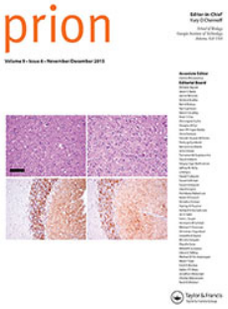

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The roles of the conserved tyrosine in the β2-α2 loop of the prion protein

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ABSTRACT. Prions cause neurodegenerative diseases for which no cure exists. Despite decades of research activities the function of the prion protein (PrP) in mammalians is not known. Moreover, little is known on the molecular mechanisms of the self-assembly of the PrP from its monomeric state (cellular PrP, PrP^{C}) to the multimeric state. The latter state includes the toxic species (scrapie PrP, PrP^{Sc}) knowledge of which would facilitate the development of drugs against prion diseases. Here we analyze the role of a tyrosine residue (Y169) which is strictly conserved in mammalian PrPs. Nuclear magnetic resonance (NMR) spectroscopy studies of many mammalian PrP^{C} proteins have provided evidence of a conformational equilibrium between a 3_{10}-helical turn and a type I β turn conformation in the β2-α2 loop (residues 165–175). In vitro cell-free experiments of the seeded conversion of PrP^{C} indicate that non-aromatic residues at position 169 reduce the formation of proteinase K-resistant PrP. Recent molecular dynamics (MD) simulations of monomeric PrP and several single-point mutants show that Y169 stabilizes the 3_{10}-helical turn conformation more than single-point mutants at position 169 or residues in contact with it. In the 3_{10}-helical turn conformation the hydrophobic and aggregation-prone segment 169-YSNQNNF-175 is buried and thus not-available for self-assembly. From the combined analysis of simulation and experimental results it emerges that Y169 is an aggregation gatekeeper with a twofold role. Mutations related to 3 human prion diseases are interpreted on the basis of the gatekeeper role in the monomeric state. Another potential role of the Y169 side chain is the stabilization of the ordered aggregates, i.e., reduction of frangibility of filamentous protofibrils and fibrils, which is likely to reduce the generation of toxic species.

KEYWORDS. molecular dynamics, free energy barrier, prion protein stability, prion diseases, fibril frangibility

THE STRICTLY CONSERVED Y169 STABILIZES THE BURIED CONFORMATION OF THE 169-YSNQNNF-175 SEGMENT

Nuclear magnetic resonance (NMR) spectroscopy studies on mouse PrP^{C}[1] and a large number of vertebrate PrP^{C}[2] have shown that PrP^{C} consists of a flexible N-terminal segment and a well-structured C-terminal domain (residues 124–230 in mouse PrP). In the latter domain a 2-strand antiparallel β sheet is packed against three α helices. The loop connecting the β strand 2 to the α helix 2 (called β2-α2, i.e., residues 165–175) has been

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the subject of multiple NMR investigations. According to the NMR studies of mouse PrP, the β2-α2 loop contains a 3_{10}-helical turn (residues 165-PVDQ-168) at 37°C, presents local conformational polymorphism at 20°C, and assumes a type I β turn conformation (at residues 167-DQYS-170) in the single-point mutants Y169G and Y169A of the strictly conserved Y169.[1–4] On the basis of the NMR spectroscopy data and in vivo results, a mainly physiological role of Y169 was hypothesized.[2]

Recently, we have carried out molecular dynamics (MD) simulations of the globular domain of mouse PrP to analyze the flexibility of the β2-α2 loop with particular emphasis on the strictly conserved Y169 which is located in the central segment of the loop.[5] Two independent simulation protocols were used for calculating the free energy profile of the transition from the 3_{10}-helical turn to the type I β turn conformation. Both protocols show that the free energy barrier of this transition is higher by about 2.5 kcal/mol for the wild type than the Y169G mutant. Furthermore, the wild type Y169 has a higher free energy barrier than the Y169A, Y169F, R164A, F175A, and D178A single-point mutants (**Fig. 1**). The MD simulation results show that the 3_{10}-helical turn conformation is significantly more stable than the type I β turn conformation only for the wild type, which is a consequence of the tertiary contacts of the Y169 side chain in the 3_{10}-helical turn conformation.[5,6] These contacts are a favorable stacking of the Y169 aromatic ring with the phenyl ring of F175, and a hydrogen bond of its hydroxyl group with the side chain of D178, which in turn is involved in a favorable ionic interaction with the side chain of R164 (**Fig. 2, left**). Analysis of the MD trajectories show that the transition of the β2-α2 loop to type I β turn increases the solvent-exposure of the hydrophobic stretch 169-YSNQNNF-175 (see fig. 7 of ref. 5). It is important to note that this hydrophobic segment has high sequence similarity to the fibril-forming GNNQQNY heptapeptide from the yeast prion protein Sup35.[7] Thus, the MD simulations indicate that the wild type Y169 side chain acts as an aggregation gatekeeper by stabilization of the buried conformation of the hydrophobic stretch 169-YSNQNNF-175.

In the following sections, in vitro and in vivo data are interpreted, whenever possible, on the basis of the MD simulation analysis of Y169 and the residues that interact with it in PrP^C.

IN VITRO DATA: WHY DOES THE WILD TYPE PRP CONVERT MORE EFFICIENTLY THAN NON-AROMATIC MUTANTS AT RESIDUE 169?

The in vitro conversion assay is a cell-free protocol that starts with the incubation of PrP^C from cell lysate (the substrate) with prion-infected brain homogenates (the seeds), and is followed by multiple cycles of sonication.[8,9] It is important to note that the in vitro conversation assay unnaturally amplifies the formation of seeds as it involves nearly 150 cycles of sonication during 24 hours (where each cycle consist of 10 minutes of incubation followed by 5 seconds of sonication[8]). Thus, the influence of mutations on the frangibility of non-toxic (proto)fibrillar aggregates is masked, at least in part, by the multiple sonication events. With this assay it was shown recently that non-aromatic residues reduce the amount of proteinase K-resistant PrP (detected by Western blotting) by about 75% whereas the Y169F and Y169W mutants behave like wild type.[10] These in vitro data have been interpreted on the basis of the steric zipper model[11] which suggests that only aromatic residues at position 169 are compatible with the ordered (proto)fibrillar state. It is not possible to directly compare the fibril-based interpretation of these experimental data with the molecular dynamics simulations because the simulation results report only on the monomeric state of PrP^C. Concerning the latter state, the molecular dynamics simulations provide strong evidence that replacement of Y169, even by the conserved mutation Y169F, results in an increased exposure of the hydrophobic stretch 169–175. It is likely that the higher accessibility of this segment in the monomeric and

FIGURE 1. MD simulation results.[5] (Top) Profile of the free energy (potential of mean force, PMF) along an interatomic distance that reports on the conformational transition of the β2-α2 loop. The distance is measured between the Cα atoms of residues 165 and 168. The dashed vertical line at a distance value of 0.82 nm discriminates between the 2 conformations of the loop. (Bottom) Representative snapshots of the 2 conformations of the β2-α2 loop. Note that the 3_{10}-helical turn (left) consists of residues 165-PVDQ-168 while the type I β turn (right) is formed by residues 167-DQYS-170. The distance used for the PMF profile is shown by a dashed line. These two panels are modified and reprinted with permission of the ACS.

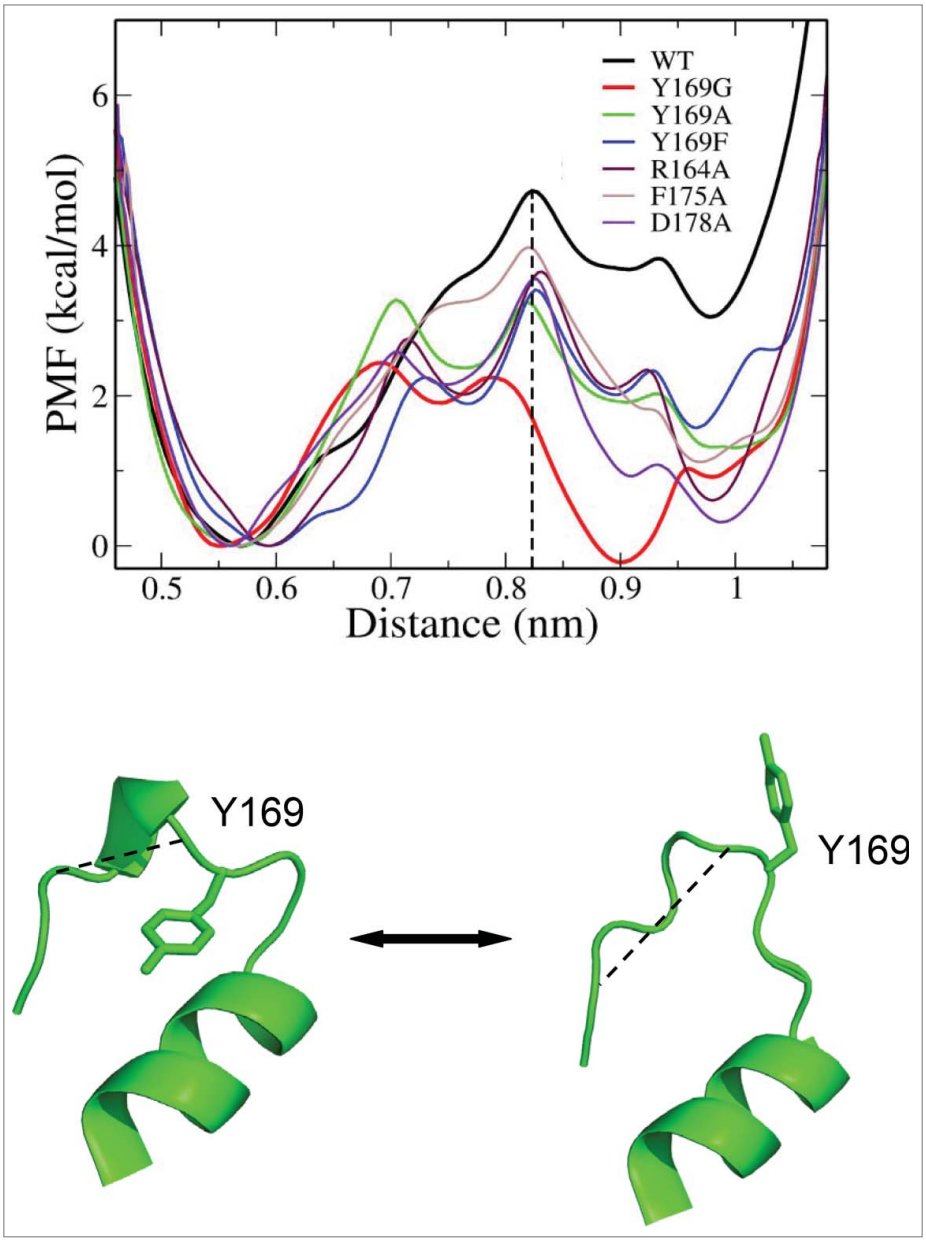
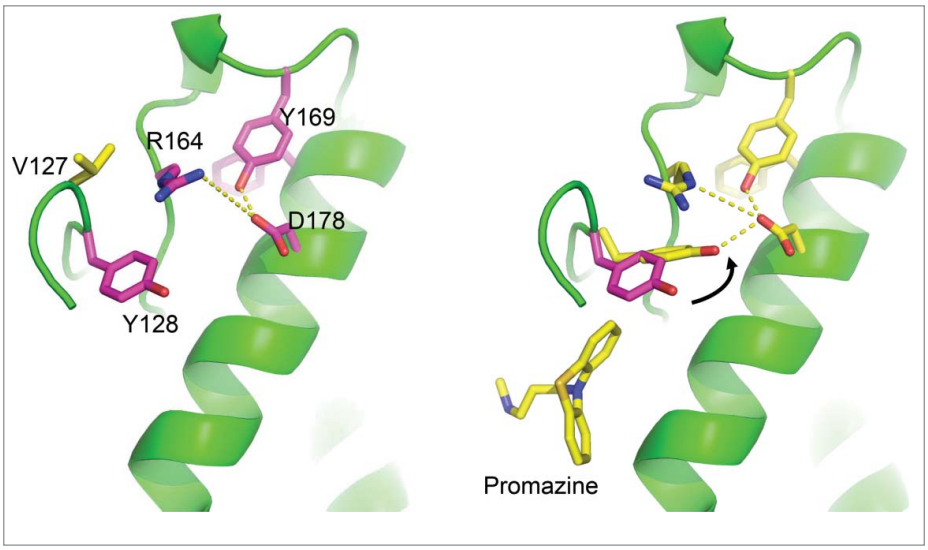


FIGURE 2. Interactions of Y169 in the 3_{10}-helical turn conformation of the β2-α2 loop. (Left) The X-ray structure of mouse PrP (PDB code 4H88) is shown by a ribbon model. The residues mentioned in the text are labeled and hydrogen bonds are shown by dashed lines. The orientation of the V127 side chain (yellow) for the G127V mutant was obtained by manual modeling. Note that the side chain of F175 is located below the phenol ring of Y169, and it is not labeled to avoid overcrowding. (Right) The X-ray structure of the complex with promazine (yellow) shows the displacement of the Y128 side chain (black arrow) which results in an additional hydrogen bond between the side chains of Y128 and D178 as well as cation-π interactions with the positively charged side chain of R164 (PDB code 4MA7).



oligomeric states renders single-point mutants at position 169 more sensitive (i.e., less resistant) to proteinase K digestion than the wild type Y169. Importantly, upon density-gradient centrifugation, proteinase K-sensitive species of $PrPS^{Sc}$ are present in lower density fractions which indicates that they consist of oligomeric aggregates of PrP molecules.[12-14]

KURU: WHY DOES THE G127V MUTANT PREVENT PRION DISEASE?

Mortuary cannibalism, practiced by the Fore population in Papua New Guinea until the late 1950s, induced an epidemic prion disease (neurodegenerative disorder) called Kuru.[15] The PrP variant G127V, identified among unaffected members of the Fore population, was interpreted as a resistance factor due to positive evolutionary selection.[15] A recent in vivo study has demonstrated that the V127 variant prevents prion conversion and shows a dose-dependent inhibition of wild type PrP propagation.[16] Remarkably, the single-point mutant G127V is as protective against prion disease as deletion of PrP. **Figure 2, left**, shows that the putative orientation of the V127 side chain restricts the flexibility of R164. Since the guanidinium group of R164 is involved in favorable electrostatic interactions with the D178 side chain, the reduced orientational disorder of R164 is likely to increase the stability of the hydrogen bond between the side chains of D178 and Y169. Thus, the in vivo data on the human variant V127, which is intrinsically resistant to prion conversion, provide further evidence of the importance of the structural stability of the 3_{10}-helical turn in the β2-α2 loop.

DRUGS: WHY DO TRICYCLIC PHENOTIAZINES INHIBIT PRION CONVERSION?

As of today, there are no pharmacological approaches to prevent or hinder the degenerative

neurological disorders related to PrP^{Sc}. Several small molecules as well as antibodies have been shown to have potential as antiprion compounds but none of them has been successful in clinical trials. Structural studies of the binding to PrP^{C} of 2 drugs used in the clinics for other diseases, promazine and chlorpromazine, have shown that these phenotiazine derivatives occupy a hydrophobic pocket proximal to the strand β2 and the helix α2.[17] **Figure 2, right**, shows that this pocket is adjacent to the residues that interact directly with the Y169 side chain. It is important to note that in the complex with promazine (PDB code 4MA7,[17]) the side chain of Y128 is displaced with respect to the X-ray structure in the complex with the POM1 antibody (PDB code 4H88[18]). The orientation of Y128 in the promazine-bound structure results in favorable cation-π interactions with the guanidinium of R164 and an additional hydrogen bond between the Y128 hydroxyl group and the D178 carboxyl. These favorable interactions provide further stabilization to the network of polar interactions involving Y128, R164, D178 and Y169.[17] Thus, the biophysical analysis of the binding of phenotiazine compounds confirms the importance of the structural stability of the β2-α2 loop, and the central role of the Y169 side chain.

WHY IS D178N LINKED TO HUMAN PRION DISEASES?

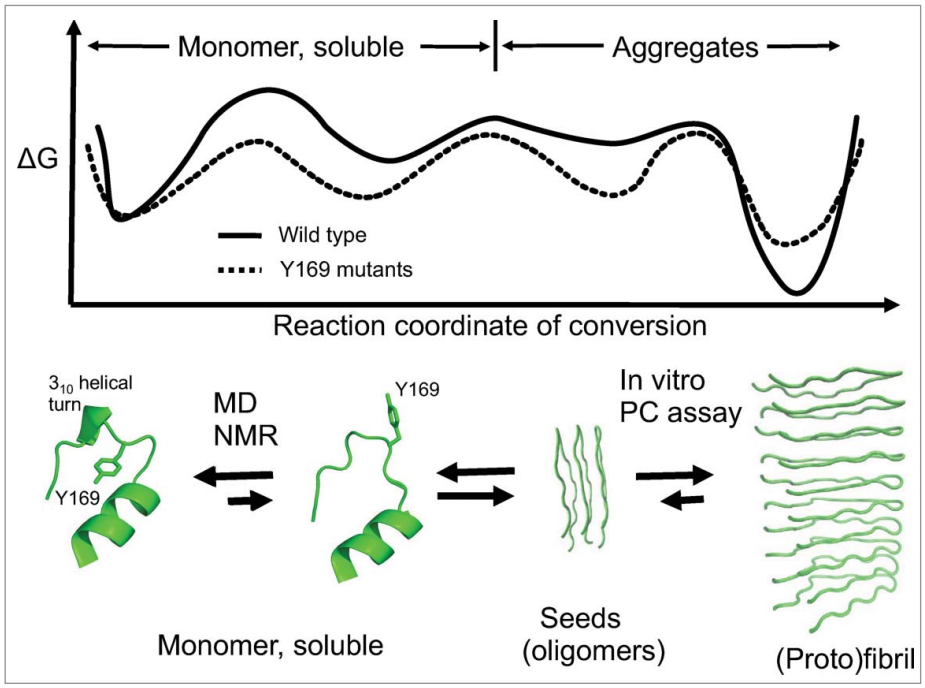
The single-point mutation D178N in PrP has been associated to fatal familial insomnia (FFI) and a subtype of familial Creutzfeldt-Jakob disease (CJD) depending on the DNA polymorphism at codon 129.[19] The NMR data[20] and our MD simulations[5,6] suggest that the side chain of D178 stabilizes the β2-α2 loop (**Fig. 1**) by the hydrogen bond with the Y169 hydroxyl (**Fig. 2**). Thus, it is likely that the D178N mutation weakens the polar interaction with Y169. Moreover, asparagine is more aggregation prone than aspartate[21] so that the D178N mutant is expected to have a lower barrier to the formation of toxic oligomers. While the reduced structural stability of PrP^{C} is, at least in part, a consequence of the D178N mutation, the different phenotypes observed for M129 and V129, i.e., FFI and CJD, respectively, are probably due to differences in the self-assembled species that are most toxic.

CONCLUSIONS

Our MD simulations report only on the monomeric, soluble state of PrP. On the other hand, MD generates a picture of the intrinsic plasticity of the prion protein in the 1-microsecond time scale with femtosecond resolution. Moreover, as a major advantage with respect to experimental methods, the MD simulations provide atomistic detail, i.e., ideal spatial resolution. The main simulation result is that residue Y169, which is strictly conserved in mammalian prion proteins, stabilizes the 3_{10}-helical turn conformation of the β2-α2 loop more than the single-point mutants Y169G, Y169A, Y169F, R164A, F175A, and D178A.[5]

Taken together, the MD simulations results and available experimental data (in vitro and in vivo) suggest that the strictly conserved Y169 is an aggregation gatekeeper with a twofold role (**Fig. 3**). In the monomeric state (left part of **Fig. 3**), the Y169 side chain stabilizes a network of favorable interactions which shifts the equilibrium of the β2-α2 loop toward the 3_{10}-helical conformation with respect to the single-point mutants Y169F, Y169A, and Y169G, where the latter mutation is most destabilizing. Stabilization of the 3_{10}-helical conformation, and the associated burial of the 169-YSNQNNF-175 segment, might not be the only beneficial role of the strictly conserved Y169. The steric zipper model, used to interpret the results of the in vitro prion conversion assay, indicates that aromatic residues at position 169 provide structural stability to the ordered aggregates.[10] Thus, the accumulating evidence on the reduced infectivity of stable (proto) fibrils[22,23] suggests that Y169 stabilizes nontoxic species. In other words, non-toxic filamentous protofibrils and/or fibrils of wild type PrP are likely to be less frangible and thus generate less propagons (i.e., seeds) than mutants of Y169 (right part of **Fig. 3**). In this context, it has to be noted that in Alzheimer's

FIGURE 3. The dual role of Y169. The schematic picture of the free energy profile of the self-assembly of PrP illustrates the stabilization effects of Y169 (solid line) compared to its mutants (dashed line). The free-energy barrier to exit the 3_{10}-helical turn conformation in the monomeric state (first barrier on the left) and the barrier from the (proto)fibril state to the seeds (right) are higher for the wild type than the Y169 mutants. The height of these free-energy barriers reflect the structural stability of the 3_{10}-helical turn conformation and reduction of (proto)fibril frangibility, respectively. The transition within the monomeric state (left) has been analyzed by NMR spectroscopy[2-4] and MD simulations,[5] while the in vitro prion conversion (PC) assay[10] reports on the formation of proteinase K-resistant PrP upon multiple cycles of sonication of (proto)fibrils (right). The cartoons of monomeric structures of the β2-α2 loop and multimeric assemblies represent schematically the minima of the free-energy profile and are shown to help with the interpretation of the reaction coordinate of conversion which in reality is much more complex than a single one-dimensional variable.



disease oligomeric assemblies are more infectious than large amyloid plaques.[24] Moreover, the amount and density of plaques does not show a significant correlation with the severity of the symptoms of dementia.[25]

In conclusion, stabilization of the 3_{10}-helical turn conformation in the monomeric state and hindering frangibility of the (proto)fibrillar state are 2 possible reasons of the conservation of Y169 in mammalian PrPs. Other possible roles of the Y169 side chain might be the direct inhibition of nucleation of toxic oligomeric states and/or an essential part in the physiological function(s) of the prion protein.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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