Disruption of the X-loop turn of the prion protein linked to scrapie resistance

Alexander D. Scouras1,2 and Valerie Daggett1,2,3,4

1Department of Biochemistry, University of Washington, Seattle, WA 98195-5013, USA, 2Computational Molecular Biology Program, University of Washington, Seattle, WA 98195-5013, USA and 3Department of Bioengineering, University of Washington, Seattle, WA 98195-5013, USA

To whom correspondence should be addressed.
E-mail: daggett@uw.edu

Received January 25, 2012; revised January 25, 2012; accepted January 30, 2012

Edited by Alan Fersht, Senior Editor, PEDS

The prion diseases are a class of neurodegenerative diseases caused by the misfolding and aggregation of the prion protein (PrP) into toxic and infectious oligomers (PrPSc). These oligomers are critical to understanding and combating these diseases. Differences in the sequence of PrP affect disease susceptibility, likely by shifting the tolerance of the protein for adaptation to PrPSc conformations and/or the recognition event between PrPSc and PrP prior to conversion of the PrPSc. We selected two sets of PrPSc-resistant mutant sequences for solvated atomistic molecular dynamics simulation to investigate the structural basis of resistance. The first group involved mutation in the X-loop (residues 164-171) resulting from selective breeding of sheep. The second group included eight mutants in mice identified by random mutagenesis targeting helix C followed by screening in cell cultures. Multiple simulations were performed of 14 different mutant and control constructs under different pH conditions for a total of 3.6 μs of simulation time. The X-loop formed a stable turn at neutral pH in wild-type PrP from both species. PrPSc-resistant mutations disrupted this turn even though only one of the mutants is in the X-loop. The X-loop is compact and buried in our previously described spiral models of PrPSc-like oligomers. On the basis of the findings presented here and in the context of the spiral oligomer model, we propose that expansion of the X-loop disrupts protofibril packing, providing a structural basis for resistance.

Keywords: prion disease/resistance to disease/species barrier/X-loop

Introduction

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are fatal neurodegenerative diseases causing ‘spongy’ holes in the brain correlated with the deposition of abnormally folded forms of the prion protein (PrP) (Aguzzi and Calella, 2009). These diseases are due to the misfolding and aggregation of a protein into a toxic and infectious oligomer (Silveira et al., 2005). In some cases PrP forms amyloid fibers and plaques like those seen in other amyloid diseases (Caughey and Lansbury, 2003). PrP is expressed primarily in neural and lymphoid tissues of vertebrates, and over 30 species of mammals are known to be susceptible to TSE infection. The infectious ‘scrapie’ particles (PrPSc) have the same primary sequence as the normal benign, endogenous form of PrP (PrPC), but PrPSc is an oligomer enriched in β-sheet structure. Therefore, transmission and susceptibility to disease are dependent on the structure of PrPSc and its interactions with PrPC, as well as the stability of PrPC. Several strains of prion diseases have been identified, differing in pathology, cross-species infectivity and structure, indicating that multiple distinct and stable aggregate conformations exist to propagate the independent strains (Wadsworth and Collinge, 2011).

The amino acid sequence of PrP can influence susceptibility to TSEs, particularly when the sequences of PrPSc and PrPC are different, such as in cross-species infection, which can give rise to a species barrier. It is believed that sequence variations can lead to different conformations and/or aggregation surfaces, which in turn may restrict the ability of a sequence to adopt a disease-causing structure or to participate in templated conversion of PrPSc by PrPSc (Aguzzi and Calella, 2009). Broadly, this study probes the relationships between sequence, structure and disease. We approached these issues by selecting disease-resistant PrP sequences and performing molecular dynamics (MD) simulations of PrPC at a range of pH values, as low pH induces conversion to PrPSc in vitro (Swietnicki et al., 1997; Gerber et al., 2008) and in vivo (Arnold et al., 1995; Godsave et al., 2008) although some conversion appears to occur at neutral pH due to interactions with infectious particles and in the cytoplasm for some variants (Laszlo et al., 1992; Aguzzi and Calella, 2009). Sequence-linked differences in the behavior of PrPC may highlight structural regions critical for binding to PrPSc and propagation of the scrapie isoform, as well as the basis of resistance.

There are several natural and artificial mutations in mouse and sheep that confer resistance to PrPSc infection. The earliest-recorded prion disease was sheep scrapie in the 1800s (Caughey and Lansbury, 2003; Sigurdson and Aguzzi, 2007). Selective breeding has generated over 55 scrapie-resistant mutations (resistant to classical scrapie strains). Three of the best-characterized alleles are known as ARQ (wild type), ARH and ARR (in order of increasing resistance), with each letter reflecting the sequence at residues 133, 151 and 168, respectively (i.e. ARQ is the wild-type allele Ala133/Arg151/Gln168, human numbering, Fig. 1). These three proteins were chosen for study here because of their similarity in sequence (variation only at residue 168) as well as availability of NMR structures for the ARH and ARR mutants (Laszlo et al., 1992) for our simulations (ARQ was generated from the ARH structure). With respect to mutations conferring resistance in mice, Ott et al. (2008) generated a mouse PrP mutation library via error-prone
The X-loop is in a packing interface between monomers in the protofibril models. Disruption of the X-loop would prevent the tight packing and discourage aggregation, providing a possible molecular explanation for scrapie resistance in these mutants.

**Methods**

Starting structures for simulation were prepared starting from the NMR structures 1XYX (Gossert et al., 2005) (mouse), 1XYW (Gossert et al., 2005) (elk), 1XYU (Lysek et al., 2005) (sheep ARH allele) and 1Y2S (Lysek et al., 2005) (sheep ARR allele). Mouse PrP was simulated with residues 109–231 whereas the elk and sheep PrP simulations use the full PrPSc fragment (residues 90–231). PrP is unstructured up to residue 127 and the preceding residues are not included in these NMR structures, so we modelled the missing residues in each structure from a model provided by T.L. James and S. Farr-Jones that was used to generate the hamster PrP NMR structure 1B10 (James et al., 1997). The sheep ARQ variant was generated from the ARH structure because no comparable NMR structure was available. Mutations for each mouse mutant, the sheep ARQ variant, and deviations from the hamster sequence in residues 90–126 were performed and using the mutageneus tool in PyMOL (DeLano, 2002), which was also used to make the graphical protein figures.

All simulations were performed using our in-house simulation package in lucem molecular mechanics (ilmm) (Beck et al., 2000–2012, 2005; Beck and Daggett, 2004). Proteins were solvated to a depth of at least 10 Å with flexible waters (Levitt et al., 1997) in a periodic box. Simulations used the Levitt et al. (1995) force field using the microcanonical (NVE, constant number of particles, volume and total energy) ensemble with all atoms represented. Mouse PrP was simulated in triplicate at pH 7 and 3 for 20 ns and a temperature of 298 K. Elk and sheep PrP were simulated with five replicates at approximate pH values of 7, 5 and 3 for 50 ns and a temperature of 298 K. The elk and sheep simulations were longer to ensure that the longer unstructured N-terminus had equilibrated. Note that the pH is approximate; the pH was set by protonation of His, Asp and Glu residues as appropriate for each pH based on the standard pKₐ of the free amino acid. Consequently, ‘pH 7’ refers to His⁰, Glu⁻, Asp⁺; ‘pH 5’ refers to His⁺, Glu⁻, Asp⁺; and ‘pH 3’ refers to His⁺, Glu⁰, Asp⁰. Naturally, this approach to modelling the pH represents a pH range. In total 3570 ns, or 3.6 μs, of simulation time is presented here. Structures were saved every picosecond for analysis; the first 10 ns of each simulation were excluded to allow for equilibration.

**Results**

**Sheep mutants**

Polymorphisms at several residues in PrP confer resistance to scrapie in sheep (Sigurdson and Aguzzi, 2007; Aguzzi and Calella, 2009). The best characterized are the wild-type ARQ, ARH and ARR alleles, which show increasing resistance to scrapie from susceptible in ARQ, resistant in ARH and essentially immune in homozygous ARR/ARR animals.
The X-loop retained a stable, helical turn in several simulations (Fig. 2A and B). A stable X-loop was first observed in the elk PrP NMR structure and was thought to contribute to the unusual chronic wasting disease strain of PrPSc in that species (Sigurdson and Aguzzi, 2007). For this reason, we also performed elk simulations for comparison. The elk X-loop sequence is 164-RPVDQYNN-171, which is most similar to sheep ARQ, differing only at residue 170. In the NMR structures, the elk PrP X-loop had a Ca-RMSD of 1.2 Å to the sheep ARH allele and 0.9 Å to the ARR allele. The elk NMR structure had the main chain hydrogen bond Pro165–Gln168; a hydrogen bond between Pro165–Tyr169 also formed during the simulation. Formation of these interactions determines the conformation of the X-loop and together these two interactions define an ‘elk-like turn’ (Fig. 2A).

The structure of the protein changed little during the elk simulations (Fig. 3A). The elk simulations had over 55% occupancy of the elk-like turn at pH 7 and 5, increasing to 72% at pH 3 (Fig. 3B and C). Each hydrogen bond (<2.6 Å) was individually formed over 75% of the time and Pro165–Tyr169 had 94% occupancy at low pH. The scrapie susceptible wild-type ARQ sheep protein behaved similar to elk, as expected from their sequence similarity, with both turn-defining hydrogen bonds formed 52%, 51% and 69% of the time at pH 7, 5 and 3, respectively (Fig. 3B and C). In the resistant alleles at pH 7, ARH retained 53% occupancy but ARR decreased to 35%, and the turn was lost in both alleles at reduced pH.

Not only were the hydrogen bonds lost in the resistant sheep alleles, but the turn also became extended (Fig. 3D). In elk and sheep ARQ simulations, the distance between α-carbons of residues 165 and 168 stayed in the range of 5.5–6.2 Å, which is typical of helical segments and turns. The average distance was 6.0 Å at pH 7 in ARH but it jumped to 8.2 Å and then 9.2 Å at pH 5 and 3, respectively; values similar to extended structures and β-strands. ARR averaged 6.7, 8.7 and 7.9 Å at pH 7, 5 and 3, respectively. As the turn became more extended, the frequency of contacts between side chains across the turn dropped (Fig. 3E), which correlated with increased resistance (Fig. 3F).

The change of conformation appeared to result from differential interactions between residues Asp167 and Arg164 (Figs 2B and 4). At neutral pH, Gln and His at residue 168 stabilized the X-loop turn via contacts with Arg164. However, positively charged residues in position 168, such as Arg or His at reduced pH, had electrostatic repulsion with Arg164, and instead interacted with Asp167 and disrupted the turn (Fig. 4).

Mouse mutants

An experimental PrP library was generated by Ott et al. (2008) with 20 dominant-negative mutations conferring up to 75% resistance to PrP infection. We selected six of the most potent single mutants and two potent triple mutants for simulation (see Fig. 3G for the list of residues, Fig. 3F for RML susceptibility to PrP infection, and Fig. 1 for placement of mutations in the structure). Each mutant was simulated multiple times at pH 3 and 7, with the results at pH 7 displayed in Fig. 3.

In wild-type mouse simulations at neutral pH, a stable turn formed, although it was distinct from the elk-like turn (Fig. 2). This ‘mouse-like turn’ only formed the Pro165–Gln168 hydrogen bond (Figs 2A, 3H and I) because of a re-orientation of Tyr169 away from helix B (Fig. 5). The mouse single mutants, although largely targeting helix C, universally disrupted the X-loop turn (Fig. 3J–K). The only construct
Fig. 3. Properties of the X-loop for sheep/elk (left column) and mouse (right column) simulations. For sheep, the columns are ordered with elk as a reference, followed by the three alleles in order of increasing resistance. For mouse, the order is wild-type (WT) reference, S170N/N174T control, six mutants in increasing resistance, and the two cysteine mutants. The mouse data displayed are for the pH 7 simulations. (A, G) Co-RMSD are taken at 1-ns intervals between each simulation and a reference set with the best defined turn: either elk at pH 3 or WT mouse (excludes RMSD between time points within a simulation within the reference set). Error bars are ± SD n = 33620 or 42025 for sheep/elk, n = 726 or 1089 for mouse. (B, H) Main-chain hydrogen bond frequencies for the first bond in the elk and mouse turns (residues 165–168) and (C, I) the second hydrogen bond in the elk turn (residues 165–169). (D, J) Cα-distance is measured between the turn-defining residues 165 and 168. For reference, distances below 5.5 Å are common in α-helices and over 8.5 Å are common in β-sheets. (E, K) Side-chain contacts between Arg164 and the polymorphic residue Gln168 in sheep and mouse mutants. (F) The relative resistance to each PrP disease strain is indicated below each respective allele or mutant. The points displayed in many of the plots are average values for each simulation in the set.
Low pH disrupted the turn in all simulations (data not shown). The mediating contacts of the disruption at both low and neutral pH were changes in residues Tyr169, Gln172, Tyr218 and Tyr226. In wild-type mouse, Tyr169 packed against Gln172 and Tyr218 nearly 100% of the time. In the mutants, Tyr 169 shifted down helix C to contact Tyr226 with 30–80% occupancy, reducing the Tyr169–Tyr218 contact to ~70% and the Tyr169–Gln172 interactions dropped below 20%. Tyr218 then shifted to the main chain of Tyr169, increasing that contact from <10% to 20–80%. The net effect of having Tyr218 shift its interactions to the main chain was to disrupt the structure of the X-loop, making it more extended such that Tyr169 shifted down to the bottom of the HC helix. The structural changes are displayed for Q219K in Fig. 5.

Each mouse mutant (except for Y218C/Y226H/D227G) reached a similar structural endpoint (Fig. 3G), but by different mechanisms. As in sheep, Q168R shifted residue 168 away from Arg164 to Asp167, which opened up the turn. S222P caused a kink in helix C such that it rotated by one-quarter turn and bent closer to the X-loop. Q218R and Q219R directly strengthened the 172–219 contact while disrupting the Tyr169–Gln172 contact. Y226H was the most distant mutation from the X-loop and appeared to have a water-mediated effect on the X-loop. Y218C removed one of the core tyrosines discussed above, destabilizing Tyr169.

Discussion

Natural and experimentally derived mutations in PrP can confer resistance to sheep scrapie and the mouse-passaged scrapie strain RML/Chandler (Caughey and Lansbury, 2003). Here, we have performed MD simulations of 14 different mutants and control constructs to investigate their effect on PrPC structure and to gain insight into the molecular basis of resistance. Wild-type sheep and mouse sequences both formed a stable turn over residues 164–171 between the second β-strand and the second α-helix of the protein, which we call the X-loop. The turn formed at pH 7, 5 and 3 in wild-type elk and sheep but only at pH 7 in mouse. Resistant mutations disrupted this turn. Our results suggest that a compact X-loop is necessary for aggregation and formation of the infectious protofibrillar species in these strains.

The results presented here suggest that propagation and inhibition of PrPSc may occur in either of two stages. The resistant ARH and ARR sheep mutations and the seven single mouse mutations investigated here perturb the structure of the X-loop. These constructs may prevent the initial formation of the infectious oligomer and/or efficient packing onto the surface of a PrPSc oligomer. Of the eight mouse mutants tested, only the Y218C/Y226H/D227G triple mutant did not show turn disruption in our simulations. In fact, the turn formed two hydrogen bonds with the main chain carbonyl of Tyr169, extending and disrupting the X-loop. Consequently Tyr169 moved down to interact with Tyr226. In most mutants, Tyr169 moves down to contact Tyr226.

of the set that retained the turn was the triple mutant Y218C/Y226H/D227G, which behaved similar to wild type, although interestingly the single mutants Y218C or Y226H alone each disrupted the turn. We also substituted the elk X-loop sequence S170N/N174T into mouse PrP as a negative control against the possibility that any mutation would disrupt the turn. These residues have been shown both experimentally (Gossert et al., 2005) and in simulations by another group (Gorfe and Caflisch, 2007) to form a turn in the X-loop. In our simulations, this construct formed the turn and was similar, in behavior, to wild-type mouse PrP (Fig. 3G–J).

Discussion

Natural and experimentally derived mutations in PrP can confer resistance to sheep scrapie and the mouse-passaged scrapie strain RML/Chandler (Caughey and Lansbury, 2003). Here, we have performed MD simulations of 14 different mutants and control constructs to investigate their effect on PrPSc structure and to gain insight into the molecular basis of resistance. Wild-type sheep and mouse sequences both formed a stable turn over residues 164–171 between the second β-strand and the second α-helix of the protein, which we call the X-loop. The turn formed at pH 7, 5 and 3 in wild-type elk and sheep but only at pH 7 in mouse. Resistant mutations disrupted this turn. Our results suggest that a compact X-loop is necessary for aggregation and formation of the infectious protofibrillar species in these strains.

The results presented here suggest that propagation and inhibition of PrPSc may occur in either of two stages. The resistant ARH and ARR sheep mutations and the seven single mouse mutations investigated here perturb the structure of the X-loop. These constructs may prevent the initial formation of the infectious oligomer and/or efficient packing onto the surface of a PrPSc oligomer. Of the eight mouse mutants tested, only the Y218C/Y226H/D227G triple mutant did not show turn disruption in our simulations. In fact, the turn formed two hydrogen bonds with a stronger Arg164–Gln168 contact, as in elk (Fig. 3H–K). This is surprising because two of its substitutions, Y218C and Y226H, each broke the turn in isolation. The mechanism of action of this mutant may be different from the others. Ott et al. (2008) noted that the triple mutants had greatly reduced cell-surface expression of PrPSc, leading them to suspect alternative disulfide-bridge formation, as residue 218 is exactly one rung down on helix C from the native disulfide bridge (Ott et al., 2008).
PrPC NMR data are typically acquired near pH 5 for most susceptible to interspecies transmission (Cartoni, Aguzzi, 2007). A structured X-loop has now also been seen in bank voles (Christen, 2010), which are highly susceptible to horizontal transmission, such as grooming (Sigurdson and Tipler, 2008), yet prion diseases have never been observed in these species. There is speculation that the X-loop, and in particular residue 170, plays a critical role as a ‘molecular switch’-modulating prion transmission (Sigurdson et al., 2009, 2010), and our work and that of others (Gorfe and Callisch, 2007) shed light on how the sequences affect the structure of the loop.

To address how different conformations of the X-loop may factor into transmission at a more detailed level, we consider the loop in the context of an oligomeric model. In previous work (DeMarco and Daggett, 2004; Scouras and Daggett, 2008), we created spiral protofibril models using PrPSc-like structures generated by simulation of PrPSc at low pH to trigger conversion. These models have a compact, elk-like X-loop, as shown for the bovine structure (Scouras and Daggett, 2008) in Fig. 6. In the protofibril model, the turn is buried between two monomers and makes contact (<5 Å distance) with 35 neighboring residues. An expanded turn or loss of the turn would disrupt the packing of the adjacent monomers and thereby discourage incorporation into the protofibril, suggesting a direct basis for resistance in these mutants. Ott et al. (2008) provided several possible explanations for the observed resistance including the idea that the mutants are ‘defective’ monomers that, after incorporation into infectious seeds, prevent further oligomerization. Our results are consistent with this idea and provide a structural origin for the advantageous ‘defect’.

Overall, our results illustrate the importance of a well-ordered X-loop at a range of pH values for susceptibility to PrPSc infection. This finding applies specifically to resistance against sheep scrapie and the RML/Chandler prion strains (which was originally derived from scrapie). It extends both to conditions in cell culture (for mouse mutants) and in vivo (for sheep polymorphisms), which could differ in glycosylation and other factors. We also demonstrate the power of MD simulations for study of prion structure under varying conditions, particularly for discerning conformational dynamics that current experimental techniques are unable to achieve. Future work will explore the counter examples, equine and tammar wallaby PrP, which have well-structured X-loops but appear to be resistant to prion diseases, as well as the design of new resistant variants.

**Acknowledgements**

The authors are grateful for financial support from the National Institutes of Health (GM 81407). PEDS Board Members are Joost Schymkowitz and Elizabeth Meiering. PEDS Senior Editor is Alan Fersht.

**References**

Disruption of the X-loop turn of the prion protein linked to scrapie resistance


