Molecular dynamics simulation of human prion protein including both N-linked oligosaccharides and the GPI anchor

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Although glycosylation appears to protect prion protein (PrP C) from the conformational transition to the disease-associated scrapie form (PrP Sc), available NMR structures are for non-glycosylated PrP C only. To investigate the influence of both the two N-linked glycans, Asn181 and Asn197, and of the GPI anchor attached to Ser230, on the structural, dynamical and electrostatic behavior of PrP, we have undertaken molecular dynamics simulations on the C-terminal region of human prion protein HuPrP(90–230), with and without the three glycans. The simulations used the AMBER94 force field in a periodic box model with explicit water molecules, considering all long-range electrostatic interactions. The results suggest the structured part of the protein, HuPrP(127–227) is stabilized overall from addition of the glycans, specifically by extensions of Helix-B and Helix-C and reduced flexibility of the linking turn containing Asn197, although some regions such as residues in the turn (165–170) between Strand-B and Helix-B have increased flexibility. The stabilization appears indirect, by reducing the mobility of the surrounding water molecules, and not from specific interactions such as H bonds or ion pairs. The results are consistent with glycosylation at Asn197 having a stabilizing role, while that at Asn181, in a region with already stable secondary structure, having a more functional role, in agreement with literature suggestions. Due to three negatively charged SiaLe x groups per N-glycan, the surface electrostatic properties change to a negative electrostatic field covering most of the C-terminal part, including the surface of Helix-B and Helix-C, while the positively charged N-terminal part PrP(90–126) of undefined structure creates a positive potential. The unusual hydrophilic Helix-A (144–152) is not covered by either of these dominant electrostatic fields, and modeling shows it could readily dimerize in an anti parallel fashion. In combination with separate simulations of the GPI anchor in a membrane model, the results show the GPI anchor is highly flexible and would maintain the protein at a distance between 9 and 13 Å from the membrane surface, with little influence on its structure or orientational freedom.

Key words: human prion protein/N-linked glycan/GPI anchor/molecular dynamics/conformation

Introduction

Prion protein (PrP) is associated with an unusual class of neurodegenerative diseases, which includes scrapie in sheep; bovine spongiform encephalopathy (BSE) in cattle; and kuru, Creutzfeldt-Jacob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familiar insomnia (FFI) in humans (Prusiner, 1996; Edenhofer et al., 1997; Ironside, 1998). According to the protein-only hypothesis (Prusiner, 1982, 1998) the disease is caused by an abnormal form of the 250 amino acid PrP, which accumulates in plaques in the brain. The protein component of this disease-associated form of PrP (PrP Sc) differs from the normal cellular form (PrP C) only in its 3-D structure, and FTIR and CD spectra indicate it has a significantly increased content of β-sheet conformation compared with PrP C (Pan et al., 1993; Horwich and Weissman, 1997). PrP C is a secreted cell surface glycoprotein with still unknown function, which cycles between the cell surface and endocytic compartments (Shyg et al., 1993; Vey et al., 1996; Harris, 1999). The accumulation of PrP Sc occurs in late endosomes and lysosomes (Arnold et al., 1995). The conformational change between PrP C and PrP Sc in animals and humans is strongly associated with mutations in the PrP gene and leads to a protease-resistant remnant consisting of the residues 90–231, which appears to be the minimum unit correlated with infectivity. So far, 17 disease-associated mutations of PrP are known for humans (Ironside, 1998; Zuegg and Gready, 1999), most of which occur in the second half of the C-terminal region.

The mature form of PrP C, made up of residues 23–231, is anchored to the cell membrane via a glycosyl-phosphatidylinositol anchor (GPI anchor) at its C terminus (Stahl et al., 1987, 1992; Baldwin et al., 1993), and has one disulfide bridge (Cys110-Cys114, human numbering). In addition, mammalian PrP contains two consensus sites for N-linked glycosylation, at Asn181 and Asn187 of human PrP (HuPrP) and Syrian hamster PrP (ShPrP), and at Asn180 and Asn186 in murine PrP (MoPrP). Studies of the N-linked glycans of MoPrP (Stimson et al., 1999) and ShPrP (Endo et al., 1989; Rudd et al., 1999a) have shown that both sites are occupied and that they contain up to 60 different but overlapping sets of sugars, including charged sialyl-Lewis x (SiaLe x) epitopes, which are suggested to serve as cell-surface recognition molecules (Eggers et al., 1989; Bevilacqua et al., 1991).
NMR structures of the full-length and N-terminally truncated forms of recombinant MoPrP (Riek et al., 1996; Billetter et al., 1997; Riek et al., 1997; Riek et al., 1998), ShPrP (Donne et al., 1997; James et al., 1997; Liu et al., 1999), and HuPrP (Hosszu et al., 1999; Zahn et al., 2000) have revealed that the whole N-terminal segment PrP^{23–126} is flexibly disordered and that only the C-terminal part PrP^{127–230} possesses a defined 3-D structure. The structurally well-defined part of PrP consists of three α-helices and a small two-stranded antiparallel β-sheet. All NMR studies could identify the three α-helices, although with slightly different lengths, but showed differences for the antiparallel β-sheet, which was not always as well defined (Donne et al., 1997; James et al., 1997; Hosszu et al., 1999).

The biological roles of oligosaccharides of glycoproteins are as diverse as their structures, and include cell surface signaling, cell recognition, intracellular trafficking, secretion, stabilizing and protecting tertiary protein structure, regulating activity of enzymes, and clearance and turnover of the glycoproteins (Varki, 1993). In addition, oligosaccharides are so flexible in their 3-D structures that even NMR experiments can rarely define their structural and dynamical properties unambiguously (Peters and Pinto, 1996). Recent reports indicate combining NMR and x-ray crystallography can help to identify the structures of oligosaccharides on proteins, at least for the first five to six sugar residues (Rudd et al., 1999b). The great variety of possible functions combined with the structurally flexible nature of oligosaccharides makes elucidation of structure–activity relationships very difficult, especially given the fact that the protein has different regions with distinct electrostatic properties, with a high number of charged residues on the surface of PrP and the membrane model which included just the GPI anchor. Finally, we have compared the dynamic and structural behavior of the glycans when bound to PrP with results of simulations of their free, solvated forms. This allows assessment of any restrictions on the flexibility of the glycans by the protein environment.

### Results

#### Model for the N-linked glycans and GPI anchor

The two N-linked glycans were considered to be the same for both N-glycosylation sites, Asn^{181} and Asn^{197}, and to consist of the largest and most charged oligosaccharide possible. Thus, the model used for the oligosaccharide, as shown in Figure 1, consists of a tetrantennary glycan with two negatively charged sialyl-Lewis^a^ epitopes (NeuNAcα(2–3)Galβ(1–4)(Fuc-α(1–3))GlcNAc), one negatively charged NeuNAcα(2–6)Galβ(1–4)GlcNAc group and one neutral Galβ(1–4)GlcNAc group as the four antennas. This model, with an overall charge of –3 e.u., can be considered the glycan from which all the oligosaccharides found by experiment

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**Fig. 1.** Schematic representation of the oligosaccharide models for the N-linked glycan (NGlyc) and the GPI anchor (GPI). Open circles, Man; solid diamonds, GlcNAc; open pentagons, Fuc; open squares, Gal; solid ovals, NeuNAc; open hexagons, mIlno; solid squares, GalNAc; shaded diamonds, GlcN; circled Ps, PO_2^-; shaded triangles, Ethanolamine; filled circles with lines, α-anomer; open circles with lines, β-anomer. Roman numbering indicates the residues number. Arabic numbers indicate the linkage type of the glyclosidic linkage, while numbers in parenthesis indicate the linkage type used by analogy to the rat Thy-1 protein (McConville and Ferguson, 1993).
NGlyc197 shows the highest RMSD (5.1 Å). Secondary structure part (11.8 Å) (see Table I). Of the three oligosaccharides, still considerably lower than the RMSD of the flexible N-terminal protein backbone of the structured part (2.1 Å) but the RMSDs of the oligosaccharides in due to different conformations of the N-terminal part of the protein (PrP90-126) only. The RMSDs of the oligosaccharides in the glycosylated model is higher for the glycosylated model, the RMSDs of the structured part of the protein PrP127-227 are the same in both simulations (see Figure 2). The higher RMSD of the glycosylated model is due to different conformations of the N-terminal part of the protein (PrP90-126) only. The RMSDs of the oligosaccharides in glyco-HuPrP are between 3.7 and 5.1 Å, i.e., higher than the RMSD of the protein backbone of the structured part (2.1 Å) but still considerably lower than the RMSD of the flexible N-terminal part (11.8 Å) (see Table I). Of the three oligosaccharides, NGlyc197 shows the highest RMSD (5.1 Å). Secondary structure analysis, shown in Figure 3, indicates increased stability of the α-helical structure for the glycosylated model. The two C-terminal α-helices, B and C, show increased lengths which resemble more the NMR structure of ShPrP (Liu et al., 1999), although Helix-C is still shorter by 3 residues at its C-terminal end. In addition, no splitting of Helix-C could be seen in the glycosylated model in contrast to the HuPrP model (Zuegg and Gready, 1999). The flexibility of the backbone, shown in Figure 3 as standard deviation (SD) of the φ and ψ backbone torsion angles and in Figure 4 as the radius of the coil tracing the positions of the Cα atoms, decreases significantly for the residues around the N197 glycosylation site in Turn-D, but remain similar throughout the other parts of the protein. However, the Turn-C region, PrP165-170, in the glyco-HuPrP model shows a slightly increased flexibility. The average structures for both simulations are also very similar (see Figure 4), even though the RMSD of the backbone atoms between both structures is calculated to be 2.5 Å. The main differences between the two structures are of slightly different conformations for turns A, C, and D. Turn-D is at the N197 glycosylation site and, therefore, influenced by NGlyc197, but the other two turns are not directly influenced by the glycans. The intraprotein interactions are also similar for both models. The three major salt bridges, Glu146↔Arg208, Arg164↔Asp178 and Arg156↔Glu196 (Zuegg and Gready, 1999), are present in both simulations for more than 90% of the time (see Table II). The exception is the salt bridge Arg156↔Glu196, between Helix-A and Turn-D, for which the occupancy drops in the glyco-HuPrP simulation to 78.4 %, due to the proximity of the N197 glycan.

<table>
<thead>
<tr>
<th>Table I. MD simulation conditions and results</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Atoms</td>
</tr>
<tr>
<td>Molecular mass (kDa)</td>
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<tr>
<td>Cl-, Na+ ions</td>
</tr>
<tr>
<td>Water molecules</td>
</tr>
<tr>
<td>Box size (Å)</td>
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<tr>
<td>Simulation time (ps)</td>
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<tr>
<td>RMSDb (Å)</td>
</tr>
<tr>
<td>Structured (127–227)</td>
</tr>
<tr>
<td>NGlyc181, NGlyc197</td>
</tr>
<tr>
<td>GPI</td>
</tr>
<tr>
<td>Secondary structure (°)</td>
</tr>
<tr>
<td>β-Strand</td>
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<tr>
<td>H-Bonded turn</td>
</tr>
</tbody>
</table>

*Corresponding average values over the whole simulation.

**RMSD of protein backbone atoms, or heavy atoms of the oligosaccharides. α and ψ backbone torsion angles for protein, and φ, ψ and α backbone torsion angles of glycosidic linkage.
The salt bridges in the Helix-A region on the other hand, Asp144↔His140, Asp144↔Arg148, Asp147↔Arg151 and Glu146↔Arg208, which involve only neighboring residues (e.g., ± 4), are somewhat reorganized, with some increasing and others decreasing their occupancies. Differences in the occupancies between this work and previous work (Zuegg and Gready, 1999) are due only to a longer simulation time (2300 ps compared with 720 ps). The H-bond network is also similar between the two simulations with similar overall occupancies between this work and previous work (Zuegg and Gready, 1999) are due only to a longer simulation time except for a small amount of 3_{10}-helical structure occurring transiently.

Table II. Salt bridges from the simulations of HuPrP and glyc-HuPrP, shown as percentage occupancy during the whole simulation

<table>
<thead>
<tr>
<th>Salt bridges</th>
<th>HuPrP</th>
<th>glyc-HuPrP</th>
</tr>
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<tbody>
<tr>
<td>Asp144 ↔ His140 (τ₃)</td>
<td>66.9</td>
<td>36.4</td>
</tr>
<tr>
<td>Asp144 ↔ Arg148 (τ₄)</td>
<td>68.5</td>
<td>26.7</td>
</tr>
<tr>
<td>Glu146 ↔ Arg208 (τ₄)</td>
<td>97.5</td>
<td>100.0</td>
</tr>
<tr>
<td>Asp147 ↔ Arg151 (τ₄)</td>
<td>9.6</td>
<td>94.0</td>
</tr>
<tr>
<td>Asp147 ↔ His140 (τ₃)</td>
<td>71.9</td>
<td>83.8</td>
</tr>
<tr>
<td>Glu152 ↔ Arg148 (τ₄)</td>
<td>1.4</td>
<td>57.2</td>
</tr>
<tr>
<td>Asp178 (τ₄) ↔ Arg164 (τ₃)</td>
<td>90.4</td>
<td>98.2</td>
</tr>
<tr>
<td>Glu196 (τ₃₀) ↔ Arg156 (τ₄)</td>
<td>96.9</td>
<td>78.4</td>
</tr>
<tr>
<td>Glu207 (τ₄) ↔ Asp147 (τ₄)</td>
<td>95.9</td>
<td>89.7</td>
</tr>
<tr>
<td>Gln211 (τ₄) ↔ His177 (τ₃)</td>
<td>23.4</td>
<td>—</td>
</tr>
<tr>
<td>Gln221 (τ₄) ↔ Arg220 (τ₄)</td>
<td>25.8</td>
<td>37.8</td>
</tr>
</tbody>
</table>

*The average secondary structure elements, corresponding to the glyc-HuPrP simulation, are shown in parentheses. Underlined residues are residues for which mutation is known to be associated with CJD, GSS, or FFI in humans.

The salt bridges in the Helix-A region of the other hand, Asp144↔His140, Asp144↔Arg148, Asp147↔Arg151 and Glu146↔Arg208, which involve only neighboring residues (e.g., ± 4), are somewhat reorganized, with some increasing and others decreasing their occupancies. Differences in the occupancies between this work and previous work (Zuegg and Gready, 1999) are due only to a longer simulation time (2300 ps compared with 720 ps). The H-bond network is also similar between the two simulations with similar overall number of H-bonds (see Table III).

The flexible N-terminal part of PrP, PrP90-126, shows high flexibility and no unique structure in both models. For the glyc-HuPrP model this region exhibits a greater conformational change, including a higher RMSD and increased flexibility of the backbone torsion angles, with an average SD of 31.5°, compared with 26.5° for the HuPrP model. Both N-linked glycans appear to influence the position of this N-terminal part, with a different orientation compared with the simulation without oligosaccharides, and with no evidence of α-helical structure in the PrP111-118 region (Zuegg and Gready, 1999) except for a small amount of 3_{10}-helical structure occurring transiently.

Structure and dynamics of glycans

As may be seen in Figure 4, the simulation of the glycosylated HuPrP model showed no unique orientation for either NGlyc chain. With a RMSD of the heavy atoms of NGlyc181 and NGlyc197 from the starting conformation of 4.9 and 5.1 Å, respectively, and an average SD of the glycosidic linkage torsion angles (φ and ψ, and ω in the case of a 1→6 linkage) of 17.2° and 21.3°, respectively, both N-glycans exhibit high flexibility. These SD values for the torsion angles are similar to the value of 20.1° from the simulation of the free NGlyc model in solution (see Table I). Nevertheless, the N-linked glycans seem to be slightly restricted by the protein, as the core of the glycans, formed by Man^{VI},Man^{IV},GlcNAc^{III},GlcNAc^{II}, Asn^{180}, seems to have reduced flexibility compared with the simulation of the solution structure, especially for the usually highly flexible 1→6 linkage of Man^{VI},Man^{V} (see Figure 5). Compared with the core structure, the four antennas of the NGlyc models have high flexibility, especially both SiaLe^X groups of the N^{197}-linked glycan show high flexibility compared with those in the NGlyc181 glycan. Generally, NGlyc197 shows higher flexibility than NGlyc181, as can be seen in the higher SD of the torsion angles.

The GPI anchor shows similar behavior to that of the NGlyc structures. In the glyc-HuPrP simulation the SD for the torsion angles of the GPI anchor model is 28.1°, similar to that for the solvated model (solv-GPI) with 29.6°. The heavy-atom RMSDs 3.7 and 4.0Å, respectively, are also similar. As for the NGlyc chains, a core structure with reduced flexibility can be identified in the GPI anchor, consisting of Man^{VI},Man^{IV},GlcNAc^{III},GlcN^{II}, Man^{V}, Man^{IV}, H6α, H3β, and H4β. This part also showed some NOE interactions in NMR experiments with solvated forms of similar GPI anchors (Homans et al., 1999; Weller et al., 1994). The average distances in the glyc-HuPrP and solv-GPI simulations for mH6α→GlcN^{III},H1 and Man^{IV},H1→GlcN^{III},H4, shown in Table IV, are in good agreement with the experimental values. The remaining NOE interactions are not quite as consistent, as
the branching at ManIV of the GPI anchors used in the NMR experiments differs significantly from that used in the simulations. The linkage to PrP, Ser230-EaaVIII-PO2VII-ManVI, is less flexible than in the solv-GPI simulation but still too flexible to give a unique overall conformation for the GPI anchor. When coupled with the highly flexible C-terminal part of PrP, PrP228–230, this makes it impossible to identify a precise orientation for the anchor point into a membrane.

**Orientation and interaction between glycans and protein**

In the glyc-HuPrP simulation, all the glycans, NGlyc and GPI, showed only a few interactions with the protein core of PrP. The H-bond analysis in Table III shows that throughout the simulation fewer than two hydrogen bonds on average occur between each N-linked glycan and the side chains of the protein. With an average of 1.4 H bonds, the GPI anchor has even less contact with the protein side chains. For all glycans in the model, most H bonds occur between the glycan and the solvent, and within each glycan. Interaction between the glycans was observed only for NGlyc181 and NGlyc197, with an average of 2.6 H bonds. One consequence of the weak direct interactions between glycans and protein is that there are few differences in the solvent accessibility of the protein side chains between the HuPrP and glyc-HuPrP models (see Figure 6). For a few residues, such as Phe198 and Thr199 in Turn-D and Ile184 in Helix-B, the solvent accessibility is changed to totally buried. For other residues, such as Asp178, Lys185, Thr192, Thr193, and Lys194 in Helix-B, the solvent accessibility is changed to less frequently exposed. Thus, the glycans restrict direct access to the protein only in a few places on the surface.
But a weaker restriction on the accessibility can be seen in cases where only one water molecule is between the glycan and the protein. Although analysis of the distances between the glycan and protein shows only three direct interactions, considering a distance which would accommodate just one water molecule (distance < 6.5 Å) indicates that NGlyc181 covers several residues in Helix-B and some in the turn before Strand-A, while NGlyc197 covers nearly all the residues of Turn-D and some at the beginning of Helix-C. The glycan residues involved in this steric cover of the protein include the first few residues next to the linkage site to the protein, namely ManIV-GlcNAcIII-(FucII-)GlcNAcI-Asn181/197. In addition to these core glycans, one of the SiaLex groups, NeuNAcXI-GalX-(FucIX-)GlcNAcVIII, of both NGlyc chains is in direct or water-bridged contact with the protein. While this shielding of PrP by the glycans might be “weak” if it were neutral, analysis of the electrostatic potential covers all the surface of Helix-B and Helix-C and a small part of Helix-A (see Figure 7).

The GPI anchor, on the other hand, covers only the surface of the last three residues of HuPrP, and makes no direct contact with the protein. This cover involves only the first 2–3 GPI residues next to the linkage to the protein, i.e., Ser230-EaVIII-PO2VII-(ManX-)ManVI. The GPI glycan occupies the space around the C-terminal end of Helix-C opposite the two N-linked glycans, and, as it has several negatively charged residues, it extends the negative electrostatic potential to the end of Helix-C.

<p>| Table III. Average number of H bonds throughout the simulation of the HuPrP model with and without the oligosaccharides (HuPrP and glyc-HuPrP, respectively), the free NGlyc and GPI models in water (solv-NGlyc and solv-GPI) and the GPI model attached to a model membrane with choline (cho-GPI) or ethanolamine (eap-GPI) as the head group of the membrane monomer |</p>
<table>
<thead>
<tr>
<th>Model</th>
<th>HuPrP</th>
<th>NGlyc</th>
<th>GPI</th>
<th>Memb</th>
<th>Wat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bb</td>
<td>Sc</td>
<td>Asn181</td>
<td>Asn197</td>
<td></td>
</tr>
<tr>
<td>HuPrP</td>
<td>202.7</td>
<td>40.6</td>
<td>0.7</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>glyc-HuPrP</td>
<td>212.3</td>
<td>37.1</td>
<td>83.3</td>
<td>6.8</td>
<td>191.6</td>
</tr>
<tr>
<td>solv-NGlyc</td>
<td>98.3</td>
<td>2.6</td>
<td>0.0</td>
<td>0.6</td>
<td>35.4</td>
</tr>
<tr>
<td>solv-GPI</td>
<td>89.8</td>
<td>34.9</td>
<td>5.5</td>
<td>33.3</td>
<td>76.7</td>
</tr>
<tr>
<td>cho-GPI</td>
<td>34.5</td>
<td>9.2</td>
<td>78.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eap-GPI</td>
<td></td>
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</tbody>
</table>

\*Analysis of the H bonds is split into protein backbone (Bb) and side chain (Sc) atoms, GPI anchor (GPI), N-linked glycan (NGlyc), membrane (Memb), and Water (Wat).

<p>| Table IV. Comparison between simulations of the GPI anchor and NMR experiments of similar GPI structures, showing corresponding NOE constraints from the NMR experiments I (Homans et al., 1989) and II (Weller et al., 1994), and average distances from the simulations |</p>
<table>
<thead>
<tr>
<th>NOE-constraints (Å)</th>
<th>Average distance in simulations (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I ( ^a )</td>
</tr>
<tr>
<td>mIno(^{2} )H6 ↔ GlcN(^{1} )H1</td>
<td>2.6</td>
</tr>
<tr>
<td>Glc(^{2} )H4 ↔ Man(^{4} )H1</td>
<td>2.4</td>
</tr>
<tr>
<td>Man(^{4} )H3 ↔ Man(^{4} )H1</td>
<td>1.8–2.7</td>
</tr>
<tr>
<td>Man(^{4} )H2 ↔ Man(^{5} )H5</td>
<td>1.8–2.7</td>
</tr>
<tr>
<td>Man(^{5} )H1 ↔ Man(^{5} )H5</td>
<td>2.5</td>
</tr>
<tr>
<td>Man(^{5} )H2 ↔ Man(^{5} )H1</td>
<td>2.3</td>
</tr>
</tbody>
</table>

\( ^a \)mIno\(^{2} \), Glc\(^{1} \)-Man\(^{4} \)-Gal-\( \cdots \)-Man\(^{5} \)-PO\(^{2} \)-Gal-\( \cdots \).

\( ^b \)(mIno\(^{2} \)-Glc\(^{1} \)-Man\(^{5} \)-Gal-\( \cdots \)-Man\(^{5} \)-PO\(^{2} \)-Gal-\( \cdots \)).
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Fig. 4. Stereoview of the average structure of HuPrP (black) and glyc-HuPrP (gray) from the simulations. The protein structure, including HuPrP from Lys104 to Ser230 only, is shown as a coil of the Cα trace, where the thickness of the coil represents the fluctuations of the Cα atoms. The thicker the coil the more flexible the structure is during the simulation. Also shown is the disulfide bridge (Cys179-Cys214). The N-glycans, NGlyc181 and NGlyc197, and the GPI anchor are shown as ball-and-stick representations for snapshots every 100 ps. The picture was generated using MOLMOL (Koradi et al., 1996) and POVRAY (http://www.povray.org).

Fig. 5. SD of the backbone and glycosidic linkage torsion angles (φ and ψ, and ω in case of a 1–6 linkage) of the NGlyc and GPI models for MD simulations of glyc-HuPrP and of the solvated glycans (solv-NGlyc and solv-GPI), shown as cylinders between the residue symbols (see Figure 1 for description of symbols), where the radius of the cylinder indicates the magnitude of the SD. The radii are grouped into ranges of SD: <5°, <10°, <25°, <35°, and <50°. Dotted boxes identify the core of the glycans with reduced flexibility.

flexible as in the simulations of the free GPI glycan in solution or the GPI glycan in the glyc-HuPrP model. The average SD values of the linkage torsion angles are 31.2° and 27.7° (Table I) for the cho-GPI and the eap-GPI simulations, respectively, values similar to the other simulations. In both simulations, the GPI-anchor glycan has reduced flexibility in the first residues from the membrane linkage, with a unique conformation for the membrane-PO2I-mInoH6-ManIV residues (see Figure 8; compare with Figure 5), but with an average distance between mInoH6→GlcNIII-H1 similar to the other simulations and
The GlcN III-ManIV linkage, on the other hand, shows a different conformation compared with the other simulations or NMR experiments. For both simulations, the remaining part of the GPI-anchor glycan has...
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high flexibility, especially in the \(-\text{PO}_{2}\text{VII-}E\alpha\text{VIII}\) part which is linked to PrP in the glyc-
\(H_u\)PrP model. The main difference between the two models is the distance of this ethanolamine
group from the surface of the membrane. For the cho-GPI model, the distance ranges from 5.5 to 13.0 Å with an average of 8.8 Å, whereas for the eap-GPI model it ranges from 9.5 to 14.8 Å with an average of 12.3 Å. All simulations were carried out by fixing the position of the fatty acids, including that to which the GPI glycan is attached.

Orientation of PrP with respect to the membrane
To find a possible orientation of \(H_u\)PrP with respect to the membrane, we tried to combine directly the structures of the glyc-
\(H_u\)PrP simulation with the structures of the two membrane simulations, cho-GPI and eap-GPI, by aligning only the GPI anchor. But, unfortunately, all resulting conformations had part of the \(H_u\)PrP structure overlapping the membrane. Therefore, models were generated by taking the GPI-anchor core structure, \(\text{Man}^{\alpha}\text{-Man}^{\beta}\text{-GlcN}^{\alpha}\text{-mno}_{\text{PO}}^{\alpha}\text{-membrane}\), from one of the membrane simulations, in this case from cho-GPI, and the remaining part of the GPI anchor from the glyc-
\(H_u\)PrP simulation. We then varied the torsion angles of the Ser\(^{230}\)-
\(E\alpha\text{VIII-PO}_{2}\text{VII}\) group which links to \(H_u\)PrP, in such a way as to get structures with reasonable properties, e.g., no clashes between \(H_u\)PrP and the membrane. The first conformation we were able to generate was a structure in which the GPI anchor had approximately the same orientation with respect to PrP as in the glyc-
\(H_u\)PrP simulation (A in Figure 9). In this conformation, the PrP is lying down on the membrane with its Helix-A close to the membrane and the two NGlyc chains facing in the opposite direction. The N-terminal part of \(H_u\)PrP is, thus, close to the membrane as well. As the N-terminal region of PrP has an overall positive electrostatic potential (see Figure 7) this might result in an unfavorable orientation. The second type of conformation we generated is based on several concepts: that GPI-anchored proteins have a tendency to form multimers on the surface of membranes (Ferguson, 1992; Vaughan, 1996; Maxfield and Mayor, 1997; Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998); that a dimer of PrP has been reported (Priola et al., 1995); that the first step in the propagation of \(PrP^{SC}\) is suggested to be complex formation between \(PrP^{SC}\) and \(PrP^{C}\) (Prusiner, 1991) and that the primary binding site for such a complex is suggested to be the region PrP\(^{119-141}\) (Warwick, 1997; Horiiuchi and Caughey, 1999); and that Helix-A may also act as a hydrophilic seed for such dimerization (Morrissey and Shakhnovich, 1999).
resulting conformation, shown in Figure 9B, has PrP standing on the membrane, with the GPI glycan forming an extension to Helix-C and with the two NGlyc chains even further away from the membrane. Figure 9B shows a possible dimer in which the two Helix-A’s are in antiparallel orientation. This antiparallel aggregation would allow the formation of four salt bridges between the two helices (Morrissey and Shakhnovich, 1999). In addition, the two C-terminal regions of the PrPSc-PrPC binding region (Horiuchi and Caughey, 1999), PrP135–141 (see pp in Figure 9B), are also in close proximity in an anti-parallel orientation. A similar region was used as the major interaction site between the monomers in the proposed dimer model of Warwicker (1997). The N-terminal parts of the HuPrPs are oriented in opposite directions, and are further away from the membrane than in the lying-down conformation. The NGlyc chains would not interfere with Helix-A aggregation, but would create a negative electrostatic potential all around the dimer, with two positive potentials from the N-terminal parts of PrP oriented parallel to the membrane but in opposite directions.

Discussion

Mammalian PrP contains two consensus sites for N-linked glycosylation, Asn181 and Asn197 in HuPrP. Deactivating the N-glycosylation site at Asn180 (MoPrP) by mutating Thr182→Ala, led to intracellular accumulation of PrP, whereas deactivating the N-glycosylation site at Asn196 by a Thr198→Ala mutation, showed no intracellular accumulation, but reduced delivery of PrP to the plasma membrane (Rogers et al., 1990; Lehmann and Harris, 1997). Both mutations showed properties of a PrPSc-state form (Lehmann and Harris, 1997), but only one of the two mutations, Thr183→Ala in HuPrP, is actually known to be disease-related in humans (Ironside, 1998). Apart from being important for cell trafficking, the N-glycosylations have been suggested to have an influence on the folding process and structural stability of PrP (Harris, 1999). Studies with the Asp178→Asn mutation, which is also associated with CJD and FFI in humans (Ironside, 1998) and is close to the Asn181 glycosylation site, suggested that the instability of the mutant PrP is partially corrected by N-glycosylation (Petersen et al., 1996). This stabilization is further suggested to be influenced by the amino acid at position 129 (Met or Val). It has been suggested that PrP has an intrinsic tendency to acquire PrPSc-like properties and protease resistance, and that the N-glycan chains might protect it against these changes (Lehmann and Harris, 1997). This suggestion is based on experiments that indicate correct folding of PrP is essential for correct processing and biosynthetic transport of the protein: for example eliminating the disulfide bridge (Capellari et al., 1999; Ma and Lindquist, 1999; Yanai et al., 1999) resulted in PrP being trapped in the
endoplasmic reticulum (ER) where it accumulated in a PrPSc-like form (Daude et al., 1997; Hedge et al., 1998). Glycosylation is able to protect PrP against incorrect folding in the ER, but is not necessarily required for biosynthetic transport (Lehmann and Harris, 1997). For example, analysis of PrPSc revealed different glycosylation forms ranging from di-, to mono- to non-glycosylated PrP (Caughey et al., 1989). Even though different prion strains sometimes display distinctive glycosylation patterns (Harris, 1999), no straightforward relationship could be identified between the different glycosylations and the natural cycling of PrP between cell surface and endosomes, or the formation of PrPSc in late endosomes.

Detailed chemical analysis of the carbohydrate chains of PrPSc purified from scrapie-infected hamster brain showed that both N-glycosylation sites of PrP are occupied, and that a mixture of bi-, tri-, and tetra-antennary complex-type oligosaccharide chains is present, with about 70% of the terminal galactose residues linked to sialic acid (Endo et al., 1989). More detailed analysis of PrPSc from scrapie-infected mouse showed similar oligosaccharide structures as for hamster PrP (Rudd et al., 1999a; Stimson et al., 1999). In addition, the analysis revealed that the termini of the oligosaccharides consist of Lewisia (Le) and sialyl Lewisia groups (SiaLe), Galβ(1–3)Galβ(1–4)GlcNAc and NeuNAc(2–3)Galβ(1–4)GlcNAc, respectively. The structures of all these different oligosaccharides, 60 altogether, seem to derive from one tetra-antennary oligosaccharide complex by partial degradation. Both N-glycosylation sites are found to have the same oligosaccharide complex, although with slightly different degradation stages. Even though all detailed structural studies of the oligosaccharides of PrP have been made on PrPSc, the cellular form PrP, it is suggested to have a similar type of oligosaccharide composition (Haraguchi et al., 1989). A recent, more detailed study indicated that PrPSc and PrPSc contain the same set of oligosaccharides, but with different relative proportions of individual glycans (Rudd et al., 1999a).

In this work we investigated the possible influence of the N-linked oligosaccharides and the GPI anchor on the protein structure of human PrP (HuPrP). To achieve this, we compared the trajectories from a molecular dynamics (MD) simulation of HuPrP with all oligosaccharides (glyc-HuPrP) with those from a simulation of HuPrP without any of the glycans (HuPrP). Previous MD simulations (Zuegg and Greardy, 1999) showed that the structures of a homology model of HuPrP and the NMR structure of ShPrP are highly sensitive to the electrostatic environment, and that only correct treatment of the long-range electrostatic interactions resulted in a stable structure. As both N-glycans can carry up to three negatively charged SiaLex groups, which would change the electrostatic environment of HuPrP significantly, investigating their effect on the protein structure appeared necessary. For the fully glycosylated HuPrP model, a N-glycan model was generated, which can be considered as the glycan from which all the oligosaccharides found experimentally (Endo et al., 1989; Rudd et al., 1999a; Stimson et al., 1999) could be derived by partial degradation. However, as the exact branching structure could not be determined in the experiments the model has some uncertainties, for example the distribution of the SiaLe groups among the branches. Similarly, the GPI-anchor model used also has some uncertainty, as experiments could neither identify the type of glycosidic linkage nor the anomeric type in one branch (Stahl et al., 1987, 1992; Baldwin et al., 1993).

In the MD simulation of the glyc-HuPrP model, both C-terminal α-helices, Helix-B and Helix-C, not only have increased length compared with the simulation of non-glycosylated HuPrP, but also the split in Helix-C observed in the HuPrP simulation does not occur. In addition, residues in Turn-D, between Helix-B and C, showed reduced variation in the backbone torsion angles. On the other hand, residues in Turn-C, between Strand-B and Helix-B, showed increased variation of the backbone torsion angles during the simulation, as did the residues in the N-terminal part, PrP90–126. Taken together, the results suggest that most of the structured part of the protein, HuPrP127–237, experiences a stabilizing effect from addition of the glycans. The perturbation to Turn-C by the presence of the glycans is not associated with any direct interaction between Turn-C and NGlyc or with the GPI anchor. NMR experiments of MoPrP121–231 (Billeter et al., 1997), ShPrP90–211 (James et al., 1997), and HuPrP (Hosszu et al., 1999) show variations in the lengths of all three α-helices, similar to the differences found between our glycosylated and nonglycosylated HuPrP models. Although the C-terminal part of PrP forms a defined structure very quickly (Wildegger et al., 1999), the differences found in the NMR experiments may be attributed to the lack of glycans. As absence of the glycans results in higher flexibility of the protein backbone, different sequences might adjust to this potential instability differently, some in Turn-D and some in Helix-C. Our simulation of the glycosylated HuPrP model suggests that both N-glycans are able to stabilize PrP in these regions.

The N-terminal part of HuPrP, HuPrP90–127, changes its dynamical behavior to more flexible on glycosylation. However, this may be due to the fact that its starting conformation was randomly chosen as NMR experiments show it is disordered and, thus no structural data are available. Hence, using a different starting conformation might result in different structural and dynamical behavior of the N-terminal part. Despite these uncertainties, the simulations suggest that glycans have some structural influence on the N-terminal part of HuPrP, especially on the residues immediately before the first strand, HuPrP120–130, which are close to NGlyc181. It is possible that this part would form a defined structure under the influence of the glycans, but unfortunately the simulation time of ~2 ns is far too short to see development of any significant secondary structure.

Interestingly, the influence of the glycans on the protein appears not to be due to specific direct interactions, such as H bonds or salt bridges. Indeed, each glycan has only one to two H-bond interactions with the protein. The influence of the glycans seems to be more indirect, by reducing the mobility of the surrounding water molecules. Throughout the simulation, some parts of the glycans in both NGlyc chains are not more than one water molecule away from the protein. Thus, both N-glycans together cover all residues in Turn-D and all surface-facing residues of Helix-B. This includes Lys194, which is reported to be a cleavage point for trypsin only when the glycan at the Asn197 site is small (Stimson et al., 1999). In addition, NGlyc181 covers one residue in Helix-A and NGlyc181 some residues just before the first strand. The extent of the area covered by the N-glycans depends, of course, on their conformations. The current simulations can be considered only as an estimate for the nature and extent of the glycan cover, due to uncertainties in the glycan models used and the fact that a full investigation
of the conformational space of the glycans would require simulations 100 times longer (Peters and Pinto, 1996). However, as the main feature of the N-glycan cover is its negative electrostatic potential, produced by the charged \( \text{SiaLex} \) groups, its extent is less sensitive to conformational details of the glycans. The negative electrostatic field extends over the whole surface of Helix-B and Helix-C onto the opposite side of \( \text{PrP} \) to Helix-A. Even though the N-glycans do not sterically cover Helix-C, the negative field extends to the C-terminal end of Helix-C because of negatively charged residues on the surface of Helix-C and because of the \( \text{PO}_4 \) group in the GPI anchor. The field has a positive counterpart at the N-terminal part of the \( \text{HuPrP} \) model, which has several positively charged residues. The orientation of the dipole moment depends entirely on the position of the N-terminal part, which in all simulations and NMR experiments shows no defined structure. In between the two dominant fields is Helix-A, which has a quite unusual nature. Helix-A, \( \text{PrP}^{144-152} \), with an amino acid sequence of \( \text{DYEDRYERE} \), is entirely hydrophilic and is stabilized by the salt bridges \( \text{Glu}^{166\rightarrow\text{Arg}^{208}} \), between Helix-A and Turn-D, and \( \text{Arg}^{156\rightarrow\text{Glu}^{196}} \), between the neighboring Turn-B and Turn-D. Interestingly, \( \text{Arg}^{208} \) is a residue whose mutation into His is known to be associated with inherited forms of human disease (Irons, 1998). The charged surface residues of Helix-A makes it a good candidate for aggregation in either a parallel or anti parallel orientation, forming in each case two salt bridges (Morrissey and Shakhnovich, 1999). As Helix-A is not covered by either of the dominant electrostatic fields, it is, therefore, electrostatically accessible. An antiparallel aggregation would not disrupt its structure, and would lead to the \( \text{HuPrP} \) dimer as shown in Figure 9B, with an extended cover by the N-glycans on top and on the side and with the N-terminal parts sticking out in opposite directions from the middle. A PrP dimer seems to occur naturally (Priola et al., 1995) or, at least, be the starting point for the conversion from \( \text{PrP}^\text{C} \) to \( \text{PrP}^\text{Sc} \) (Prusiner, 1991; Warwicker, 1997). By contrast, a parallel aggregation of Helix-A would disrupt the helical structure and lead to \( \beta \)-nucleation, which has been suggested to form the mechanism for the conversion of \( \text{PrP}^\text{C} \) to \( \text{PrP}^\text{Sc} \) (Morrissey and Shakhnovich, 1999). Interestingly, a mutation of \( \text{Arg}^{208} \) would disrupt the salt bridge between Helix-A and Turn-D and might, thereby, facilitate the transformation of Helix-A into \( \beta \)-sheet conformation, necessary for the parallel aggregation. On the other hand, our model suggests that the N-glycans are not favorable for such an orientation, even though such aggregation could include major rearrangements in the N-terminal part of the \( \text{structured} \) portion of PrP, and, thus, would lead to different locations and relative orientations of the residues of the former Helix-A region, such as opposite Helix-B and Helix-C as in a proposed model of \( \text{PrP}^\text{Sc} \) (Huang et al., 1996). However, in addition to inferences from our model, experiments showed that binding of antibody specific for the Helix-A region is not able to inhibit the first step in the formation and aggregation of \( \text{PrP}^\text{Sc} \), namely the binding of \( \text{PrP}^\text{Sc} \) to \( \text{PrP}^\text{C} \) (Horiuchi and Caughey, 1999).

The antibody-binding experiments also showed that antibody specific for the region \( \text{PrP}^{218-231} \) is able to inhibit the binding of \( \text{PrP}^\text{Sc} \) to \( \text{PrP}^\text{C} \) (Horiuchi and Caughey, 1999). This region is in the vicinity of the \( \text{protein X} \) binding region (Telling et al., 1995; \( \text{Glu}^{168}, \text{Gln}^{172}, \text{Ile}^{215}, \text{and Glu}^{219} \), where \( \text{protein X} \) is suggested to play a role during \( \text{PrP}^\text{Sc} \) propagation (Kaneko et al., 1997a). Even though this region, involving Turn-C and the end of Helix-C, is not entirely covered by the N-glycans the proximity of the negative electrostatic potential would not favor an aggregation in this region. As both N-glycans and the GPI anchor do not influence the formation of the \( \text{PrP}^\text{Sc}-\text{PrP}^\text{C} \) complex (Kaneko et al., 1997b) and \( \text{PrP} \) lacking Turn-C is still able to generate a \( \text{PrP}^\text{Sc}-\text{like} \) form (Muramoto et al., 1996), the region around Strand-A, \( \text{PrP}^{119-138} \), has been suggested to form the potential binding site in \( \text{PrP}^\text{Sc}-\text{PrP}^\text{C} \) complex formation (Horiuchi and Caughey, 1999). In addition, this region includes some of the residues important for the species barrier for disease transmission (Kocisko et al., 1995; Schätzl et al., 1995), and was used as the primary binding site for a dimer model (Warwicker, 1997). In our solvated model glyc-\( \text{HuPrP} \) (Figure 4), this region is not influenced by the oligosaccharides and is exposed to the solvent. The monomer model of the membrane-bound \( \text{HuPrP} \) (Figure 9A) shows this region is close to the membrane. On the other hand, the dimer model (Figure 9B) has part of this proposed \( \text{PrP}^\text{Sc}-\text{PrP}^\text{C} \) binding region, \( \text{PrP}^{15-141} \) next to each other. The earlier proposed dimer model (Warwicker, 1997) used the \( \text{PrP}^{130-136} \) region as the primary binding site, but this resulted in a different orientation, in which both C-terminal ends were pointing in opposite directions, thus making it impossible for both monomers to be bound to the membrane by GPI anchors. Our dimer model can be seen as a possible model not only for a reported naturally occurring dimer (Priola et al., 1995), but also as a possible dimer starting point in \( \text{PrP}^\text{Sc} \) propagation. The monomer model shows the N-terminal part of \( \text{PrP} \) closer to the membrane, more in agreement with experiments suggesting that this part makes strong interactions with the membrane (Morillas et al., 1999), but due to the uncertainty of the structure and its high flexibility, an orientation of this N-terminal part towards the membrane is not excluded in the dimer model.

The mutation experiments which deactivated each N-glycosylation site (Lehmann and Harris, 1997), showed that the Asn\(^{181} \) site seems to be associated more with a cell-trafficking role, whereas the Asn\(^{197} \) site seems to stabilize the PrP structure. Structures from the glyc-\( \text{HuPrP} \) simulation show \( \text{NGlyc}^\text{181} \) affecting an area which already has a stable secondary structure, whereas \( \text{NGlyc}^\text{197} \) affects an otherwise more flexible area of a turn by reducing its flexibility. This is consistent with Asn\(^{181} \) glycosylation having a more functional role. A similar suggestion has been made in comparing \( \text{PrP} \) with the \( \text{PrP} \) like protein doppel (Dpl), where only the site analogous to Asn\(^{111} \) is conserved in the Dpl sequence (Asn\(^{111} \); Moore et al., 1999). Note, however, that \( \text{NGlyc}^\text{181} \) contributes more to the negative electrostatic potential covering of \( \text{HuPrP} \), as \( \text{NGlyc}^\text{197} \) is more solvent exposed. The second N-glycosylation site of Dpl at Asn\(^{199} \) would be in Turn-C which, assuming a similar 3-D structure, would result in an N-glycan oriented on the same side of the protein as Asn\(^{111} \) but closer to the C-terminal part of the protein. In addition, this site is closer to Asn\(^{111} \) than Asn\(^{197} \) is to Asn\(^{181} \) in \( \text{PrP} \), thus restricting the available space for oligosaccharides, and suggesting that the N-glycans of Dpl might be a different size compared with the \( \text{PrP} \) N-glycans, and cover Helices B and C at the C-terminal part of the protein more than in the Turn-D region as in \( \text{PrP} \).

The model for the N-glycans used in this work represents the largest and the most charged glycan possible. In addition, both
N-glycosylation sites were considered to be occupied by the same glycan. Partial degradation of the N-glycans would, of course, change their properties, especially cleavage of the charged NeuNAc residues would reduce the negative electrostatic potential. Detailed analysis of the N-glycans of PrP showed substantial heterogeneity in their structure (Endo et al., 1989; Rudd et al., 1999a; Stimson et al., 1999). It has also been reported that PrPSc strains, encoding distinct disease phenotypes, are associated with different patterns of glycosylation (Collinge et al., 1996; Mastrianni et al., 1999; Somerville, 1999). However, our work showed that even the most charged N-glycans do not have a major influence on the conformational structure of HuPrP, and that most of the important parts of PrP9-C with respect to proposed mechanism for PrPSc formation (N-terminal part, Strand-A, Turn-A, and Turn-C), are accessible even with the largest possible N-glycans. Recent experiments showed a different glycosylation pattern between PrP9-C and PrPSc, which correlated with differences in the activity of N-acetylglucosaminyltransferase III and suggested that some cells forming PrPSc undergo changes that diminish the activity of an enzyme in the glycosylation pathway (Rudd et al., 1999a). Also, different protein conformations can be attributed to different PrPSc strains (Caughey et al., 1989; Hill et al., 1997; Safar et al., 1998), with some conformational differences being correlated with concentrations of Cu2+ and Zn2+ ions (Wadsworth et al., 1999). Taken together, these results suggest that the structure of PrPSc is more variable than the PrP9-C structure, and that the structure of PrPSc is more sensitive to particular aspects of the environment, such as the presence of metal ions or glycosylation, or the more general status of the PrPSc forming cell.

The GPI anchor appears to have only a minor influence on the structure of HuPrP, in contrast to the report for the Thy-1 protein (Barboni et al., 1995). Its role seems to consist entirely in attaching PrP to the membrane. Simulations of the GPI anchor in a membrane showed that it keeps the protein at a distance between 9 and 13 Å from the membrane surface, depending on the type of membrane-head group. This distance is enough to maintain several water shells between the membrane surface and the protein and is, thus, enough to guarantee a high degree of freedom for the movement and orientation of PrP. This orientational freedom is assisted by the structurally flexible linkage group, ethanolamine, and by the C-terminal end of the protein which does not have any interactions with the rest of the model and is highly flexible.

In summary, it has been shown that the N-linked glycans do reduce the flexibility of the protein backbone in some parts of PrP, such as residues in the turn between Helix-B and Helix-C and within those helices, but perturb it in other parts to increase the flexibility, such as residues in the turn between Strand-B and Helix-B. But the main influence of the N-glycans appears to come from its negative charges, generating a negative electrostatic field which covers the whole surface of Helix-B and Helix-C. In addition, the simulations show that the GPI anchor has little influence on the structure of PrP. Its flexible structure guarantees a high degree of freedom for the orientation of PrP, but at the same time keeps the protein 9–13 Å above the membrane surface. A possible orientation of HuPrP could be generated which takes into account the unique nature of Helix-A and the reported PrPSc-PrP binding regions, allowing HuPrP to form a homodimer on the membrane.

Materials and methods

Force field parameter set

All molecular dynamics simulations were carried out using the Amber 5 package (Pearlman et al., 1995; Case et al., 1997) and the all-atom Amber94 force field (Cornell et al., 1995). For the oligosaccharides, the Glycam93 force field (Woods et al., 1995) was adapted to the Amber94 force field. The Glycam93 parameter set was originally generated by adding 3 atomtypes (anomeric carbon atom for each anomer and the glycosidic oxygen) to the standard protein Amber91 force field (Weiner et al., 1986). In cases where the parameters in Glycam93 for the relevant tetrahedral atom and the ethertype oxygen atom were the same as those in the Amber91 parameter set, the parameters were changed to the corresponding Amber94 parameter set values. These comprised all the van der Waals parameters and most of the dihedral parameters. For the other cases, which included mostly bond distance and bond angle parameters, the parameters were set to the original Glycam93 parameter set values. The main difference between Amber91 and Amber94 parameters is the absorption of the H-bond energy term into the van der Waals potentials (Cornell et al., 1995). Therefore, partial charges of all sugar residues have been recalculated in the same way as for the Amber94 parameter set, namely by fitting the electrostatic potential, calculated with the ab initio method HF/6-31G* using GAUSSIANS94 (Frisch et al., 1994), with the RESP program (Cornell et al., 1993). Default conformations for the sugar residues have been used, using the C1 conformation for Fucα, the C2 conformation for NeuNACα and the C1 conformation for all remaining sugars. In the same way, partial charges have been generated for the new non-sugar residues myo-inositol, ethanolamine, choline and diacylglycerol. Default bonding parameters for these residues have been used.

Model building for glyc-HuPrP

The HuPrP model was generated as described previously (Zuegg and Greedy, 1999), and consists of a homology model of human PrP9-C based on the domain structure of Syrian hamster PrP (James et al., 1997). The HuPrP model had its ionizable residues set to their solution ionization state at pH 7, as calculated with the program TITRA (Petersen et al., 1997). The starting models for the N-linked glycans (NGlyc) and GPI-anchor (GPI) oligosaccharides were generated by assuming a 1C1 conformation for Fucα, a 2C2 conformation for NeuNACα and a 1C1 conformation for all remaining sugar residues, and changing only the glycosidic linkage torsion angles to produce a model with no steric clashes. Several minimization steps were required in the building process to obtain a low energy conformation of the models. The model oligosaccharides were attached to the HuPrP model, one N-linked glycan model at each Nδ atom of Asn181 and Asn197, and one GPI model at the backbone C atom of Ser230. The GPI model was terminated at the PO2I group with a CH3 group. The resulting model, glyc-HuPrP, has 3376 atoms and a molecular mass of 25.3 kDa, compared with the HuPrP model with 2180 atoms and a mass of 16.0 kDa. In order to use the particle mesh Ewald (PME) method (Darden et al., 1993) for the calculation of long-range electrostatic interactions in the simulations, the system has to be neutralized. As in the previous work (Zuegg and Greedy, 1999), this was achieved by adding Na+ and Cl− ions to the
system. For the glyc-\textit{HuPrP} model, 20 \(\text{Na}^+\) and 15 \(\text{Cl}^-\) ions were added with the CION program in the AMBER 5 package, compared with 12 \(\text{Na}^+\) and 13 \(\text{Cl}^-\) ions added to the \textit{HuPrP} model. Both models were immersed in a rectangular box of pre-equilibrated TIP3 water molecules (Jorgensen et al., 1983), of dimensions of 66 \(\times\) 63 \(\times\) 58 Å, containing 5143 water molecules for the \textit{HuPrP} model, and 68 \(\times\) 86 \(\times\) 78 Å with 10321 water molecules for the glyc-\textit{HuPrP} model.

**Model building for solvated glycans**

To analyze any structural and dynamical restrictions of the glycans due to the protein, simulations were carried out with free glycan models. The same NGlyc model as used as a starting model for glyc-\textit{HuPrP} was capped at the first GlcNAc\textsuperscript{1} with a NH-CO-CH\textsubscript{3} group, neutralized by adding 3 \(\text{Na}^+\) ions with the CION program, and immersed in a box of water (solv-NGlyc). Similarly, a solvated, free GPI-anchor glycan model, solv-GPI, was generated by replacing the protein backbone with a CO-CH\textsubscript{3} group on one side and the membrane glycerol with a CH\textsubscript{3} group on the other side. After adding one \(\text{Na}^+\) ion to neutralize the model, the system was immersed in a box of water. Both solvated models were simulated with the same MD parameters as the glyc-\textit{HuPrP} and glyc-\textit{HuPrP} models.

**GPI anchor and membrane model**

For simulations of the GPI anchor attached to a membrane, two different models were generated differing only in the head group of the membrane monomer. The membrane was generated as a monolayer of a short C\textsubscript{10}, fatty acid using 1,2-diveralanyl-phosphatidyl-ethanolamine (eap-GPI) or 1,2-diveralanyl-phosphatidyl-choline (cho-GPI) as the monomer. The GPI-anchor model was attached to the membrane by replacing one of the head groups with the GPI glycan. The GPI-anchor model was then covered by a cap of water molecules, containing 1244 and 1107 water molecules for eap-GPI and cho-GPI, respectively.

**MD simulations**

All MD simulations were performed using the SANDER module in the AMBER package. Systems were equilibrated by minimization and short constant pressure simulations as described in the previous work (Zuegg and Gready, 1999). In the simulations, Newton’s equations of motion were integrated with a step size of 1 fs, with lengths of all bonds involving hydrogen atoms constrained using the SHAKE algorithm with a relative tolerance of 5 \(\times\) 10\textsuperscript{-6} Å. A pair-list to calculate non-bonded interactions was generated every 50 simulation steps. The temperature of the system was controlled to be 300 K using two independent Berendsen thermostats (Berendsen et al., 1984), one for the solute and one for the solvent, and with coupling times \(\tau_{\text{Salt}} = 0.5\) ps and \(\tau_{\text{Water}} = 0.75\) ps.

For all models in a box of water, \textit{HuPrP}, glyc-\textit{HuPrP}, solv-Nglyc, and solv-GPI, the PME method was used to calculate the electrostatic interactions, using grid sizes which produce a grid spacing of \(\sim 1\) Å. These simulations were carried out using periodic boundary conditions and constant volume, and removing the overall translational and rotational motion of the system every 100 time steps.

For the models with a water cap, cho-GPI and eap-GPI, no PME or periodic boundary conditions could be used. Instead, the electrostatic interactions were calculated by truncating the interaction at a distance of 8 Å, with the water cap being restrained to its cap-like form. In addition, the fatty acids of the membrane model were restrained to their starting positions, in order to maintain the shape of the monolayer.

**Analysis**

NMR experiments on ShPrP\textsuperscript{90–231} revealed a highly flexible N-terminal part with only the C-terminal part having a defined secondary structure (James et al., 1997). Therefore, all the root-mean-square deviation (RMSD) analysis of the structure was calculated not only for the complete model (PrP\textsuperscript{90–230}), but also for the flexible part (PrP\textsuperscript{90–126}) and the structured part (PrP\textsuperscript{127–237}) separately. Analysis of molecular trajectories was done with the program CARNAL in AMBER 5, including structural alignment and calculation of the RMSDs of the structures, and also torsion angle analysis. Analysis of the secondary structure was done with the DSSP program (Kabsch and Sander, 1983). Salt bridges were defined by the distance between the positively and negatively charged heavy atoms. For Arg residues, all three nitrogen atoms of the side chain, N\textsubscript{\textalpha}, N\textsubscript{\textbeta}, and N\textsubscript{\textgamma}, were used. A salt bridge was deemed present if the distance between the two heavy atoms less the corresponding van der Waals radii was less than 1.5 Å. Calculation of the electrostatic potential was carried out with the DELPHI program (Gilson and Honig, 1987) in INSIGHTII-98 (1998, Molecular Simulations Inc., San Diego, CA), using default van der Waals radii and the same partial charges as in the simulation with AMBER. For model manipulation and visual analysis, INSIGHTII was used. The pictures were generated using MOLDSCRIPT (Kraulis, 1991), RASTER3D (Merritt and Bacon, 1997), MOLMOL (Koradi et al., 1996) and POVRAY (http://www.povray.org). The calculations and analysis were carried out on SGI Power-Challenge (SGI-PC) and Fujitsu VPP300 (VPP) supercomputers, and SGI Indigo2 and Octane workstations.

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**Abbreviations**

PrP, prion protein; \textit{HuPrP}, human prion protein; ShPrP, Syrian hamster prion protein; \textit{MoPrP}, mouse prion protein; Dpl, doppel protein; CJD, Creutzfeld-Jacob disease; GSS, Gerstmann-Sträussler-Scheinker syndrome; FFI, fatal familiar insomnia; ER, endoplasmic reticulum; GPI, glycosyl phosphatidylinositol; Man, D-mannose; GlcNAc, N-acetyl-D-glucosamine; GlcN, D-glucosamine; Gal, D-galactose; GalNAc, N-acetyl-D-galactosamine; NeuNAc, N-acetyl-D-neuraminic acid; Fuc, L-fucose; mIono, n-myo-inositol; E, ethanolamine; SiaLe\textsubscript{a}, sialyl Lewis\textsubscript{a}, NeuNAcα(2–3)Galβ(1–4)(Fucα(1–3)GlcNAc; MD, molecular dynamics; PME, particle mesh Ewald; RMSD, root mean square deviation; SD, standard deviation.


