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Simulations of Membrane-bound Diglycosylated Human Prion Protein Reveal Potential Protective Mechanisms against Misfolding

Short Title: Protective Mechanisms against Prion Propagation

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Abstract

Prion diseases are associated with the misfolding of the prion protein (PrP) from its normal cellular form (PrP^{C}) to its infectious scrapie form (PrP^{Sc}). Posttranslational modifications of PrP *in vivo* can play an important role in modulating the process of misfolding. To gain more insight into the effects of posttranslational modifications on PrP structure and dynamics and to test the hypothesis that such modifications can interact with the protein, we have performed molecular dynamics simulations of diglycosylated human PrP^{C} bound to a lipid bilayer via a glycophosphatidylinositol anchor. Multiple simulations were performed at three different pH ranges to explore pH effects on structure and dynamics. In contrast to simulations of protein-only PrP^{C}, no large effects were observed upon lowering the pH of the system. The protein tilted toward the membrane surface in all of the simulations and the putative PrP^{Sc} oligomerization sites became inaccessible, thereby offering a possible protective mechanism against PrP^{Sc}-induced misfolding of PrP^{C}.

Abbreviations used

MD	Molecular Dynamics
TSE	Transmissible spongiform encephalopathy
PrP	prion protein
PrP^{C}	cellular prion protein, biologically active form
PrP^{Sc}	scrapie prion protein, infectious conformer
CJD	Creutzfeldt-Jakob disease
BSE	bovine spongiform encephalopathy
GPI	Glycosylphosphatidylinositol
NMR	Nuclear Magnetic Resonance Spectrometry
DSSP	Dictionary of secondary structure of proteins
SASA	Solvent accessible surface area

Introduction

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases associated with misfolding of the prion protein (PrP). TSEs include a range of diseases in humans and other mammals, such as Creutzfeldt-Jakob disease (CJD) and bovine spongiform encephalopathy (BSE or mad cow disease). These diseases are caused by the misfolding of the prion protein from its native conformation (PrP^{C}) to a disease-associated form (PrP^{Sc}) (Van der Kamp and Daggett, 2009; Prusiner et al., 1998; Gambetti et al., 2003). PrP^{C} is a glycosylphosphatidylinositol (GPI) anchor membrane-bound protein and it is usually diglycosylated at Asn181 and Asn197 (DeArmond et al., 1997, 1999). The native structure of PrP^{C} consists of a flexible N-terminal region (residues 23-127) and a structured C-terminal globular domain (residues 128-228). There are three helices – HA (residues 144-154), HB (residues 173-194), and HC (residues 200-228) – and two β-strands – S1 (residues 128-131) and S2 (residues 161-164) – in the structured globular domain (Figure 1). PrP^{C} is presented at the cell surface of neurons, and it has been implicated in signal transduction (Mouillet-Richard et al., 2000; Linden et al., 2008; Resenberger et al., 2011) and metal metabolism (Burns et al., 2003; Millhauser, 2007; Klewpatinond et al., 2008; Singh et al., 2009).

The central hypothesis of prion diseases is that they are protein-only diseases, whereby the prion protein is the only agent required for propagation and transmission of the disease (Prusiner et al., 1998). Recombinant PrP, which lacks glycans and the GPI anchor, is the primary construct used in experimental studies. However, non-protein moieties affect the disease process *in vivo*. The N-linked glycans influence PrP expression, distribution (within regions of the brain and among different types of neuronal cells) and deposition of PrP^{Sc} *in vivo* (DeArmond et al.,

1997, 1999). The glycans have also been observed to modify the conformation of PrP^{C} and/or affect the affinity of PrP^{C} for a particular strain of PrP^{Sc} (Rudd et al., 1999; DeMarco and Daggett, 2005; Collinge et al., 1996; Safar et al., 1998). Nonetheless, based on the protein structure alone, recombinant PrP^{C} appears to be a good model for biologically relevant forms of PrP^{C}, because the structures of diglycosylated human PrP^{C} and recombinant PrP^{C} are similar, as indicated by circular dichroism (CD) and Nuclear Magnetic Resonance Spectrometry (NMR) (Hornemann et al., 2004).

Most of the molecular dynamics (MD) simulation studies of PrP only focus on the protein-only portion (PrP_{prot}), i.e. the simulations include neither the posttranslational modifications nor the membrane environment. Various computational studies have been performed on PrP_{prot} at acidic pH ranges (Alonso et al., 2001; Cheng and Daggett, 2014; Van der Kamp and Daggett, 2010). Experiments indicate that low pH cellular environments, such as endocytic organelles and lipid rafts, play a role in misfolding (Borchelt et al., 1992; Arnold et al., 1995). In order to provide a more realistic simulation environment of PrP^{C} to determine the pH effects on misfolding, it is necessary to include the non-protein moieties in simulations.

Thus far there has been only one membrane MD simulation study of GPI-anchored diglycosylated PrP^{C} in which both PrP_{prot} and the full scrapie-competent construct (i.e. with the N-terminus built on to the NMR fragment) including the membrane, glycans and GPI anchor (PrP_{mem}) were simulated (DeMarco and Daggett, 2009). In this case the simulations were performed at neutral and low pH at 25°C and they began with the protein perpendicular to the membrane to allow for interactions between the two to evolve naturally. However, the PrP_{mem} simulations were only 15 ns. Here we build upon that work by performing multiple, longer

simulations that include the glycans, GPI anchor and lipid membrane at 37°C to better explore the conversion pathway of PrP^{C} and its structural dynamics under more physiological conditions. We performed simulations of the system in triplicate at low, mid, and neutral pH. Each PrP_{mem} simulation was 80 ns long, increasing sampling time from 30 ns to 720 ns, and we have 750 ns of control free PrP^{C} MD at this same temperature and pH regimes, for a total of ~1.5 μs. With these new simulations we have both confirmed and extended our earlier findings and observed interactions between the protein and non-protein moieties that potentially modulate conversion of PrP^{C}. We found that the structured C-terminal domain of PrP is more stable with glycans and membrane than without such non-protein moieties. The glycans did not form contacts with the membrane, but the Asn181 glycan formed contacts with the flexible N-terminus of PrP. In addition, PrP tilted towards the membrane such that the putative oligomerization sites on the protein were shielded by the membrane surface. Our findings shed light on why conversion of PrP^{C} to PrP^{Sc} is more efficient with the bare recombinant protein than with glycosylated, membrane-bound PrP^{C}.

Materials and Methods

Starting structures of PrP_{prot} and PrP_{mem}

For PrP_{prot}, the starting coordinates for the globular region (residues 125-228) were obtained from the human NMR structure (Zahn et al., 2000) (PDBID: 1QLX). The NMR structure was then extended to include residues 90-230; this longer construct is necessary because the N-terminal residues not present in the NMR structures are critical to infectivity and the shorter NMR construct is not scrapie competent. In order to model residues 90-230 of PrP,

the missing N-terminal residues were added to the structure such that the N-terminus extended away from the protein to avoid bias, as described previously (Van der Kamp and Daggett, 2010; DeMarco and Daggett, 2009). To construct PrP_{mem} (Figure 1A), non-protein moieties of the system were added to the PrP_{prot} system, as described previously (DeMarco and Daggett, 2009). Briefly, two 13-residue glycans were attached to Asn181 and Asn197. A triantennary glycoform scaffold common to mouse PrP^{C} and PrP^{Sc} was chosen (Stimson et al., 1999). This glycan is representative of human N-glycans (Varki et al., 2003) (Figure 1B). This glycan contains one sialic group and the acid was deprotonated in all simulations as its pKa is ~2.5, which is below the pH regimes investigated here. The GPI anchor was attached to the C-terminus of PrP. Further details regarding building of the glycan and GPI components are provided by DeMarco and Daggett (2009). The GPI anchor was embedded in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) membrane bilayer, which consists of 335 lipid-molecules. The lipid bilayer was modelled based on the structure built by the Schulten group (Heller et al., 1993). POPC was chosen as is has been identified as the most abundant lipid around PrP (Brugger et al, 2004).

MD simulations

For both PrP_{prot} and PrP_{mem} simulations, the starting structures were solvated in a pre-equilibrated rectangular water box (Levitt et al., 1997) with a density of 0.993 g/ml, which is the experimental density for water at 37 °C (Kell, 1967). Detailed methods of the PrP_{prot} simulations have been described previously (Van der Kamp and Daggett, 2010). For the PrP_{mem} system simulations, the periodic box, which extended >20 Å from any solute atom above and below, had

the following dimensions: 94 × 98 × 147 Å. Neutral, mid, and low pH ranges were modelled by altering protonation states of histidine, aspartate, and glutamate residues, as described previously (Van der Kamp and Daggett, 2010). Unfortunately pKa values are not available for these groups, so the pH ranges fall in the following approximate ranges: neutral, pH ≥ ~6; mid, 4 ≤ pH ≤ 6; and low, 3 ≤ pH ≤ 4. These ranges are approximate and individual pKas can certainly differ from the 'standard' values, but this approach has correctly modeled pH-dependent conformational behavior in a variety of systems, including the prion protein. The Levitt et al. (1995) force field was employed for all MD simulations, and simulations were performed at 37 °C using *in lucem* molecular mechanics (*il*mm) (Beck et al., 2000-2017) and standard procedures (Beck and Daggett, 2004). The lipid (Smolin et al, 2008) parameters have been presented previously and they were derived to be consistent with our force field. For the PrP_{mem} system, triplicate simulations were performed for each pH using a different random number seed, and each simulation was performed for 80 ns. Performing multiple independent simulations improves sampling over a single simulation (e.g. 3 x 80 ns should provide better sampling and improved probability of observing conformational transitions compared with a single simulation of 240 ns). For the PrP_{prot} system, five simulations were performed for each pH regime, and each simulation was 50 ns long. In total we have ~1.5 μs of sampling: 720 ns for PrP_{mem} and 750 ns for PrP_{prot}.

MD simulation analysis

Analyses such as, solvent accessible surface area (SASA), dictionary of secondary structure of proteins (DSSP), Cα root-mean square deviation (RMSD), and Cα root-mean square

fluctuation (RMSF) were performed with *il*mm as previously described (Van der Kamp and Daggett, 2010). NOE restraint data (BMRB 4641) were obtained from human PrP E200K (Zhang et al., 2000). NOE restraint satisfaction of PrP_{mem} simulations was measured for the combined simulations in each pH regime with *il*mm. An NOE restraint was considered satisfied if the $<r^{-6}>$ weighted distance between the nearest protons associated with the NOE was less than the upper bound or 5 Å, whichever is greater. The HC tilt angle was measured between a vector fit through Cα atoms of residues 200-214 on HC and the x-y plane. The x-y plane was an approximation of the membrane surface. Heavy atom contacts were considered intact when two carbon atoms were ≤ 5.4 Å apart or when other interacting heavy atoms were ≤ 4.6 Å apart. The population of a contact (percentage time present over the simulation) was averaged across simulation replicates for each pH regime. The 40-80 ns of PrP_{mem} simulations and the 10-50 ns time periods of PrP_{prot} simulations were used to calculate the average contact occupancies.

Statistics

Data are presented as the mean and associated standard deviation. Overall statistics over time amounted to analysis of 1,500,000 time points (or structures). Statistical properties and frequencies over the specific time periods provided in the previous section included 40,000 samples for each simulation.

Results and Discussion

The starting structure of the PrP_{prot} system (bare protein in water) was created using the human NMR PrP^{C} structure (PDB:1QLX). The missing N-terminal residues 90-127 were added

to the structure, as they are important to PrP conversion and pathology (Swietnicki et al., 1997; Luhrs et al., 2006). These N-terminal residues were built to extend away from the structured globular domain to avoid bias in sampling of the N-terminus, as described previously (Van der Kamp and Daggett, 2010; DeMarco and Daggett, 2009). The PrP_{mem} system (including glycans, the GPI anchor and the membrane), in addition to the NMR structure, was created by placing PrP on a phosphatidylcholine (POPC) lipid bilayer (Figure 1A). The GPI anchor was embedded in the lipid bilayer and attached to the C-terminus of the PrP. Glycans were attached to the glycosylation sites at Asn181 and Asn197. Simulations of both the PrP_{prot} and PrP_{mem} systems were performed at neutral, mid and low pH. The pH environment was modeled by altering the protonation states of charged residues such that low pH corresponds to pH below the pKa of Asp and Glu (approximately pH 4), mid pH corresponds to pH above 4 and below the pKa of His, and neutral pH is above the pKa of His (see Materials and Methods). All simulations were performed at 37 $^{\circ}$C.

Misfolding in Protein-only PrP^C simulations

PrP_{prot} simulations (without GPI anchor, glycans and membrane) at neutral, mid and low pH yielded structures that deviate significantly from the starting structure. The Cα root-mean square deviation (RMSD) was used to detect gross conformational changes in simulations. The average Cα RMSD for the globular domain (residues 128-228) for neutral, mid and low pH simulations were 2.12 ± 0.5 Å, 2.59 ± 0.38 Å and 2.96 ± 1.27Å, respectively, showing increasing structural changes upon lowering the pH. One of the major contributors to the increase in Cα RMSD at low pH was the HA helix region: the HA helix along with its preceding loop (S1-HA

loop) was displaced from its native position, resulting in significant disruption in hydrophobic contacts (Figure 2A,B). Such a conformational change was also observed in previous simulations of human PrP pathogenic mutants (Van der Kamp and Daggett, 2010; Cheng and Daggett, 2014; Chen et al., 2010) and low pH simulations of human PrP (Van der Kamp and Daggett, 2010). Hydrophobic packing is crucial to protein stability, and disruption of the S1-HA loop packing increases the aggregation tendency of PrP. In addition, detachment of the S1-HA loop from the HC helix is necessary for PrP^{Sc} fibrillation, as indicated in both *in vitro* (Eghiaian et al., 2007; Hafner-Bratkovic et al., 2011) and *in vivo* (Hafner-Bratkovic et al., 2011) disulfide engineering experiments. These experiments support our earlier hypothesis that PrP^{C} misfolding involves the exposure of the hydrophobic S1-HA loop, with the formation of a new hydrophobic strand that is a site for PrP^{Sc}-oligomerization. We denoted this oligomerization site as the E4 strand (Figure 2B), since it consistently forms an extended structure in simulations of various PrP species at low pH (Alonso et al., 2001; DeMarco and Daggett, 2004; DeMarco et al., 2006; Scouras and Daggett, 2008).

In addition to the E4 strand, another nonnative strand (denoted E1) forms at the N-terminus in the PrP_{prot} simulations (Figure 2C). The E1 strand was recruited by the native strand S1, and our previous simulations also have formed this E1 strand, which ranges from residues 113-122 (DeMarco and Daggett, 2004; Scouras and Daggett, 2008; Alonso et al., 2001). We hypothesized that the formation of the E1 strand is another hallmark of PrP misfolding, and that the E1 strand is also a site for PrP^{Sc}-oligomerization. Our results are in agreement with experiments indicating that the PrP peptides within residues 106-127 of the N-terminus aggregate and form β-sheet-rich oligomers and fibrils (Lee et al., 2008; Silva et al., 2003; Walsh

et al., 2010; Cheng et al., 2011). Interestingly, a recent crystal structure of PrP in complex with a PrP^{Sc}-inhibitory nanobody also has an E1 strand consisting of N-terminal residues 118-122 (Figure 2C) (Abskharon et al., 2014), lending further support to the MD-derived misfolded structures and our prediction and characterization of this strand before the structure was determined (Alonso et al., 2001).

In order to interpret our MD-derived misfolded PrP^{Sc} structures in the context of the oligomerization process, we have previously proposed the spiral model. In the spiral model, MD-derived misfolded PrP^{Sc} structures were assembled by docking the E1 strand of one monomer to an adjacent neighbor at the E4 strand, and in this way the monomers are arranged along a three-fold screw-axis (Figure 2D). The spiral model has been previously validated by various experimental data (DeMarco and Daggett, 2004; DeMarco et al., 2006), and recent experimental data on PrP^{Sc} soluble oligomers lend further support to our model (Cheng and Daggett, 2017, in preparation).

Although misfolding was observed in the PrP_{prot} simulations, experiment indicates that membrane-bound PrP^{C} is more resistant to misfolding, since neither acidic treatment (Baron et al., 2002) nor denaturants (Lin et al., 2013) have been shown to induce misfolding and oligomerization of membrane-bound PrP^{C}. Furthermore, unlike bare recombinant PrP, membrane-bound PrP^{C} appears to be resistant to PrP^{Sc}-induced misfolding unless it is released from membranes by phospholipase C digestion (Baron et al, 2002; Baron and Caughey, 2003). We hypothesize two possible mechanisms by which the posttranslational modifications and the membrane environment contribute to the misfolding-resistance: (1) the glycans and membrane environment provide contacts that stabilize the native structure of PrP^{C}; and (2) the glycans and

membrane environment shield the putative oligomerization sites, strands E1 and E4, thereby providing a steric barrier to misfolding and oligomerization. In the following results from our PrP_{mem} simulations, we evaluate the stability of PrP using Cα RMSD, Nuclear Overhauser Effect Crosspeak (NOE) satisfaction and contact analyses. Details regarding the contacts and relative positions between PrP and the glycans, and between PrP and the membrane, are also provided. We conclude by discussing how the glycans and the membrane may contribute to protective mechanisms against PrP misfolding.

Conformational changes in PrP_{mem} simulations

Gross conformational changes of PrP in the PrP_{mem} simulations are reflected in the Cα RMSD relative to the starting structure. In all pH regimes, the average Cα RMSDs of the globular domain of PrP (residues 128-228) were within the range of 1.5-2.0 Å (Figure S1), reflecting the retention of the native-like globular domain structure. The Cα root-mean square fluctuations (RMSF) about the average structure after equilibration reflect the mobility of the protein. The Cα RMSF values of the residues in the globular domain were less than 2 Å in all pH regimes, whereas those of the residues in the flexible N-terminus were in the range of 2 – 5 Å (Figure S2).

NOE satisfaction by PrP_{mem} simulations

The PrP structures in the PrP_{mem} simulations were compared to the Nuclear Overhauser Effect crosspeaks (NOE) reflecting the proximity of protons throughout the structure by comparing with the NOE restraints of the human PrP E200K mutant (Zhang et al., 2000), which is structurally similar to wild type human PrP, using a previously described method (Van der

Kamp and Daggett, 2010). The NOE restraints are available for residues 125-230, which cover the structured, helical domain. Structures from the neutral, mid, and low pH PrP_{mem} simulations satisfied 88% of the 2641 NOEs, in all pH regimes. Therefore, PrP structures from our PrP_{mem} simulations are similar to the NMR structures of human recombinant PrP regardless of the pH environment. This is in agreement with CD experiments that confirm that PrP^{C} retains a helix-rich native structure when bound to POPC vesicles (Sanghera and Pinheiro, 2002).

Acidic pH effects on PrP_{mem} simulations

There were contact differences in acidic and neutral pH simulations (Table S1). The mid pH simulations lost the Glu152-His155 contact when the His140-Asp147 as well as other interactions formed. As for the low pH simulations, only two residue contacts were significantly disrupted: Leu125-Ile182 and Tyr157-Phe198. The Leu125-Ile182 contact was affected primarily by the loss of a hydrogen bond between side chains of Tyr128 and Asp178 upon protonation of the Asp. As for the Tyr157-Phe198 contact, Phe198 became more exposed and lost hydrophobic packing with Tyr157. As a consequence of these changes residues 154-157 at the C-terminus of HA lost helicity in both the mid and low pH simulations compared to neutral pH (Table S1). These minor structural and intramolecular contact changes were all directly affected by the protonated residues at mid and low pH. These contact changes were localized and did not have a significant impact on the structure, as indicated by the globular Cα RMSD (Figure S1). These results are consistent with experiment, which indicates that low pH misfolding is inefficient for membrane-bound PrP (Baron et al., 2002).

PrP_{mem} vs PrP_{prot}: Major differences in intramolecular interactions

There were three PrP regions with significant contact differences between PrP_{mem} and PrP_{prot} simulations: the Phe198 hydrophobic pocket, the native sheet packing with the HB and HC helices, and the packing between HC and the S1-HA loop. A list of relevant contact occupancies are provided in Table S2. Overall, the contacts in the PrP_{mem} simulations were much more stable than those in $PrP_{prot.}$ These three areas that differ are discussed in detail below.

In the PrP_{mem} simulations, Phe198 was buried and its average side chain solvent accessible surface area (SASAs) for neutral, mid, and low pH simulations was only 4, 13, and 19 $Å^2$, respectively. On the other hand, the SASA of Phe198 in the PrP_{prot} simulations was at least three to four times greater (Figure S3). As Phe198 became exposed, the contacts between Phe198 and Met206 were lost in the mid and low pH PrP_{prot} simulations. The exposure of Phe198 was accompanied by the formation of the nonnative His187-Asp202 salt bridge. The side chains of His187 and Asp202 occupied the native side chain position of Phe198 (Figure 3A). This nonnative salt bridge kept the side chain of Phe198 in a solvent exposed conformation, which is consistent with our previous PrP_{prot} simulations (Van der Kamp and Daggett, 2010). On the other hand, in all PrP_{mem} simulations, Phe198 retained stable core contacts with neighboring residues, including His187 and Met206. The side chain motion of Phe198 was deterred by the bulky glycan attached to Asn197. As a result, the side chain of Phe198 remained buried in between HB and HC in all PrP_{mem} simulations.

In all PrP_{mem} simulations, the native S2 strand was tightly packed to HB and HC, such that Thr183 formed a stable contact with Gln160 (Figure 3B). The residue contact between Gln160 and Met213 on HC was populated on average >80% of the time in the neutral and mid

pH PrP_{mem} simulations (Table S1), but this interaction was less stable in the low pH simulations (36% occupancy). On the other hand, all of the PrP_{prot} simulations, regardless of pH, showed a significant loss in both Gln160-Thr183 and Gln160-Met213 contacts. Other than the packing between S2 and the helices HB and HC, there were two hydrogen bonds that contributed to the stability of this region: Tyr128-Asp178 and Tyr162-Thr183. The side chain of Tyr128 on S1 stabilized the native sheet by hydrogen bonding to the side chain of Asp178 on HB (Figure 3B). As for the side chain of Thr183, it hydrogen bonded with the backbone amide of Tyr162 (Figure 3B). These hydrogen bonds were more prevalent in PrP_{mem} simulations than PrP_{prot} simulations. Overall, the native sheet in PrP_{mem} formed stable packing interactions and hydrogen bonds while such interactions were lost in the PrP_{prot} simulations.

Contacts in the S1-HA loop were more variable due to the conformational flexibility of this loop. One of the relatively robust contacts formed in PrP_{mem} simulations was Arg136-Val209, in which the hydrophobic portion of the side chain of Arg136 packed against the side chain of Val209 on HC (Figure 3C). In PrP_{prot}, however, this contact was diminished and highly variable across simulations. Such loss of contacts between S1-HA loop and HC was expected, since the HA helix of the low pH PrP_{prot} simulations was highly mobile and occasionally detached from HC (Figure S4).

Interactions between the membrane and the flexible N-terminus

The flexible N-terminus (residues 90-127) formed contacts with the membrane surface in the PrP_{mem} simulations (Figure S5). As can be seen in Figure S5, The N-terminus made extensive contacts with the membrane with respect to both the number of residues involved and the extent

of time that contacts were formed in all simulations at mid and low pH. This was also the case in two of the three simulations at neutral pH. As an illustration of the particularly prevalent interactions, His96 and Lys101 each formed long-lasting salt bridges with the phosphate groups on the membrane surface at low and mid pH (Figure 4A,B). These two positively charged residues are within residues 90-110, which represent one of the charged clusters of PrP (Wang et al., 2010). These charge clusters are important for electrostatic interactions involved in membrane association (Boland et al., 2010). At acidic pH, PrP binds to anionic lipids with higher affinity (Morillas et al., 1999). The pH effect was observed in our simulations, as reflected in the heightened interactions with the membrane at mid and low pH (Figure S5), and illustrated more specifically for a stable salt bridge between the doubly-protonated His96 with a phosphate group on the membrane (Figure 4A).

Interactions between the glycans and the flexible N-terminus

The N-terminus of PrP interacted with the Asn181 glycan via Lys residues, but not with the Asn197 glycan. For example, at neutral pH, Lys101 formed stable contacts with the Asn181 glycan (Figure 5A), preventing the N-terminus from forming contacts with the membrane (Figure S5). At mid pH, Lys104 also formed stable contacts with the Asn181 glycan in a similar conformation (Figure 5B). At low pH, Lys104 bound to the Asn181 glycan in a conformation different from that of the neutral and mid pH simulations, but with the same effect (Figure 5C).

Such consistent interactions between lysine residues and the Asn181 glycan were surprising and heretofore unrecognized. However, it is known that the charge clusters at the flexible N-terminus of PrP are important for binding glycosaminoglycans (GAGs), such as

heparin, chondroitin sulfate, and hyaluronic acid (Pan et al., 2002). Given the similar constituents of GAGs and glycans, it is reasonable to expect that the charge cluster of the flexible N-terminus can bind to the glycans, as indicated in our simulations. Glycosylation of PrP impedes the conversion efficiency in *in vitro* conversion assays (Priola and Lawson, 2001). While these large glycans can sterically inhibit PrP^{Sc}-induced misfolding, our results suggest that glycans can also bind to N-terminal lysine residues, thereby deterring the N-terminus from forming the E1 strand, which is a hallmark for misfolding in our MD-simulated conversion. Other than retardation of the misfolding process, glycans also play a complicated role in prion propagation (Zahn et al., 2000). Given that the glycans can form stable interactions with the flexible N-terminus of PrP, different glycans may induce different N-terminal conformations, thus influencing strain-specific prion propagation, which is sensitive to conformational changes in PrP^{Sc}. Another complication is that while the glycans appear to stabilize and deter conversion of PrP^{C}, the glycans are also involved in recognition and targeting of PrP^{Sc} to PrP^{C} glycoforms in different regions of the brain (DeArmond et al., 1999). Unfortunately, the PrP^{C} simulations presented here cannot directly address potential PrP^{C}-PrP^{Sc} interactions, but our results suggest that the glycans have a number of effects on PrP^{C} as well as its interactions with PrP^{Sc}. While speculative, PrP^{Sc} binding may compete with favorable glycan-PrP^{C} interactions; MD simulations investigating these possible mechanisms are in progress.

Tilting of the structured C-terminal domain on the membrane

In all of the PrP_{mem} simulations, PrP tilted toward the membrane. For example, the angle between HC and the membrane surface dropped from 90 to ~30º by the end of the simulations

(Figure 6A,B). This tilting motion placed the putative E1 and E4 oligomerization sites on or very near the membrane surface. Exposure of residues 135-140 (the E4 region) was probed, and in low pH simulation 1, as an example, its exposure dropped from ~500 $Å^2$ to < 200 $Å^2$ by 45 ns. This significant reduction was due to the tilting motion of PrP, which buried the E4 epitope (Figure 6B). This tilted conformation was stable and buried the E4 epitope around 45 ns and remained as such until the end of the simulation. The other putative oligomerization site, the E1 strand, was formed only in low pH simulation 3 (Figure 7). However, with a 30^o tilt, the E1 strand was buried on the membrane surface. We hypothesize that the tilting motion of membrane-bound PrP^C retards PrP^{Sc}-induced misfolding by masking putative oligomerization sites on PrP^C (Figure 8). Experiments broadly support this hypothesis, given that membrane-bound PrP is more resistant to misfolding than free PrP (Baron et al., 2002; Baron and Caughey, 2003). In addition, an antibody that targets residues 138-141 shows significantly decreased binding of membrane-bound PrP^C compared with soluble PrP^C (Leclerc et al., 2003), consistent with steric occlusion of E4 in the MD simulations.

Conclusions

Overall, the PrP_{mem} simulations were extremely stable and preserved a native-like PrP^C structure in all pH regimes, unlike the PrP_{prot} simulation of free unglycosylated PrP^C. In most of the PrP_{mem} simulations, the flexible N-terminus formed sporadic contacts with the membrane except for a few lysine and histidine residues in the N-terminus that formed stable salt bridges with the phosphate groups on the membrane. The glycans may modulate the misfolding process by interfering with E1 formation at the N-terminus through direct contacts with lysine residues at

the N-terminus. The tilting motion of membrane-bound PrP buried the E1 and E4 regions, both of which are putative oligomerization sites. This led us to hypothesize that the tilted conformation of membrane-bound PrP^{C} protects critical regions of PrP^{C} and deters PrP^{Sc}-induced misfolding, explaining why recombinant PrP^{C} converts more readily.

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Figure Legends

Figure 1. PrP_{mem} system used for MD simulations. (A) Cyan and purple colored surfaces are the glycans attached to Asn181 and Asn197, respectively. The GPI anchor is represented as the light-blue surface. HA (residues 144-154), N-terminus (residues 90-127), S1 and S2 (residues 128-131 and 161-164) are colored in cyan, green, and yellow, respectively. The HB (residues 173-194) and HC (residues 200-228) helices are in blue. Remaining loop regions are colored gray. The carbon and oxygen atoms of the phosphatidylcholine (POPC) molecules are indicated as thin cyan and red lines, respectively. Nitrogen and phosphorus atoms are indicated by blue and brown spheres, respectively. The water is not displayed. (B) Schematic depiction of individual residues in the glycan attached to Asn 181 and Asn 197.

Figure 2. Misfolding in PrP_{prot} simulations. (A) Left: starting structure. Right: helix HA structure for the last 25 ns of all PrP_{prot} simulations at 1 ns granularity. Structures were aligned to the starting structure at the stable core residues 174-186 and 200-219, as shown in blue. Cyan, orange, and red cartoons represents structures from neutral, mid and low pH simulations, respectively. (B) Left: starting structure. Right: mid pH simulation 3 at 49.9 ns. Hydrophobic residues 134, 137, 139, 141, 205, 209 and 213 are shown with translucent surface and stick representations of the side chains. E4 region is colored in orange. (C) Formation of the E1 strand (in red). Left: crystal structure (PDBID: 4KML (Abskharon et al., 2014)). Right: a representative MD-derived misfolded structure from PrP_{prot} simulations. (D) An MD-derived converted PrP^{Sc} structure was used to construct a threefold screw-axis spiral model. The oligomerization site occurs between the E1 and E4 strands of the adjacent monomers. The structures are not to scale with panels A-C.

Figure 3 Differences between PrP_{mem} and PrP_{prot} simulations. Structures are taken from mid pH simulations. Relevant residue contact occupancies are displayed. Standard deviations are computed for both PrP_{mem} and PrP_{prot} (For each pH, n = 3 for $PrP_{mem,}$ and n = 5 for PrP_{prot}). (A) Phe198 hydrophobic pocket. The black oval indicates the nonnative salt bridge that formed in PrP_{prot} simulation. (B) Native sheet packing with HB and HC. The black ovals indicate the loss of hydrogen bonds in PrP_{prot} simulations. (C) S1-HA loop packing with HC. The S1-HA loop detaches from HC and loses hydrophobic packing in $PrP_{prot.}$

Figure 4. Contacts between N-terminus and the membrane surface at mid and low pH. Contacts between residues 90-117 and the membrane are shown on the right. (A) Structure of membrane-bound PrP in mid pH simulation 3 at 80 ns. His96 formed a stable salt bridge with phosphate head group on the membrane. (B) Structure of membrane-bound PrP in low pH simulation 2 at 80 ns. Lys106 formed stable salt bridge with phosphate head group on the membrane.

Figure 5. Contacts between the Asn183 glycan and lysine residues at the flexible N-terminus. Contacts between residues 90-117 and the Asn181 glycan are shown in the right column. Relevant lysine residues, *N*-acetylneuraminate (NeuNAc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), and fucose (Fuc) are shown as sticks. (A) Neutral pH simulation 3 at 80 ns. (B) Mid pH simulation 2 at 80 ns. (C) Low pH simulation 3 at 80 ns.

Figure 6. PrP tilting on the membrane protecting the E4 putative oligomerization site. (A) HC tilt angle for PrP_{mem} simulation 1 (blue), 2 (green) and 3 (orange) at neutral, mid and low pH. (B) Before and after the tilting motion in low pH simulation 1.

Figure 7. Buried E1 epitope putative oligomerization site. (A) Structure of the membrane-bound PrP in low pH simulation 3 at 80ns. The orange epitope is E1, which is buried on the membrane surface in grey. (B) Secondary structure of the simulation over time.

Figure 8. Schematic of PrP conversion. Without glycans and the membrane environment, PrP_{prot} misfolds and both E1 and E4 strands are accessible, allowing for oligomerization. As for PrP_{mem}, the oligomerization sites E1 and E4 are buried or near the membrane surface, which would interfere of PrP^{Sc} to PrP^{C} and propagation of PrP^{Sc}.