Structural plasticity of the cellular prion protein and implications in health and disease

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Two lines of transgenic mice expressing mouse/elk and mouse/horse prion protein (PrP) hybrids, which both form a well-structured $\beta 2$ - $\alpha 2$ loop in the NMR structures at 20 °C termed rigid-loop cellular prion proteins (RL-PrP^C), presented with accumulation of the aggregated scrapie form of PrP in brain tissue, and the mouse/elk hybrid has also been shown to develop a spontaneous transmissible spongiform encephalopathy. Independently, there is in vitro evidence for correlations between the amino acid sequence in the $\beta 2$ - $\alpha 2$ loop and the propensity for conformational transitions to disease-related forms of PrP. To further contribute to the structural basis for these observations, this paper presents a detailed characterization of RL-PrP^C conformations in solution. A dynamic local conformational polymorphism involving the $\beta 2$ - $\alpha 2$ loop was found to be evolutionarily preserved among all mammalian species, including those species for which the WT PrP forms an RL-PrP^C. The interconversion between two ensembles of PrP^C conformers that contain, respectively, a 3_{10} -helix turn or a type I β -turn structure of the $\beta 2$ - $\alpha 2$ loop, exposes two different surface epitopes, which are analyzed for their possible roles in the still evasive function of PrP^C in healthy organisms and/or at the onset of a transmissible spongiform encephalopathy.

prion protein stability | protein dynamics | conformational equilibrium | NMR line shape analysis

Transmissible spongiform encephalopathies (TSEs) include Creutzfeldt-Jakob disease in humans, scrapie in sheep and goats, bovine spongiform encephalopathy in cattle, and chronic wasting disease (CWD) in elk and deer (1–3). A common feature of these diseases is the conversion of the cellular form of the prion protein (PrP^C) found in healthy organisms into aggregated isoforms (PrP^{Sc}), which are deposited primarily in the brain of the diseased individuals (1, 4). Despite extensive investigations, the physiological function of PrP^C in healthy organisms as well as the mechanistic aspects of its pathophysiological role remain elusive (4–9). Although the PrP^{Sc} form found in diseased tissue has been intensively studied, other approaches underline the importance of PrP^C as a potential target for TSE prevention and medical intervention after outbreak of the disease (10–12), with a special focus on rigid-loop cellular prion proteins (RL-PrP^C) (11, 13–15), which are investigated in this paper.

A common PrP^C fold for a globular domain formed by the polypeptide segment of residues 125–228 in mouse PrP (mPrP) [see Schätzl et al. (16) for the numeration in different species], with three α -helices and a short two-stranded antiparallel β -sheet, has been observed for the cellular prion proteins of all mammalian species studied so far (17–27). For WT PrP of most species, parts of the backbone amide group NMR signals of residues in a loop between a β -strand, $\beta 2$, and a helix, $\alpha 2$, are not observable in NMR spectra recorded with aqueous solutions at pH 4.5 and 20 °C at a ¹H resonance frequency of 500 MHz (or higher) because of line broadening by conformational exchange; therefore, the $\beta 2$ - $\alpha 2$ loop in these PrP^Cs is poorly defined in NMR structures determined under these conditions. This result is also obtained for mPrP^C, where the observed line broadening can be rationalized by conformational exchange between two precisely defined, locally

different structures (28). There are, by now, numerous model simulations focused on the $\beta 2$ - $\alpha 2$ loop in PrP^C (29–33), and continuation of work along these lines will greatly benefit from a more comprehensive platform of experimental data.

The aforementioned solution parameters of pH 4.5 and T = 20 °C had initially been selected for providing long-time stability of the recombinant PrP^Cs, and they were used in our laboratory as standard conditions for 750 MHz NMR screens for the presence of the PrP^C fold in prion proteins from a wide variety of species. This screen revealed a feature in the elk prion protein, where all backbone amide group NMR signals of the $\beta 2$ - $\alpha 2$ loop are observable at 20 °C and a ¹H resonance frequency of 750 MHz; the $\beta 2$ - $\alpha 2$ loop is, therefore, well-defined in the NMR structure (23). This observation attracted special interest in the context of the CWD crisis of deer and elk in North America (34–37) and was followed up by structural studies of WT and specifically designed variant prion proteins (24–26, 28, 38), in vivo experiments with transgenic mice (11, 13, 14, 39, 40), and in vitro studies of correlations between the $\beta 2$ - $\alpha 2$ loop amino acid sequence and the propensity of PrP^C to undergo transitions to PrP^{Sc}-related conformations (41–43). In connection with animal experiments relating to CWD, the term RL-PrP^C was introduced for PrP^Cs forming a structurally well-defined $\beta 2$ - $\alpha 2$ loop in the solution NMR structure at 20 °C (39). The WT prion proteins of bank vole, wallaby, and horse were then found to also form an RL-PrP^C structure (24–26). Transgenic mice expressing a mouse/elk hybrid RL-PrP^C differing from mPrP by the amino acid replacements S170N and N174T developed a spontaneous TSE (11, 13), and a mouse/horse hybrid RL-PrP^C with the single-residue exchange D167S was shown to present with spontaneous spongiform degeneration (14).

This paper investigates the $\beta 2$ - $\alpha 2$ loop in the presently known RL-PrP^Cs using NMR studies of structure, intramolecular conformational equilibria, and intramolecular rate processes. The resulting data are surveyed for all presently investigated RL-PrP^Cs from which the designed variant of mPrP mPrP [Y225A,Y226A](121–231) (25), representing an RL-PrP^C with the same $\beta 2$ - $\alpha 2$ loop sequence as WT mPrP^C, was selected to illustrate details of this database, which then provides a platform for an evaluation of the biological implications of the $\beta 2$ - $\alpha 2$ loop polymorphism.

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Data deposition: The atomic coordinates of the NMR structure of mPrP[Y169A,Y225A,Y226A](121–231) at 20 °C have been deposited in the Protein Data Bank (www.pdb.org, PDB ID code 2L1K) and the chemical shifts in the BioMagResBank (www.bmrb.wisc.edu, accession no. 17087).

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Fig. 1. Temperature and ^1H resonance frequency dependence of 2D ^{15}N , ^1H -HSQC NMR spectra of a designed RL-PrP^C variant of the mouse prion protein, mPrP[Y225A,Y226A](121–231). (A) Contour plots of 750 MHz spectra at 5 °C, 10 °C, 15 °C, and 20 °C. The resonances of residues 167–171 and 175 are highlighted, with blue circles around observed signals and red circles indicating anticipated empty peak positions. (B) Temperature variation of 1D cross-sections along $\omega_2(^1\text{H})$ from 750 MHz spectra recorded with 5° intervals over the range of 5 °C to 45 °C. Shown are data for A133, which represents the behavior of those residues that are not affected by the $\beta 2$ – $\alpha 2$ loop conformational exchange, and residues 170, 171, and 175. For S170 and F175, the vertical scale was increased twofold relative to A133. (C) ^1H resonance frequency dependence of 1D cross-sections along $\omega_2(^1\text{H})$ recorded at 20 °C. For the residues 170, 171, and 175, the vertical scale was increased fourfold relative to A133.

Results and Discussion

Impact of Temperature and Proton Resonance Frequency Variation on the NMR Spectra of RL-PrP^Cs. Based on earlier observations that N-terminal elongation does not measurably affect the conformational behavior of the globular domain in PrP^Cs (19, 20, 44), the present experiments were performed with PrP constructs of residues 121–231. The temperature dependence of the 750 MHz 2D [¹⁵N, ¹H]–heteronuclear single-quantum coherence (HSQC) spectrum of uniformly ¹⁵N-labeled mPrP[Y225A,Y226A](121–231) reveals that no correlation peaks of S170, N171, and F175 are seen in the contour plots at 5 °C, 10 °C, and 15 °C (Fig. 1A), and the cross-sections through the NMR signals of these three residues (Fig. 1B) document that the peak intensity is very low, even at 20 °C. These three signals also show further line broadening at higher ¹H resonance frequencies (Fig. 1C). The RL-variant mPrP[Y225A,Y226A](121–231), thus, has qualitatively the same

dependence of the ^{15}N - ^1H correlation signals on the ^1H frequency and the temperature as previously observed for mPrP (121–231) (28), with the important difference that the temperature where line narrowing enables observation of all these NMR signals at 750 MHz is shifted to lower temperature (i.e., to about 20 °C compared with about 40 °C for the WT protein).

The protein mPrP[Y225A,Y226A](121–231) mimics WT wallaby (*Macropus eugenii*) PrP as a protein with RL-PrP^C behavior (25), although it differs from wallaby PrP in the $\beta 2$ – $\alpha 2$ loop amino acid sequence. It is so far only the second prion protein with RL-PrP^C behavior that contains the same $\beta 2$ – $\alpha 2$ loop sequence as mPrP, the other one being mPrP[F175A](121–231) (38). Similar behavior as for mPrP[Y225A,Y226A](121–231) (Fig. 1) was observed for WT wallaby, horse, bank vole, and elk PrP and the RL variants of mPrP(121–231) with the amino acid substitutions V166A, D167S (as in horse), D167S/N173K (as in horse PrP), S170N (as in elk and bank vole PrP), S170N/N174T (as in elk PrP), F175A, and Y225A (Fig. 2). For all RL-PrP^Cs, the resonances of residues 170, 171, and 175 were not detectable below 15 °C, and the residues 167–169 showed pronounced line broadening when lowering the temperature from 15 °C to 5 °C. In Fig. 2, mPrP(121–231) and its variants with the amino acid substitutions Y169F and Y226A represent the large group of mammalian PrPs with disordered $\beta 2$ – $\alpha 2$ loop at 20 °C (17–22, 27). We, thus, have two groups of PrP^Cs, within which all proteins show essentially identical temperature variation of the NMR spectra. Notwithstanding the different transition temperatures, the proteins with WT mPrP behavior and the RL-PrP^Cs share closely similar behavior. In the following section, the common origin of the temperature-dependent line broadening in both classes of PrPs is documented by NMR studies of conformational exchange in the family of RL-PrPs and an NMR structure determination of a variant prion protein representing the lowly populated molecular species involved in this exchange.

Fig. 2. Survey of the line broadening behavior of $\beta 2$ - $\alpha 2$ loop backbone amide signals of WT mPrP(121-231) and designed mPrP(121-231) variants in 750 MHz NMR spectra at variable temperatures. At the top of *Center*, the mPrP amino acid sequence in positions 166-175, 225, and 226 is given. For the variant proteins, the differences to the WT sequence are indicated by amino acid one-letter symbols, and the conserved residues are represented by dots. In *Right*, the different proteins are identified, where ‡ indicates data from ref. 28, ◇ indicates data from ref. 38, and related WT RL-PrP^Cs are indicated in parentheses. In *Left*, exchange broadening of the amide group NMR signals in 750 MHz 2D [¹⁵N,¹H]-HSQC spectra over the temperature range 5 °C to 45 °C is indicated using the following color code for discrete temperature ranges: yellow, none of the loop residues 167-171 observed; cyan, residues 167-169 observed; red, all loop residues observed; white, no evidence for line broadening of the NMR signals of the $\beta 2$ - $\alpha 2$ loop by conformational exchange. Lines 2-9 represent RL-PrP^Cs with all $\beta 2$ - $\alpha 2$ loop signals visible at a temperature of 20 °C or higher.

NMR Structure of mPrP[Y169A,Y225A,Y226A](121–231) at 20 °C. Unlike the RL-PrPs, which show extensive exchange line broadening at temperatures below 20 °C (Fig. 2), variants derived from RL-PrPs where Tyr at position 169 has been substituted with Ala or Gly show no evidence of conformational exchange in the β 2– α 2 loop (Fig. 2). Here, mPrP[Y169A,Y225A,Y226A](121–231) was selected for a structure determination, because we hypothesized, based on previous observations with mPrP (28), that it might mimic the behavior of the minor species in the β 2– α 2 loop polymorphism of mPrP[Y225A,Y226A](121–231), which is an RL-PrP (25).

At the outset of the structure determination, tentative NMR assignments for most of the residues outside of the β 2– α 2 loop were obtained by reference to the assignments of mPrP[Y225A,Y226A](121–231) and mPrP[Y169A](121–231) (25, 28). These assignments were confirmed and extended to the β 2– α 2 loop using a 3D HNCA spectrum and the three 3D heteronuclear-resolved [^1H , ^1H]-NOESY spectra that were recorded to collect conformational constraints for the structure determination. Based on the resulting nearly complete resonance assignments, the NMR structure was determined using the ATNOS/CANDID/DYANA protocol (45–47). A survey of the input used and the statistics of the structure calculations are given in Table 1, and the structure is shown in Fig. 3.

Similar to mPrP[Y225A,Y226A](121–231), the NMR structure of mPrP[Y169A,Y225A,Y226A](121–231) represents a typical PrP^C fold, with an antiparallel two-stranded β -sheet of residues 128–131 and 161–164 and three α -helices comprising the residues 144–153, 172–190, and 200–227, where α 1 and α 2 end with

Table 1. Input for the structure calculation and characterization of the energy-minimized NMR structure of mPrP[Y169A,Y225A,Y226A](121–231)

Quantity	Data*
NOE upper distance limits	2,700
Intraresidual	605
Sequential	693
Medium range	752
Long range	650
Dihedral angle constraints	108
Residual target function value, Å ²	1.54 ± 0.35
Residual distance constraint violations	
Number > 0.1 Å	27 ± 4
Maximum, Å	0.15 ± 0.06
Residual dihedral angle constraint violations	
Number > 2.0°	0 ± 0
Maximum, °	1.49 ± 1.07
Amber energies, kcal·mol ^{−1}	
Total	−4,982 ± 61
Van der Waals	−344 ± 16
Electrostatic	−5,566 ± 56
rmsd to the mean coordinates, Å [†]	
bb (125–226)	0.50 ± 0.07
ha (125–226)	0.89 ± 0.07
Ramachandran statistics, % [‡]	
Most favored	79
Additional allowed	19
Generously allowed	2
Disallowed	0

*Except for the top six entries, which describe the input for the structure calculations, the average values for the 20 energy-minimized conformers with the lowest residual DYANA target function values and the SDs among them are given.

[†]The numbers in parentheses indicate the residues for which the rmsd values were calculated; bb, backbone atoms N, C $^{\alpha}$, and C $^{\beta}$; ha, all heavy atoms.

[‡]As determined by PROCHECK (71).

Fig. 3. NMR structure of mPrP[Y169A,Y225A,Y226A](121–231) at T = 20 °C and pH 4.5. (A) Bundle of 20 energy-minimized conformers (Table 1). Each conformer is represented by a spline function through the C $^{\alpha}$ positions. Yellow, α -helices; green, β -strands; cyan, 3_{10} -helical turns; gray, nonregular secondary structure. (B) All heavy-atom presentation of the energy-minimized conformer with the lowest DYANA target function value in the bundle shown in A. The backbone is represented by a gray spline function through the C $^{\alpha}$ positions, and the stick presentations of the side chains are colored according to their global all heavy-atom displacements, D (72): cyan, D $\leq$ 0.6 Å; yellow, 0.6 Å < D $\leq$ 1.2 Å; red, D > 1.2 Å. (C) Polypeptide backbone of the segment 165–175, which includes the β 2– α 2 loop. The radius of the spline function through the C $^{\alpha}$ atoms is proportional to the mean global backbone displacement per residue among the bundle of 20 energy-minimized conformers in A. (D) $^{13}\text{C}^{\alpha}$ chemical shift deviations from the random coil values, $\Delta^{13}\text{C}^{\alpha}$, for the residues 165–175, where blue color highlights a pattern associated with type I β -hairpin structures (28).

3_{10} -helical turns (Fig. 3A–C). The β 2– α 2 loop forms a type I β -turn, which is directly manifested by the $^{13}\text{C}^{\alpha}$ chemical shifts (Fig. 3D) and thus, is different from mPrP[Y225A,Y226A](121–231), where the loop forms a 3_{10} -helical turn involving the residues 166–168 (25).

Common Features of the β 2– α 2 Loop in All Cellular Prion Proteins. A detailed analysis of correlations between exchange line broadening and chemical shift variation, based on the sequence-specific NMR assignments (28), showed that the line width dependence on the temperature and the magnetic field of the β 2– α 2 loop NMR signals in the presently investigated RL-PrP^C can be rationalized by exchange between a major structure containing a 3_{10} -helical turn, represented by mPrP[Y225A,Y226A](121–231) (25), and a less populated structure containing a type I β -turn, represented by mPrP[Y169A,Y225A,Y226A](121–231) (Fig. 4). The slow rate and the concomitant high-energy barrier for the interconversion show that this conformational exchange must involve a major structural rearrangement, including also the polypeptide backbone (48). This indication from the rate of the conformational interconversion is confirmed by the determination of the two limiting loop structures.

Despite the different temperatures at which the transition from an incomplete to a complete set of β 2– α 2 loop signals is observed (Fig. 2), comparison of RL-PrP^Cs with prion proteins showing WT mPrP behavior leads to a unified description of the cellular prion protein structures of all mammalian species studied up to now, with the following common features. (i) In all PrP^Cs, the NMR signals of the same residues are affected to the same extent by variation of the temperature and the NMR frequency, indicating that the two limiting structures linked by the exchange are closely similar or identical in all of the different proteins of both classes. (ii) The observed polymorphism

Fig. 4. Correlation of amide ^1H and ^{15}N chemical shift differences between corresponding 750 MHz 2D [^{15}N , ^1H] correlation NMR signals in mPrP[Y225A, Y226A](121–231) and mPrP[Y169A, Y225A, Y226A](121–231) and exchange broadening of the associated NMR signals of mPrP[Y225A, Y226A](121–231). The data were collected at 20 °C and pH 4.5. (A) Histogram-type plot vs. the amino acid sequence of the square of the amide proton chemical shift differences in Hz, $\Delta\nu_{1/2}^2(^1\text{H})$, between the two proteins. (B) Plot vs. the sequence of the line widths at half-height along $\omega_2(^1\text{H})$, $\Delta\nu_{1/2}^2(^1\text{H})$ in mPrP[Y225A, Y226A](121–231). (C) Same as B for mPrP[Y169A, Y225A, Y226A](121–231). (D–F) Same presentation as in A–C of data measured along the $\omega_1(^{15}\text{N})$ frequency axis. (G) Quantitative assessment [using the relation $\Delta\nu_{1/2} = 2\pi p_A p_B (\Delta\nu_{AB})^2 k^{-1}$, which describes the dependence of the line broadening $\Delta\nu_{1/2}$ because of exchange between two species A and B on the populations p_A and p_B , the chemical shift difference $\Delta\nu_{AB}$, and the exchange rate k (28)] of the hypothesis that the NMR line broadening for loop residues in mPrP [Y225A, Y226A](121–231) is because of exchange between 3_{10} -helix and type I β -turn conformations of the $\beta 2$ – $\alpha 2$ loop. The slope of the plot representing $k/(2\pi p_A p_B)$ is equal to 23,400 s^{−1}. The Pearson correlation coefficient is 0.86.

is strictly localized to the $\beta 2$ – $\alpha 2$ loop surface area of the protein. (iii) The broadening of the $\beta 2$ – $\alpha 2$ loop NMR signals at low temperatures is caused by conformational exchange between two PrP structures with different loop conformations. (iv) The more highly populated structure forms a 3_{10} -helical turn in the $\beta 2$ – $\alpha 2$ loop, and the minor species forms a type I β -turn. In conclusion, the conservation of Y169 in all mammalian prion proteins (16, 28) preserves a conformational equilibrium between two PrP^C structures containing a 3_{10} -helical turn or type I β -turn conformation of the $\beta 2$ – $\alpha 2$ loop. The importance of the tyrosyl hydroxyl group in this sequence position is emphasized by the observation that this structural polymorphism is preserved after replacement of Y169 with phenylalanine (28), whereas F169 is not found in mammalian PrPs.

PrP^Cs with a structurally well-defined $\beta 2$ – $\alpha 2$ loop in solution structures at 20 °C are often referred to as RL-PrP^Cs. The data presented in this paper now show that the high structural definition of the loop in RL-PrP^Cs results from somewhat faster conformational exchange between two limiting structures than in PrP^Cs with disordered loops under the same solution conditions. In all PrP^Cs, the $\beta 2$ – $\alpha 2$ loop is rigid on the nanosecond time-scale, which was documented by $^{15}\text{N}\{^1\text{H}\}$ -NOE measurements

with the RL variant mPrP[Y225A, Y226A](121–231) (25) and mPrP(121–231) (Fig. S1).

Biological Implications of the $\beta 2$ – $\alpha 2$ Loop Structural Polymorphism in Cellular Prion Proteins. The $\beta 2$ – $\alpha 2$ loop is part of a surface epitope that has attracted keen interest with regard to its potential role in the onset of TSEs. (i) Specific amino acid types in positions 167 and 170 have been related to high susceptibility for interspecies transmission to bank voles (49, 50) and spontaneous TSE in transgenic mice expressing designed variants of mPrP (11, 13, 14). (ii) The residue position 168 is polymorphic in sheep PrP, and the residue type in this position affects susceptibility of sheep to scrapie (51–54). (iii) For TSE-sensitive mutants of PrP^C, molecular dynamics simulations indicate a trend for solvent exposure of Y169 (29–33), leading to increased hydrophobicity of the loop surface, which has been related to increased cytotoxicity of prion proteins (55, 56). The conformations with exposed Y169 that are observed in the molecular dynamics simulations are similar to the type I β -turn loop conformation (Fig. 5). (iv) There is also the controversial Protein X hypothesis, which suggests that a surface epitope consisting of residues 168, 172, 215, and 219 is the recognition site for a not further characterized Protein X that would modulate the transition of PrP^C to alternative conformations related to the aggregated scrapie

Fig. 5. Illustration of different surface epitopes formed by residues 164–175 in the major and minor conformations of three PrP^Cs. A and A' represent the two limiting structures connected by intermediate rate conformational exchange in the designed rigid loop mPrP[Y225A, Y226A](121–231). (B and B') Limiting conformations formed by the $\beta 2$ – $\alpha 2$ loop residues in WT rigid loop bank vole PrP^C. (C and C') Limiting conformations in sheep PrP[Q168R]. In all panels, the polypeptide backbone from the end of strand- $\beta 2$ at residue 163 to the fourth turn of helix $\alpha 2$ is shown, with space-filling presentations of the side chains 164 and 171 in blue, 168–170 in functional colors, and 175 in green. A–C represent the respective structures (24, 25, 73). A'–C' have been modeled by inserting the respective amino acid replacements into the structure of mPrP[Y169A, Y225A, Y226A](121–231), which in all three examples, includes that the Ala at position 169 was replaced by Tyr with the rotameric state $\chi_1 = -74^\circ$ to minimize steric clashes.

form of the prion protein (57, 58). In addition, the strict conservation of both Y169 and the dynamic conformational polymorphism of the β 2– α 2 loop in mammals indicate that these structural features must be linked to the so far elusive physiological role of PrP^C in healthy organisms. In view of the implicated key roles of PrP^C in health and disease, a detailed characterization is needed for both β 2– α 2 loop conformers. Although the major species with the 3_{10} -helical loop structure is well-known (17–27), the corresponding minor species with the WT sequence are not accessible for detailed studies, but essential information can be inferred from the variant proteins with Y169 replaced by alanine or glycine.

In both mPrP and mPrP[Y225A,Y226A], replacement of Y169 by alanine or glycine results in a reduction of the transition temperature for thermal unfolding by 10 °C (Fig. S2 A–D). Reduced stability may contribute to increased propensity for transition to PrP^{Sc}-related structures of conformers with the β 2– α 2 loop in a type I β -turn conformation (41).

The two different backbone conformations of the β 2– α 2 loop generate surface epitopes with different solvent exposure of individual amino acid side chains. In Fig. 5, we present the two loop conformations in three representative PrPs. Quite generally, these examples illustrate that the high frequency of amino acid substitutions in the loop (16, 59) ensures that there are discrete species differences of the surface epitope in both conformations. This observation may provide a rationale, for example, for a species barrier in TSE-related processes as well as selectivity of possible intermolecular signaling processes involving PrP^C in healthy organisms (9). More detailed information resulting from inspection of Fig. 5 includes that the lesser-populated conformation exposes a larger part of the hydrophobic surface of the Y169 side chain to the solvent, which is a feature that has been correlated with increased neurotoxicity (55, 56). In the sheep PrP^C, it is interesting that R168 interacts closely with R164 in the 3_{10} -helical loop conformation, whereas it is fully solvent exposed in the β -turn conformation. The apparent steric crowding of the two positively charged side chains might promote increased population of the type I β -turn loop conformation in the sheep protein containing Arg at position 168, consistent with molecular dynamics simulations that show that the loop containing R168 takes on more extended conformations (33).

The PrP^C structures provide a basis for rational design of transgenic mice for in vivo studies of the effects of specific molecular properties of the cellular prion protein. (i) Replacement of Y169 in mPrP with phenylalanine preserves the β 2– α 2 loop polymorphism (28), and there is only a small reduction of the thermal stability by 2.6 °C (Fig. S2E). This designed variant enables studies of the impact of the hydroxyl group of Y169. (ii) Replacement of F175 in mPrP with alanine preserves the loop polymorphism, albeit with RL-PrP^C behavior, and the thermal stability is reduced by 10 °C (38). Because this protein contains the WT mPrP sequence of the β 2– α 2 loop, it enables studies of the effect of eliminating the hydrophobic stacking interactions between Y169 and F175 in mPrP, which may allow Y169 to more readily take on solvent-exposed conformations in the mPrP [F175A] variant. (iii) Replacement of Y169 in mPrP with alanine

results in high population of the type I β -turn loop conformation; there is no evidence of a dynamic polymorphism (28), and the thermal stability is lowered by about 10 °C (Fig. S2 A–D). This variant protein will provide information on the combined effects of the absence of the 3_{10} -helix conformation of the β 2– α 2 loop and the reduced thermal stability. Overall, the systematic studies of a wide variety of PrP^C structures (17–28) that are further expanded in this paper provide a wealth of insights into the structural consequences of amino acid sequence variations, which can be further correlated with in vivo and in vitro experiments probing their effects on the physiological role of PrP in health and disease.

Materials and Methods

Protein Expression and Purification. Using previously described clones (60), the uniformly ¹⁵N- or ¹³C,¹⁵N-labeled proteins analyzed in this study were prepared as described (19, 61, 62). For the NMR experiments, solutions containing 1–2 mM protein in H₂O were prepared with addition of 10 mM [d₄]-sodium acetate buffer at pH 4.5, 10% (vol/vol) D₂O, 0.02% sodium azide, and a protease inhibitor mixture (Roche).

NMR Experiments. The NMR measurements for the structure determination of mPrP[Y169A,Y225A,Y226A](121–231) were performed at 20 °C. The chemical shifts were calibrated with a coaxial insert (Norell Inc.) containing 2,2-dimethyl-2-silapentane-5-sulfonic acid in NMR buffer, because this protein precipitates on addition of 2,2-dimethyl-2-silapentane-5-sulfonic acid. An HNCA triple-resonance experiment with the uniformly ¹³C,¹⁵N-labeled protein was recorded on a Bruker DRX500 spectrometer equipped with a triply tuneable cryogenic probe head. A 3D ¹⁵N-resolved [¹H,¹H]-NOESY spectrum and two 3D ¹³C-resolved [¹H,¹H]-NOESY spectra with the ¹³C carrier frequencies in the aliphatic and aromatic regions, respectively, were recorded with a mixing time of 60 ms on a Bruker Avance900 spectrometer.

The temperature dependence of 2D [¹⁵N,¹H]-HSQC spectra was measured at 750 MHz with 1 mM solutions of the uniformly ¹⁵N-labeled proteins. The temperature was calibrated with a standard Bruker 4% MeOH/MeOD sample. Before Fourier transformation, the 2D [¹⁵N,¹H]-HSQC datasets were zero-filled to 16k and 1k points in the ¹H and ¹⁵N dimensions, respectively. ¹H^N and ¹⁵N chemical shift assignments made at the standard conditions of T = 20 °C and pH 4.5 (28) were extended to the different temperatures by interactively following the temperature dependence of the individual signals using the program CARRA (63) (www.nmr.ch). ¹⁵N(¹H)-NOE data for WT mPrP(121–231) at 37 °C were recorded at 500 MHz, with recovery and proton saturation periods of 2.0 and 3.0 s, respectively, and the reference experiment was measured with a 5.0-s recovery period (64, 65).

NMR Structure Calculation. The standard protocol of the stand-alone ATNOS/CANDID program package (46, 47), version 1.2, was used for automatic peak picking, automatic NOE assignment, and preparation of the input for structure calculations with DYANA (45). The final cycle of the calculation was started with 80 randomized conformers. The 20 conformers with the lowest residual target function values were energy-minimized in a water shell with the program OPALp (66, 67) using the AMBER force field (68). The program MOLMOL (69) was used to analyze the results of the protein structure calculations, including regular secondary structure identification with the method of Kabsch and Sander (70), and prepare the drawings of the structures.

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