests that this region is highly stable even in the presence of high concentrations of

Figure 5. Inter-residue NOEs found in the β 1-strand region of bPrP^C. (A) Normalized NOE cross-peak volumes as a function of the concentration of urea for residues located in the β 1-strand. The contour plot (inset) shows the sum of the square of the error for a global fit of the data yielding a m -value of 1.5 kJ mol⁻¹ M⁻¹ and a $[D]_{1/2}$ of 3.5M. The gray line on the graph corresponds to a denaturation curve with these thermodynamic parameters. (B) Cartoon representation of the β -sheet region of bPrP^C. The following inter-residue ¹H-¹H NOEs contacts are shown with black lines: 128.H δ -182.H δ ₁, 162.H δ -130.H δ ₂, 162.H α -130.H δ ₁, 162.H ϵ -130.H δ ₁, 162.H δ -130.H δ ₁, 128.H ϵ -182.H γ ₂, 128.H ϵ -182.H δ ₁, 128.H δ -182.H γ ₂, 162.H ϵ -130.H δ ₂, 163.H ϵ -131.H α ₁, 128.H δ -125.H α , 162.H α -130.H α , 163.H ϵ -131.H α ₂. The heavy atoms of the residues found in the β 1-strand are colored in yellow, while the remaining atoms from other regions are colored in green. The hydrogen atoms are all colored in white.

denaturant. The third region that was investigated corresponds to the residues surrounding α 1-helix (residues 138–157), which also showed the characteristics of a well stable region (Fig. 7). The global fitting of the NOEs of this region revealed an m -value of 3.6 kJ mol⁻¹ M⁻¹ and a $[D]_{1/2}$ of 5.7M, similar to the β 2-strand region mentioned earlier. Only a few NOEs vanished early in the titration for this region, mostly long-range NOEs that were already weak in the absence of urea. Since a few NOEs were surprisingly still identifiable at 8M urea, we have mapped these contacts on the structure of bPrP^C (Fig. 8). The global fitting of the eight identified NOEs revealed a m -value of

2.2 kJ mol⁻¹ M⁻¹ and a $[D]_{1/2}$ of 6.3M. Three of these NOEs were found in the α 1-helix region and another three NOEs were found between β 2-strand and α 2-helix. Interestingly, the last two NOEs found to be still present at 8M urea are between the side chains of residues V166 and Y218, linking the loop_{165–172} with the end of helix α 3. No NOE contacts were found between strands β 1 and β 2 at 8M urea.

A different analysis of the data is presented in the Supporting Information, dividing the 44 inter-residue NOEs identified into two distinct categories: the stable (Supporting Information Fig. S3) and less stable NOEs (Supporting Information Fig. S4). The stable NOEs corresponds to the NOEs having a peak volume at 5M urea larger than 50% of the original peak volume at 0M urea (typically $[D]_{1/2} > 5M$). The second group

Figure 6. Inter-residue NOEs found in the β 2-strand region of bPrP^C. (A) Normalized NOE cross-peak volumes as a function of the concentration of urea, for residues located in the β 2-strand region but not making contacts with residues located in the β 1-strand. The contour plot (inset) shows the sum of the square of the error for a global fit of the data yielding a m -value of 2.6 kJ mol⁻¹ M⁻¹ and a $[D]_{1/2}$ of 5.7M. The gray line on the graph corresponds to a denaturation curve with these thermodynamic parameters. (B) Cartoon representation of the β -sheet region of bPrP^C. The following inter-residue ¹H-¹H NOEs contacts are shown with black lines: 162.H β ₁-182.H γ ₂, 214.H α -161.H γ ₂, 161.H α -183.H γ ₂, 162.H δ -182.H γ ₂, 162.H δ -183.H γ ₂, 163.H ϵ -217.H γ , 163.H ϵ -221.H γ , 162.H δ -183.H α . The heavy atoms are colored in green and the hydrogen atoms are colored in white.

Figure 7. Inter-residue NOEs found in the $\alpha 1$ -helix region of bPrP^C. (A) Normalized NOE cross-peak volumes as a function of the concentration of urea, for residues located in the $\alpha 1$ -helix region (residues 138–157). The contour plot (inset) shows the sum of the square of the error for a global fit of the data yielding a m -value of $3.6 \text{ kJ mol}^{-1} \text{ M}^{-1}$ and a $[D]_{1/2}$ of 5.7 M . The gray line on the graph corresponds to a denaturation curve with these thermodynamic parameters. (B) Cartoon representation of the $\alpha 1$ region of bPrP^C. The following inter-residue ^1H - ^1H NOE contacts are shown with black lines: 139.H δ_1 -212.H γ , 139.H δ_1 -212.H β , 209.H α -139.H δ_1 , 139.H δ_1 -209.H γ_2 , 139.H δ_1 -208.H β , 139.H δ_1 -212.H γ , 206.H ϵ -157.H ϵ , 141.H ζ -139.H γ_2 , 141.H ϵ -139.H γ_2 , 141.H δ -139.H γ_2 , 141.H ζ -139.H δ_1 , 141.H ϵ -139.H γ_1 , 157.H ϵ -154.H α , 157.H δ -154.H α , 150.H ϵ -138.H α , 150.H ϵ -157.H ϵ , 198.H δ -157.H ϵ , 150.H δ -157.H ϵ . The heavy atoms are colored in green and the hydrogen atoms are colored in white.

contains the less stable NOEs (Supporting Information Fig. S4), corresponding to the NOEs having a peak volume at 5 M that was lower than 50% of the peak volume at 0 M urea (typically $[D]_{1/2} < 5 \text{ M}$). Again, to obtain an m -value and $[D]_{1/2}$ that describes the data best, a global fit was performed for each set of NOEs (i.e., stable and less stable) using Eq. (2). We obtained a global minimum with an m -value of $3.0 \text{ kJ mol}^{-1} \text{ M}^{-1}$ and a $[D]_{1/2}$ of 5.8 M for the stable NOEs, compared to an m -value of $4.4 \text{ kJ mol}^{-1} \text{ M}^{-1}$ and a $[D]_{1/2}$ of 2.2 M for the less stable NOEs. Most of these NOEs are located in the $\alpha 1$ -helix region, which makes strong contacts with the N-terminal portion of $\alpha 3$ -helix (Sup-

porting Information Fig. S2). This complex network of NOEs is also accompanied by stable NOEs between F198 situated in the loop before the $\alpha 3$ -helix, and M206 and Y157. On the other side of the protein, many stable NOEs can be found between $\beta 2$ -strand and the adjacent helices $\alpha 2$ and $\alpha 3$, and only a few in the $\beta 1$ -strand. With the exception of three weak NOEs found in the $\alpha 1$ -helix region, all of the less stable NOEs are between residues located in the $\beta 1$ and $\beta 2$ -strands.

We have also performed a protein-refolding experiment. Taking the sample obtained after the urea titration from 0 to 10 M urea, we buffer exchanged the NMR sample and removed most of the urea, as indicated by the presence of a low intensity peak of urea in the $1\text{D } ^1\text{H}$ spectrum. We compared the acquired 1D

Figure 8. Inter-residue NOEs of bPrP^C identified at 8 M urea. (A) Normalized NOE cross-peak volumes as a function of the concentration of urea, for NOEs still identifiable at 8 M urea. The contour plot (inset) shows the sum of the square of the error for a global fit of the data yielding a m -value of $2.2 \text{ kJ mol}^{-1} \text{ M}^{-1}$ and a $[D]_{1/2}$ of 6.3 M . The gray line on the graph corresponds to a denaturation curve with these thermodynamic parameters. (B) Cartoon representation of bPrP^C. The following inter-residue ^1H - ^1H NOEs contacts are shown with black lines: 162.H β -182.H γ_2 , 209.H α -139.H δ_1 , 161.H α -183.H γ_2 , 139.H δ_1 -212.H γ , 218.H δ -166.H γ_2 , 218.H ϵ -166.H γ_2 , 162.H δ -183.H α , 150.H ϵ -138.H α . The heavy atoms are colored in green and the hydrogen atoms are colored in white.

^1H NMR spectrum of this sample and to the original oM urea spectrum to see if the bPrP^C was refolded. Both spectra were very similar without significant changes in regards to the chemical shifts. The peak intensities were reduced, most likely due to a loss of protein during the urea extraction, but it could also suggest an irreversible state of the aggregates observed by electron microscopy (data not shown). The similarities between the two spectra indicate that the refolded bPrP^C is identical to the original starting structure, confirming that the protein primary sequence remained unmodified during the denaturation process.

Discussion

We have used ^1H NMR spectroscopy to investigate the stability of bPrP^C_{121–230} using urea as a denaturant. The analysis of the 1D and 2D ^1H NMR spectra showed that the titration curves for all residues observed were monotonic and adequately fit using a two-state unfolding model to extract residue-specific thermodynamic parameters. Using this method, we obtained individual $[D]_{1/2}$ and m -values for various residues of bPrP^C. No intermediates were stable enough, or at high enough concentrations, to be observed in any of the denaturation curves. However, the analysis revealed different stabilities for the different residues monitored, which implies that the denaturation process must involve many microscopic intermediates. Similar observations have been reported for human PrP^C,²⁹ and Syrian hamster PrP^C.³⁶ Kinetics studies of the global folding of murine PrP^C showed that PrP^C rapidly folds into its native conformation without the presence of structural intermediates,³⁷ while kinetic studies of human PrP^C^{38,39} observed a folding intermediate. It has been shown in a previous study that the denaturation curve of Syrian hamster PrP^C using urea and monitored by circular dichroism exhibits the classic sigmoidal shape associated with two-state unfolding.²⁵ However, the authors did not exclude the possibility of an undetected intermediate state.

From the 1D ^1H NMR spectra analysis, we have identified residues Y162 and Y163 as being perturbed at lower concentration of urea relative to other residues located in other secondary structures of the protein. While these two tyrosines are the most sensitive residues to urea among those examined, they also possess the two highest $\Delta G^{\circ}_{\text{N-U}}$. This implies that in the absence of denaturant, the β -sheet region is a stable region of PrP^C, but in the presence of urea is the most easily perturbed region of the protein. The NOE data also suggest that the β -sheet region is a sensitive region of bPrP^C upon addition of urea. Alternative explanations would be that some of these titrations involve close, undetected multiple steps which would lower the observed m -value, or the assumption that the $\Delta G^{\circ}_{\text{N-U}}$ is only linearly dependent upon urea concentration is inadequate. These observations are

reflected in the discrepancies found in the literature about the stability of the β -sheet. For example, molecular dynamics simulations by DeMarco *et al.* suggest that the extension/growth of the anti-parallel β -sheet of PrP^C is the principal conformational change leading to PrP^{Sc}.¹⁸ Kachel *et al.* also reported that the β_1 and β_2 -strand structures are insensitive to the application of high pressure.²⁹ However, several other studies, experimental as well as theoretical, suggest that the β -sheet of PrP^C is one of the less stable parts of the C-terminal region of PrP. Using a collective dynamic simulation approach, Blinov *et al.* suggested that the locations of relatively low stability may be associated with the β -sheet formed by β_1 and β_2 -strands and the adjacent loops, whereas α_3 -helix appears to be a relatively stable part of the protein.²⁶ Using coarse-grained protein molecular dynamics simulations, Chebaro *et al.* found that α_1 -helix is remarkably stable and concluded that the stability of the native β -sheet is the principal region affected by the different pathogenic mutations.⁴⁰ Using molecular dynamics simulations, Hirschberger *et al.* suggested that the hydrophobic interaction between helix α_1 to helix α_3 is required for PrP^C to correctly fold into its stable native structure.⁴¹ Also using computer simulations, Barducci *et al.* found that the antiparallel β -sheet is significantly weaker in the pathogenic D178N mutant than in the wild-type PrP^C, because of hydrogen bonding network involving residues N178 with R164 and Y128 side chains.^{42,43} These simulations are experimentally supported by antibody binding essays that suggest exposure of a pathological epitope YYR during the conversion of PrP^C into PrP^{Sc}.⁴⁴ The evidence points to the YYR motif found in the β_2 -strand, which would also imply a β -sheet dissociation. Finally, a recent NMR study of the dynamics of PrP^C reported slow exchange motions in the short anti-parallel β -sheet.³¹

The analysis of our experimental results using NMR spectroscopy suggests that the β -sheet of PrP^C shows an important conformational change upon denaturation as monitored by the disappearance of key NOEs between β_1 and β_2 -strands. In general, the NOE is a complex phenomenon that can be difficult to interpret, and the disappearance of an NOE can reflect a number of factors (see Supporting Information). For example, if two residues are involved in an inter-residue NOE, but only one moves, the NOE might disappear. The loss of a NOE can reflect such change in distances, but can also result from a change in correlation time toward faster motions than the overall tumbling of the protein, which is often responsible for the loss of an intra-residue NOE, for example. Obviously, the strongest support for a conformational change in a given structural element is the disappearance of several NOEs reflecting several ^1H - ^1H distances in that region. According to our 2D NOESY analysis, the α_1 -helix region is highly resistant to urea denaturation, and the β_2 -strand region makes strong

interactions with $\alpha 2$ and $\alpha 3$ helices. Even at 8M urea, NOEs are still observable for these two stable regions, in addition to two NOEs contacts remaining present between residue V166, located in the loop_{165–172}, and Y218 located in the $\alpha 3$ -helix. These last two NOEs are part of an important network of intramolecular interactions found in PrP^C that defines the stability of the loop_{165–172} and the $\alpha 3$ -helix together. This has been the focus of a recent study by the Wuthrich group,⁴⁵ suggesting that these well-defined long-range interactions could be important for the functional specificity of PrP^C, and that this loop is a potential recognition area for the hypothetical “protein X.”

Another particular residue is F198, located in the loop_{194–200} between helices $\alpha 2$ and $\alpha 3$ [see Fig. 7(B)]. Among others, the aromatic protons of F198 make stable NOE contacts with the methyl of M206 and with the aromatic ring of Y157. These NOEs are still intense at 5M urea. The mutation of this residue (F198S) is associated with the familial Gerstmann-Straussler-Scheinker disease. It has been shown that the F198S variant of human PrP^C has a dramatically increased propensity to self-associate into β -sheet-rich oligomers.⁴⁶ The role of this aromatic ring seems to be to anchor the loop_{194–200} into the hydrophobic core PrP^C. It is reasonable to think that the substitution of this bulky hydrophobic residue for a smaller and polar residue like serine would abolish the hydrophobic interactions of the aromatic ring of F198 with the core of the protein.

One could wonder about the effect of not having the flexible N-terminal tail of bPrP^C on the stability of the protein. According to a previous study using high pressure NMR spectroscopy,²⁹ huPrP_{23–230} and huPrP_{121–230} have a similar ΔG°_{N-U} . The authors observed virtually no difference between both constructs, suggesting that the N-terminal tail does not affect the stability of the globular C-terminal domain of PrP^C.

We have quantified the stability of the C-terminal domain of the bPrP^C on a per residue basis using NMR spectroscopy. Upon urea denaturation, we have observed an early perturbation of the β -sheet region compared to the other secondary structures, suggesting a dissociation of $\beta 1$ -strand from $\beta 2$ -strand at low urea concentrations. While $\beta 1$ -strand detaches from $\beta 2$ -strand, the $\beta 2$ -strand remains strongly in contact with $\alpha 2$ and $\alpha 3$ as evidenced by the presence of strong NOE contacts between the two regions even at a high concentration of urea. We have also noticed strong hydrophobic interactions between the $\alpha 1$ -helix region and the N-terminal section of the $\alpha 3$ -helix, as well as between the loop_{165–172} with the C-terminal end of $\alpha 3$ -helix.

Materials and Methods

Protein purification

The bPrP_{121–230} gene was synthetically synthesized with codon usage optimized for *E. coli* expression. *NdeI* and *BamHI* cut sites were included at the 5' and

3' ends of the sequence to facilitate ligation into pRSETa plasmid previously digested with the same restriction enzymes. Ligations were transformed into XL-1 blue cells, and colonies were screened for insertions. The creation of pRSETa-bPrP_{121–230} was confirmed by sequencing in the forward and reverse directions. The C-terminus region of bovine PrP containing residues 121 to 230 (bPrP_{121–230}) was expressed and purified in *E. coli* based on previously described methodology for human PrP.⁴⁷ A molecular weight of $13,093 \pm 5$ Da was measured using mass spectroscopy compared to 13,097 Da for the predicted molecular weight. The purity of the protein was also verified using 1D ¹H NMR spectrum.

NMR samples

The NMR sample was prepared with ~2 mg of bPrP_{121–230} in 500 μ L of deuterated NMR buffer containing 10 mM of sodium acetate, 0.3 mM of deuterated 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS-*d*₆), and 0.012% (w/v) NaN₃. The pH of the sample was 6.3 according to the chemical shift of the acetate methyl group based on previously described methodology.⁴⁸ The urea-induced unfolding of bPrP_{121–230} was performed by adding solid deuterated urea (urea-*d*₄, Sigma-Aldrich) directly into the NMR tube. The urea concentration was increased by 1M at each titration step until 10M, because the solubility of urea in water is 10.49M at 25 °C. To account for the volume increase caused by the addition of solid denaturant, the length of the NMR sample was measured at each step, and an increased amount of urea-*d*₄ was added at each step to reach the correct molarity.

NMR spectroscopy

The NMR experiments were all performed on an 800 MHz Varian INOVA NMR spectrometer equipped with a cryogenic probe. The one-dimensional (1D) proton NMR spectra were acquired with 256 transients, a spectral width of 15.0 ppm, and a time delay of 2 s, while the two-dimensional (2D) ¹H-¹H NOESY spectra were acquired with 32 transients, 256 increments, spectral widths of 15.0 ppm for both proton dimensions, a time delay of 1 s, and a mixing time 100 ms. A total of 50 h were needed to acquire the NMR data between the time at which urea-*d*₄ was added for the first point until the acquisition of the last spectrum of the titration.

Data processing

The processing and visualization of the 1D spectra was done with the VnmrJ software v2.1B (Varian inc.). Each spectrum was processed with a line broadening of 1.5 Hz and the vertical scale (and integral scale) was adjusted according to the volume increase of the sample caused by the addition of solid urea. The peak areas were then calculated by isolating a given

resonance in a window of ~ 0.1 ppm, applying a drift correction on the baseline, and measuring the area under the curve by using the *dli* command in the Vnmrj software (display list of integral). Intensities, integral, and deconvolution techniques were used to analyze the resonances, but only the results of the peak area are reported here (see Discussion). The peak area of a given resonance was normalized to the first titration point (0M), then plotted as a function of the urea concentration and fit using the xcvfit software.⁴⁹

The processing of the 2D spectra was performed using nmrPipe.⁵⁰ Linear prediction was used in the *t2* dimension to add resolution for a maximum of half the number of acquired points. Sine functions of 90° and 75° were applied in the *t1* and *t2* dimensions, respectively, and zero-filling was used to double the number of points in both dimensions before the Fourier Transform. NMRViewJ (One Moon Scientific inc.) was used to analyze the 2D NOESY spectra and obtain the cross-peak volumes from the peak analysis tools. Before the volume measurement, a box was manually adjusted around each identified resonances to define the limits of the cross-peaks, allowing a more accurate measurement of the peak volumes.

Data analysis

Assuming a two-state model of unfolding and a linear dependency of the Gibbs energy of unfolding (ΔG_{N-U}) as a function of the concentration of urea^{21,22}:

$$\Delta G_{N-U} = \Delta G_{N-U}^{\circ} - m[D] \quad (1)$$

where ΔG_{N-U}° is the free energy of unfolding extrapolated to zero denaturant concentration, *m* is the slope at midpoint of the unfolding transition, and *[D]* is the concentration of denaturant. Since ΔG_{N-U} is equal to zero at midpoint of the denaturation, ΔG_{N-U}° is by definition equal to $m \cdot [D]_{1/2}$, where $[D]_{1/2}$ is the concentration of denaturant at midpoint of unfolding. This leads to the following formula to fit the data and directly extract the thermodynamic properties of the protein upon denaturant-induced unfolding:

$$\frac{I}{I_0} = \frac{a}{1 + \exp\{m([D] - [D]_{1/2})/RT\}} \quad (2)$$

Where *I* is the peak area or peak volume at a given denaturant concentration, *I*₀ is the peak area or peak volume at zero concentration denaturant, the *m*-value reflects the dependence of the free energy of unfolding on the denaturant concentration, *[D]* is the concentration of denaturant, $[D]_{1/2}$ is the concentration of denaturant at midpoint of unfolding, *a* is a scaling constant, *R* is the gas constant, and *T* is the temperature.

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