The intrinsic stability of the human prion β-sheet region investigated by molecular dynamics

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Human prion diseases are neurodegenerative disorders associated to the misfolding of the prion protein (PrP). Common features of prion disorders are the fibrillar amyloid deposits and the formation of prefibrillar oligomeric species also suggested as the origin of cytotoxicity associated with diseases. Although the process of PrP misfolding has been extensively investigated, many crucial aspects of this process remain unclear. We have here carried out a molecular dynamics study to evaluate the intrinsic dynamics of PrP β-sheet, a region that is believed to play a crucial role in prion aggregation. Moreover, as this region mediates protein association in dimeric assemblies frequently observed in prion crystallographic investigations, we also analyzed the dynamics of these intermolecular interactions. The extensive sampling of replica exchange shows that the native antiparallel β-structure of the prion is endowed with a remarkable stability. Therefore, upon unfolding, the persistence of a structured β-region may seed molecular association and influence the subsequent phases of the aggregation process. The analysis of the four-stranded β-sheet detected in the dimeric assemblies of PrP shows a tendency of this region to form dynamical structured states. The impact on the β-sheet structure and dynamics of disease associated point mutations has also been evaluated.

Keywords: Computer simulations; prion peptide stability; prion toxicity; neurodegenerative diseases

Introduction

Transmissible spongiform encephalopathies, also known as prion diseases, are neurodegenerative disorders associated to the misfolding of the prion protein (PrP) (Aguzzi, Baumann, & Bremer, 2008; Eichner & Radford, 2011; Prusiner, 1998). They include sporadic, infectious, and genetic forms such as the Creutzfeldt–Jacob diseases, the Gerstmann–Straussler–Scheinker syndrome, fatal familial insomnia, and kuru (Parchi, Strammiello, Giese, & Kretzschmar, 2011). A distinctive trait of prion disorders is the deposition of fibrillar amyloid aggregates of an abnormal protease resistant form of PrP (PrPSc). Prefibrillar oligomeric species arising from primary/secondary nucleation or fibrillar fragmentation have been individuated as the harmful species at the origin of the cytotoxicity associated with these diseases (Glabe, 2008; Xue, Hellewell, Hewitt, & Radford, 2010). Misfolded PrPSc forms are the infectious agents which also cause distinct heritable prion strains with different phenotypes in genetically identical animals (Collinge & Clarke, 2007; Tanaka, Collins, Toyama, & Weissman, 2006). It has been suggested that strain specific properties result from distinct multiple infectious conformation/aggregation states of PrPSc molecules.

Several aspects regarding the origin of these diseases and their transmission are still obscure. Moreover, the structural features and the molecular basis of the toxicity of amyloid-like fibers and oligomeric species associated with these disorders are highly debated (Xue et al., 2010). Nevertheless, a large consensus is found in the link between the disease and the conversion of the normal cellular PrP protein to a β-sheet-rich pathogenic form, PrPSc (Collinge, 2001).

The PrP in its native state is composed of a globular domain that extends approximately from residues 125–228 and an N-terminal flexible, disordered domain starting at the residue 23. The first 22 residues are cleaved shortly after translation. The globular domain is composed of three α-helices and a small antiparallel β-sheet formed by two β-strands (strand S1: residues 129–131 and strand S2: residues 161–163). In physiological conditions PrPSc is attached to the outer cellular membrane via a glycosylphosphatidyli-
nositol anchor. Moreover, in the mature form the protein is glycosylated at 181 and 197 asparagine residues.

The analysis of the crystal packing of globular region C-terminal domain of PrPSc has unveiled a significant propensity of the protein to form dimeric interactions (Antonyuk et al., 2009; Haire et al., 2004; Lee et al., 2010). Although the specific features of these associations depend on particular state of the protein (presence of mutations and/or formation of macromolecular complexes) and/or the crystal packing, they are invariably mediated by the β-sheet region of the globular fold. Indeed, in all the cases, these dimeric associations lead to the formation of a four-stranded antiparallel β-sheet arising from an intermolecular association of two PrP molecules, each contributing with a two-stranded antiparallel native β-sheet. It has been suggested that the formation of an intermolecular β-sheet can possibly seed the conversion of PrP into PrPSc (Riek et al., 1996; Antonyuk et al., 2009), although under normal conditions this interaction may be disfavored by a β-bulge located on strand S1 that was proposed to play a role of negatively designed element (Richardson & Richardson, 2002) for avoiding edge-to-edge intermolecular β-sheet aggregation (De Simone, Dodson, Fraternali, & Zagari, 2006). It is now clear that PrP misfolding can, however, occur along different conformational transitions (Eghiaian et al., 2007). Among the postulated pathways of misfolding, the detachment of the subdomains S1–H1–S2 and H2–H3 has been strongly supported by many experimental and theoretical evidences (De Simone, Zagari, & Derreumaux, 2007; Schwarzinger, Horn, Ziegler, & Sticht, 2006). In the native state, these two subdomains have uncoupled dynamics (Viles et al., 2001) and show a different response to pH variations (Calzolai & Zahn, 2003) and pressure (Cordeiro et al., 2004; Kuwata et al., 2002). While the detachment of PrP subdomains has been largely characterized, the subsequent conformational transitions remain elusive. In this context, it is crucial to understand the intrinsic stability of the isolated structural units within the two subdomains. It is generally accepted that the subdomain H2–H3 forms a very stable core of the protein either in native (Viles et al., 2001) or in unfolding conditions (Hosszu et al., 1999). Conversely, the subdomain S1–H1–S2 has been accounted for the conformational transitions leading to the fibrillar form of PrP (Govaerts, Wille, Prusiner, & Cohen, 2004). We have recently investigated the conformational properties of the helix H1 when isolated in solution, by pointing out that this structural element can be kinetically trapped in misfolded conformations (Camilloni, Schaal, Schweimer, Schwarzinger, & De Simone, 2012).

As the prion misfolding proceeds through the formation of significantly unfolded states, whose experimental characterization is particularly complicated, molecular dynamics (MD) simulations have been successfully applied to elucidate several aspects of the intricate folding/misfolding process (Behmard, Abdolmaleki, Asadabadi, & Jahandideh, 2011; Blinov, Berjanskii, Wishart, & Stepanova, 2009; Chen, van der Kamp, & Daggett, 2010; Colacino, Tiana, Broglia, & Colombo, 2006; De Simone, Dodson, Verma, Zagari, & Fraternali, 2005; Ma & Nussinov, 2006; O’Brien et al., 2009; Samson & Levitt, 2011; Santini, Claude, Audic, & Derreuxmaux, 2003; Tcherkasskaya, Sanders, Chynwat, Davidson, & Orser, 2003; Zhang, 2009, 2011; Zhang & Liu, 2011; Zhong & Xie, 2009). Taking into account the potential key role of β-sheet in prion aggregation/misfolding, we have undertaken MD studies to evaluate the stability of the β-sheet from the S1–H1–S2 subdomain when isolated in solution. In particular, we employed replica-exchange molecular dynamics (REMD), which allow for an enhanced and significant sampling of protein and peptide conformations (Sugita & Okamoto, 1999) to evaluate the stability of the β-sheet interface between strands S1 and S2 when isolated in solution. Our aim was to evaluate the intrinsic stability of this region outside the native tertiary context, hence mimicking destabilizing conditions associated with the unfolding of the globular domain. REMD simulations were also carried out considering the four-stranded β-sheet assembly that mediates the dimeric intermolecular association of the wild type human PrP bound to the antibody ICSM 18-Fab in the crystalline state (Antonyuk et al., 2009) (Figure 1). Interestingly, the S1 strand contains two point mutations that seem to be involved in prion diseases. In particular, this strand contains the residue 129, which is associated with the polymorphism Met→Val that is involved in the modulation of the susceptibility to prion disease (Baskakov et al., 2005; Gerber et al., 2008; Hosszu et al., 2004; Tahiri-Alaoui, Gill, Disterer, & James, 2004), and residue Gly131 whose mutation to Val has been associated with Gerstmann–Straussler–Scheinker disease (Panegyres et al., 2001). The effect of these two point mutations on the β-sheet assemblies has also been analyzed.

Materials and methods

The systems

REMD simulations have been performed on nine different β-sheet models built from the crystal structure of wt human PrP bound to the ICSM 18-Fab (PDB code 2W9E) (Antonyuk et al., 2009) (Table 1 and Figure 1).

We considered extended protein regions, which contain the wild type short β-strands (strand S1: residues 129–131, and strand S2: residues 161–163). Each filament is made up of 12 residues (A: 158–169, and B: 125–136). In particular, we considered (i) the double-stranded antiparallel β-sheet from a single PrP molecule (AB assembly), (ii) the intermolecular four-stranded antiparallel β-sheet formed by two PrP monomers related by
crystallographic symmetry (ABB'A') and (iii) the double-stranded antiparallel β-sheet formed at the dimer symmetric interface (BB') (Table 1). The symmetry-related monomer (A'B') was generated from the crystal structure PrP coordinates by applying the P6_32_2 symmetry operation (−y, −x, ½−z) followed by the translation symmetry (−1, −1, 0).

For each of the three types of assembly, both mutations G131 and M129 V were modeled. They are located at the B (B') segment, so that nine models were built (Tables 1 and 2). The M129 and G131 V mutants were generated by PyMol software. In the simulations, the N- and C-termini were kept both uncharged, because these regions are internal fragments of PrP molecule. When necessary, Cl− ions were added to neutralize the system.

### Simulation procedure

MD simulations were performed with the GROMACS software package 4.0 (Van Der Spoel et al., 2005) by using OPLS-AA force field (Jorgensen, Maxwell, & Tirado-Rives, 1996).

The models extracted from the Protein Data Bank coordinate files (AB) or generated by crystallographic symmetry (ABB'A', BB') were immersed in a cubic box filled with TIP3P water model. Periodic boundary conditions were applied. The nonbonded interactions were accounted by using the particle mesh Ewald method (grid spacing 0.12 nm) (Darden, York, & Pedersen, 1993) for the electrostatic contribution and cutoff distances of 0.9 nm for van der Waals terms. An integration time step of 2 fs was used.

The REMD protocol includes several replicas of the system evolving independently at different temperatures (T). Replica at adjacent temperatures undergo a partial exchange of configuration information. Temperatures are spaced exponentially with \( T_i = T_0 e^{ki} \), where \( i \) is the number of replicas, \( k \) and \( T_0 \) can be tuned to obtain rea-

### Table 1. Sequences of the peptides characterized in the present study.

| System     | Sequence         | Length | Water molecules | Cl− | Strands | Simulated time (ns) |
|------------|------------------|--------|-----------------|-----|---------|---------------------|
| wt ABB'A'  | PNQTEYPADEY      | 169    | 3396            | −   | 4       | 100                 |
| G131 V ABB'A' | RESASHMYYGL      | 125    | 3393            | −   | 4       | 100                 |
| M129 V ABB'A' | LGGYMIDASMR     | 136    | −               | −   | 4       | 100                 |
| wt AB      | YEDEMPPYQNP      | 158    | 2876            | −   | 2       | 100                 |

Notes: The fragments B and B' are directly involved in the intermolecular interaction. The regions in β-structure are highlighted in grey. The residues involved in point mutations are shown in bold and underlined.

### Table 2. Sampling statistics.

| System     | Average exchange frequency (%) | Atoms (n) | Water molecules | Cl− | Strands | Simulated time (ns) |
|------------|--------------------------------|-----------|-----------------|-----|---------|---------------------|
| wt ABB'A'  | 9.5                            | 764       | 3396            | −   | 4       | 100                 |
| G131 V ABB'A' | 10.0                         | 782       | 3393            | −   | 4       | 100                 |
| M129 V ABB'A' | 10.1                         | 762       | 3400            | −   | 4       | 100                 |
| wt AB      | 13.4                           | 382       | 2869            | −   | 2       | 100                 |
| G131 V AB  | 14.0                           | 391       | 2867            | −   | 2       | 100                 |
| M129 V AB  | 13.7                           | 381       | 2871            | −   | 2       | 100                 |
| wt BB'     | 13.4                           | 346       | 2876            | 2   | 2       | 100                 |
| G131 V BB' | 14.2                           | 364       | 2868            | 2   | 2       | 100                 |
| M129 V BB' | 13.5                           | 344       | 2878            | 2   | 2       | 100                 |
sonable temperature intervals to allow exchanges. In our simulations, the REMD samplings for all systems were composed of 32 replicas ranging from 296.5 to 435 K. The temperatures have been selected to ensuring homogeneous exchange frequencies between replicas. These are: 296.5, 300.5, 304.5, 308.5, 312.5, 316.5, 320.6, 324.7, 328.8, 332.9, 337.1, 341.3, 345.5, 349.8, 354.1, 358.4, 362.7, 367, 371.4, 375.8, 380.3, 385, 389.8, 394.6, 399.5, 404.4, 409.4, 414.4, 419.5, 424.6, 429.8 and 435 K. To achieve consistent exchange frequencies among the various samplings carried out, the same temperature pattern and comparable system dimensions (Table 2) have been adopted. Exchanges between neighboring temperatures are attempted every $t_{\text{swap}}$ on the basis of the Metropolis criterion. A $t_{\text{swap}}$ of 1000 MD steps (2 ps) was chosen in order to allow the kinetic and potential energy of the system to relax.

All the models, including the ones containing the mutated residues, were subjected to energy minimization steps by the steepest descent algorithm. After the initial energy minimization, the systems at each temperature have been equilibrated for 100 ps in order to equilibrate waters and initial thermodynamic properties of each replica. The production run was then started. We used

![Figure 2. Evolution of secondary structure elements in AB two-stranded β-sheet simulations. (A) wt, (B) M129 V and (C) G131 V.](image-url)
the NPT ensemble in all the simulations. The initial velocities were generated according to a Maxwell distribution at each given temperature. A velocity-rescaling thermostat was used for temperature coupling, whereas the Berendsen algorithm was used for pressure coupling.

For all the systems analyzed, the simulation time was 100 ns per each replica. As result, for each replica system a total sampling of $3.2 \mu$s ($100$ ns $\times 32$) was carried out.

The trajectories were checked to assess the quality of the simulation using GROMACS and VMD (Humphrey, Dalke, & Schulten, 1996) routines. Pictures were generated using the programs VMD and PyMol.

**Results**

**Systems and overall REMD features**

REMD simulations have been performed on two-stranded as well as four-stranded $\beta$-sheets of PrP derived from the crystallographic structure of the protein complexed to the ICSM 18-Fab (Antonyuk et al., 2009)(Figure 1(A)).

REMD technique allows for significantly enhanced sampling of conformations, due to frequent switching of simulation temperatures. During one run, the high temperature simulation segments facilitate the crossing of the energy barriers while the low temperature ones explore energy minima in detail (see ‘Materials and methods’ section).

We performed REMD runs on nine different model assemblies (Table 2). Each trajectory comprises the simulation of 32 thermal baths and has a length of 100 ns. All runs have reported significant random walks in the temperature space consistent with fairly good exchange frequencies among replicas (Table 2). These frequencies are essential for the evaluation of the simulation convergence. Each replica explored the whole temperature space by passing repeatedly throughout all thermal baths. Throughout the following sections we present the analysis of the room temperature bath (bath2 = 300.5 K) of sampling.

**AB two-stranded $\beta$-sheet of wt-PrP**

We have investigated the stability of 12-residue regions encompassing the $\beta$-strands outside the protein context. In the starting PrP structure, the short antiparallel $\beta$-sheet consists of three main- chain hydrogen bonds connecting residue 129 with residue 163 and residue 131 with residue 161 (namely: N Met$_{129}$–O Tyr$_{163}$, O Met$_{129}$–N Tyr$_{163}$, and N Gly$_{131}$–O Val$_{161}$) (Figure 1(B)). The additional H-bond, N Met$_{134}$–O Asn$_{159}$, forms a $\beta$-bulge, which in the native PrP is stabilized by a hydration site that is conserved among prions from mammals and non-mammals (De Simone et al., 2006). The dehydration might destabilize the bulge with a consequent stretching and elongation of the antiparallel $\beta$-sheet (De Simone et al., 2005).

The secondary structure composition at 300.5 K shows the persistence of the $\beta$-strand pairings indicating the remarkable stability of the starting $\beta$-sheet model (Figure 2(A)). To get a quantitative measure, we have calculated the percentage of occurrence in $\beta$-strand conformation for each residue, as assigned by the DSSP program used in the GROMACS package (Figure S1). Indeed, on average, residues 129–131 and residues 161–163 form a $\beta$-sheet in 71% of the sampled conformations. The same estimate can be derived from the analysis of the specific distances between atoms involved in the three hydrogen bonds of the original sheet. Moreover, the additional distance O Gly$_{131}$–N Val$_{161}$ has been monitored in the sampling as well as the H-bond of the $\beta$-bulge. The latter, in case of elongation of the sheet, should be replaced by N Ala$_{133}$–O Asn$_{159}$. The specific H-bond has been considered to be formed when the distance between heavy atoms is below 3.3 Å. By using this threshold, the additional H-bond O Gly$_{131}$–N Val$_{161}$ is observed in 45% of the sampled structures. The H-bond of the bulge is only present in about 9% of the structures, whereas the replacing interaction between N

![Figure 3](image-url)  
**Figure 3.** Representative structure of elongated $\beta$-sheet of AB two-stranded $\beta$-sheet model. (A) wt (B) G131 V. Only the backbone is represented. The N- and C-termini are indicated as blue and red spheres, respectively. In this structure the fragment A is colored in yellow and the fragment B in violet. The residues and H-bonds involved in elongated $\beta$-sheet are shown.
Ala<sub>133</sub>–O Asn<sub>159</sub> occurs in 14% of the structures. This indicates that in the sampling, the elongation of the original antiparallel β-sheet (Figure 3(A)) in the direction of Asn<sub>159</sub> residue is observed.

Interestingly, the conformational fluctuation of the two 12-residue segments sometimes results in the formation of a parallel arrangement of β-strands. In particular, the H-bond pattern involves Ala<sub>133</sub> bonded to both Met<sub>166</sub> and Glu<sub>168</sub> (Figure S3). The latter acidic residue is also sporadically H-bonded to Ser<sub>135</sub>.

In order to evaluate the relative abundance of the observed antiparallel and parallel arrangements, two representative H-bonds for each arrangement have been considered. For the antiparallel β-sheet the average value of the two distances, N Met<sub>129</sub>–O Tyr<sub>163</sub> and O Val<sub>161</sub>–N Gly<sub>131</sub>, has been calculated; for the parallel β-sheet the average value of the two distances, N Ala<sub>133</sub>–O Met<sub>166</sub> and O Ala<sub>133</sub>–N Glu<sub>168</sub>, has been calculated. The analysis of bath2 sampling reveals that 70.4 and 4.8% of collected structures forms antiparallel and parallel sheet, respectively. These data indicate that an extensive sampling of conformations was achieved.

**AB two-stranded β-sheet of M129V and G131V mutants**

The residue replacements are located in the B/B’ segment and are directly involved in the H-bonding interactions characterizing the AB β-sheet (Table 1). The secondary structure analysis of the AB interface reveals that, compared to the wt model, there is a slightly stabilization of the sheet in the M129 V mutant (Figure 2(B)), whereas a destabilization is observed in the G131 V mutant (Figure 2(C)).

In the G131 V containing assembly, the average occurrence of β-strand conformations for residues 129–131 and residues 161–163 at 300.5 K is 66% (Figure S1). By analyzing in detail the distance pairs characteristic of the β-sheet, the destabilization of the G131 V model appears even more evident. Indeed, only the 54% of the collected structures in the G131 V bath2 simulation presents an average distance of the main-chain H-bond pairs Met<sub>129</sub>–Tyr<sub>163</sub> lower than 3.3 Å. On the other hand, the additional H-bond distance between O Val<sub>131</sub>–N Val<sub>161</sub> is very frequently below the 3.3 Å threshold. This indicates that, when formed, the β-sheet is elongated by completing the H-bonding pattern which connects Val<sub>131</sub> and Val<sub>161</sub> of partnering strands (Figure 3(B)). This elongation can also be favored by the less persistent side-chain/main-chain H-bond between NE2 Gln<sub>160</sub>–O Val<sub>131</sub> which engages the main-chain O of Val<sub>131</sub> thus subtracting it from the β-sheet H-bonding pattern. This observation is in line to the results of MD simulations carried out on the whole mutant protein (Chen et al., 2010). The mutated β-branched Val side-chain probably disfavors the formation of the above side-chain/main-chain H-bond. In addition, compared to the wt sampled conformations, the G131 V ones exhibit a more marked loss of the H-bond of the bulge which is only present in about 2.3% of the structures. This is a further factor contributing to the elongation of the β-sheet. No parallel β-sheet arrangements are observed in the sampled conformations.

In the M129 V containing assembly, the average occurrence of β-strand conformations for residues 129–131 and residues 161–163 is 87% (Figure S1). In terms of distance pairs, the 87% of the collected structures in the bath2 simulation shows an average distance of the main-chain H-bond pairs Met<sub>129</sub>–Tyr<sub>163</sub> lower than 3.3 Å, thus indicating a slight stabilization of the original β-sheet compared to the wt assembly.

The structures of the sampling also explore parallel β-strand pairing even though different from the one described above for the wt assembly. Indeed, the 2.7% of the structures exhibits an average distance between the pair O Arg<sub>164</sub>–N Ser<sub>132</sub> and O Ser<sub>132</sub>–N Met<sub>166</sub> lower than 3.3 Å.

To summarize the AB two-stranded β-sheet simulations, it can be stated that the antiparallel β-sheet is rather preserved in the isolated peptide models with the propensity to form the β-sheet which follows the subsequent order: M129 V > wt > G131 V. Compared to the β-sheet arrangement observed in the crystal structure, the simulations show frequent elongations of the sheet. The occurrence of parallel β-sheet arrangements, completely different from the starting coordinates, indicates the achievement of an extensive sampling of conformations.

**BB’ two-stranded β-sheet of wt-PrP**

In the crystal structure of PrP bound to the Fab fragment of monoclonal antibody ICSM 18, two symmetry-related molecules interact via their two-stranded β-sheets by forming a four-stranded β-sheet (Figure 1). The novel β-strand association (BB’ sheet) involves two backbone H-bonds between Leu<sub>130</sub> and Leu<sub>130</sub> residues on adjacent strands. The backbone amide and carbonyl groups of both residues directly hydrogen bond to one another across the strands thus forming a hydrogen-bonded pair.

An extended H-bonding pattern for the antiparallel β-sheet requires the formation of H-bonded pairs consisting of residues Ser<sub>132</sub> and Tyr<sub>128</sub> on one side of the Leu<sub>130</sub> pair, as well as of residues Tyr<sub>128</sub> and Ser<sub>132</sub> on the other side (Figure 1(B)).

The evolution of secondary structure (Figure 4(A)) indicates that the B and B’ segments widely vary in their conformation. We have also evaluated the persistence of the H-bonded pairs, which reflects the presence of BB’ β-strand association, in the sampled structures. The Ser<sub>132</sub>–Tyr<sub>128</sub> pair is present in the 10% of the structures (the H-bonded pair is considered to be present when the average distance of the two backbone H-bonds is lower than 3.3 Å). This H-bonded pair is slightly more
persistent than the original Leu\textsuperscript{130} pair in the starting structure. The concomitant occurrence of the various H-bonded pairs, resulting in the elongation of the β-sheet, is frequently found in the BB’ assembly, with a representative structure shown in Figure 5.

We have also checked the presence of alternative antiparallel β-sheet pairings which display a different H-bonding pattern with respect to the original one, resulting in a shift of β-strand alignments. The system explores several alternative antiparallel conformations, each populated less than 10%; by adding these contributions a 13% of antiparallel alternative structures is obtained. We also considered the antiparallel β-sheet occurring in other crystal structures of the human PrP (Lee et al., 2010), ovine PrP (Riek et al., 1996), and of six-residue peptides (127–132) from the human PrP (Apostol, Sawaya, Cascio, & Eisenberg, 2010). In these structures the antiparallel β-sheet is characterized by either Leu\textsuperscript{130}–Tyr\textsuperscript{128}’ or Leu\textsuperscript{130}–Met\textsuperscript{129}’ pairs instead of Leu\textsuperscript{130}–Leu\textsuperscript{130}’ pairs. Our bath2 sampling includes both β-strand alignments with a preference for Leu\textsuperscript{130}–Tyr\textsuperscript{128}’ over the Leu\textsuperscript{130}–Met\textsuperscript{129}’ pair (1.7 vs. 0.2%).

A parallel β-sheet arrangement is also observed in a significant number of structures (7.5%), involving the backbone H-bonds between Tyr\textsuperscript{128}–Tyr\textsuperscript{128}’ and Leu\textsuperscript{130}–Tyr\textsuperscript{128}’.

**BB’ two-stranded β-sheet model.**

**BB’ two-stranded β-sheet of M129 and G131 V mutants**

The analysis of BB’ interface for the two mutants indicate that, compared to the wt results, the BB’ β-sheet is slightly less stable for the M129 V assembly, whereas it is significantly stabilized for the G131 V assembly. Interestingly, the order of propensity to form BB’ intermolecular β-sheet (G131 V > wt > M129 V) is inverted compared to that to form AB intramolecular β-sheet.

In M129 V, the H-bonded pair Leu\textsuperscript{130}–Leu\textsuperscript{130}’ is present in about 4% of structures. Compared to the AB interface, the BB’ interface is more dynamic and a number of other antiparallel β-strand alignments are observed. By adding all the significantly populated antiparallel β-sheet pairings, about 13% of structures are
involved in persistent antiparallel arrangements. Therefore, the difference of stability with respect to the wt results remains.

On the other hand, in G131 V simulation, the H-bonded pair Leu$^{130}$–Leu$^{130}$ is present in about 30% of structures. It is worth noting that the same percentage of structures exhibit also the other H-bonded pair Ser$^{132}$–Tyr$^{128}$ of the BB’ sheet thus indicating a stable association. Alternative antiparallel arrangements are observed and add up to about 19% of structures. The higher stability of the BB’ association compared to the wt BB’ assembly is also evident in the evolution of secondary structure elements shown in Figure 4(B).

In both mutant samplings, a small but significant number of structures (ranging from 0.8 to 1.9%) shows antiparallel β-sheets arrangements observed in other crystal structures (see the above section).

Parallel contacts have also been monitored along the room temperature trajectories. In G131 V, a significant parallel arrangement is observed in 4.5% of structures involving Leu$^{30}$–Met$^{29}$ and Leu$^{130}$–Val$^{131}$. In M129 V a more populated parallel arrangement is found in about 11% of structures involving the mutated residue, i.e. the backbone H-bonds between Val$^{129}$–Tyr$^{128}$ and Val$^{129}$–Leu$^{130}$.

To summarize the BB’ two-stranded β-sheet simulations, it can be stated that the BB’ interface is more dynamic that the AB interface and indeed the β-strand association is less persistent and more varied. The order of propensity to form BB’ intermolecular β-sheet is the following: G131 V > wt > M129 V, with a significant increase of the β-structure in G131 V simulation. The sampled conformations along the trajectories include the slightly different intermolecular pairings found in other prion crystal structures thus indicating that an adequate conformational sampling has been achieved.

**ABB’A four-stranded β-sheet**

The simulations of four-stranded β-sheets provide a picture similar to that emerged from the two-stranded β-sheet simulations. Indeed, the AB and A’B’ sheet pairings preserve their stability in the large four-stranded assembly (Figure S3). The recurring H-bonding patterns connecting the partnering strands in the β-sheet are even more persistent than in the two-stranded β-sheet simulations. However, the compared stability trend is the same, i.e. M129 V > wt > G131 V.

The behavior of the BB’ interface is also similar in the four-stranded and in the two-stranded β-sheets, with the expected reversed stability trend, i.e. G131 V > wt > M129 V. The evolution of secondary structure in Figure S3 did not reveal the stability of the BB’ strand association since the β-conformation (in red) of the B/B’ segments can be due to the β-strand association with A/A’ segments. However, if the percentage of structures exhibiting the original BB’ antiparallel arrangement, in terms of distance pairs, is calculated, the results do not change significantly in the four-stranded assembly with respect to the two-stranded BB’ assembly (data not shown).

Our analysis indicates that the A and B segments have an intrinsic tendency to form a β-sheet pairing so that the four-stranded sheet breaks into two AB and A’B’ sheets. Therefore, the initial presence of an extended four-stranded sheet does not change the behavior of the original AB β-sheet.

No significant parallel arrangements are observed in any assembly, probably due to a restricted conformational freedom of the more crowded system.

**Discussion**

Neurodegenerative diseases are diversified pathologies characterized, from the molecular point of view, by common features such as the occurrence of toxic oligomeric species and the formation of amyloid-like fibrillar deposits. In all cases, these deposits are characterized by assemblies of misfolded proteins/peptides which predominantly assume a β-structure. Despite these analogies, the process of protein misfolding and aggregation is highly diversified in different systems. Indeed, misfolding and aggregation may either require a significant unfolding of the protein involved or they may occur with minimal structural alterations. In this framework, the mammalian prion misfolding represents one of the most investigated processes despite the difficulties in characterizing transient intermediate species. Over the years, it has been shown that computational approaches can provide a significant contribution to the understanding of prion misfolding (Ma & Nussinov, 2006). As the structure of the protein undertakes major conformational transitions in the aggregation process, the characterization of intrinsic structural propensities of prion fragments has revealed to be a powerful approach (Ziegler, Viehrig, Geimer, Rosch, & Schwarzinger, 2006) of particular interest. In previous studies extensive analyses of the intrinsic conformational preferences of the fragments that assume helical conformation in the folded globular state of the protein have been reported (Camilloni et al., 2012; Costantini & Facchiano, 2009; Dima & Thirumalai, 2004; Ji, Zhang, & Shen, 2005). Here, we focused our attention on the intrinsic conformational preferences of prion fragments that constitute the sole β-sheet of the protein and in conditions mimicking a situation in which the β-sheet is isolated in solution. This scenario is consistent with the misfolding pathway based on the detachment of the subdomains S1–H1–S2 and H2–H3, which is supported by a number of experimental and theoretical evidences (Calzolai & Zahn, 2003; De Simone et al., 2007; Govaerts, Wille, Prusiner, & Cohen, 2004; Hosszu...
et al., 1999; Kuwata et al., 2002; Schwarzinger et al., 2006; Viles et al., 2001). The extensive sampling achieved through REMD simulations spanning over a wide range of temperatures (138.5 K) shows that the native antiparallel β-structure of the region is endowed with a remarkable stability. Indeed, although other states (unstructured or parallel β) are detected in the conformational ensemble, the antiparallel state accounts for 70% of the total population. This observation extends the conclusions of previous investigations which provided evidence about the stability of the β-sheet region in partially unfolded states of the PrP (Smirnovas et al., 2011). Therefore, upon unfolding, the persistence of a structured β-region may suggest that it can play an active role in the subsequent phases of the aggregation process (nucleation, formation of oligomeric toxic species, fiber formation, and elongation). It should be emphasized that exposed β-region are particularly prone to either specific or unspecific interaction processes. For this reason, it has been shown that in native globular proteins, an evolutionary negative design operates to avoid the exposure of β-sheets (Richardson & Richardson, 2002).

Moreover, it has been proposed that the exposure of β-sheets by pathological forms may generally be related to the cytotoxic effects they exert due to the high and uncontrolled reactivity of these regions (De Simone, Esposito, Pedone, & Vitagliano, 2008; Esposito, Paladino, Pedone, & Vitagliano, 2008). Along this line, it may be speculated that the exposed β-region of prion upon unfolding may be directly involved in the genesis of the related diseases.

The second major aspect of the present study was the evaluation of the intrinsic stability of the intermolecular associations of the prion β-sheet frequently observed in crystallographic investigations. These structures suggested a possible motif of dimerization of the native PrP proteins but also a putative association of PrP intermediate species in which the two-stranded antiparallel β-sheet is detached from the native interface and therefore more prone to engage intermolecular β-sheets. MD simulations clearly indicate that this intermolecular association is more labile compared to the intramolecular association of the strands observed in the native sheet. Interestingly, the analysis of the conformational ensemble emerged from the trajectory shows the presence of the diverse intermolecular pairings observed in the distinct crystallographic analyses, although only a single initial state was considered. This finding indicates that an adequate conformational sampling has been achieved during the simulation. Evidently, crystal packing and interactions between other regions of the protein dictate specific pairings observed in the crystallographic studies. The present analysis shows that there is a tendency of these fragments to aggregate without definite structural states.

Moreover, the prion fragments considered in the present analysis also embed natural mutations that play a role in pathological states. In particular, we considered prion mutations at positions 129 and 131. The human PrP has a naturally occurring polymorphism at position 129. Phylogenetically, Met is the ancestral amino acid at codon 129, and Val is a mutation observed in humans. The European population is 40% homozygous for Met129, 10% homozygous for Val129, and 50% heterozygous. The 129 polymorphism, alone as in sporadic and acquired forms of CJD (Brown et al., 1994; Palmer, Dryden, Hughes, & Collinge, 1991), or in conjunction with other gene mutations as in familial forms (genetic CJD and FFI) (Goldfarb et al., 1992; Hauw et al., 2000), modulates disease susceptibility and the clinicopathological phenotypes. Indeed, Met homozygous individuals show high susceptibility to sporadic as well as to variant CJD. M/V129 heterozygosity may confer resistance to kuru, a disease acquired by cannibalism (Mead et al., 2003). The conformational behavior of the M129V mutant is not markedly different from that displayed by the wild-type fragment in both intramolecular and intermolecular β-sheet assemblies considered here. This suggests that the beneficial effect observed in heterozygous subjects is likely related to the difficulties of building β-assemblies with mixture of similar but not identical local sequences, in line with the suggestion of Eisenberg and coworkers (Apostol et al., 2010; Apostol, Wiltzius, Sawaya, Cascio, & Eisenberg, 2011; Sievers et al., 2011). However, it should be noticed that a closer inspection of the dynamical behaviors of the two peptide variants unveils some specificities (see the results for details). This is not surprising as in vitro studies have shown a different propensity of the two polymorphs to form either oligomeric intermediates or fibrillar states (Baskakov et al., 2005; Tahiri- Alaoui et al., 2004). The second mutation we considered is G131V, a pathogenic mutation proximal to the 129 polymorphic site. For the mutant G131V we observe a significant increase of the β-structure in the intermolecular pairing. From a molecular point of view, this is not surprising taking into account the higher β-propensity of Val compared to Gly residues. This finding is also in line with the results obtained from simulations carried out on the prion folded state by other authors (Chen et al., 2010; Samson & Levitt, 2011; Santini et al., 2003). The increase in the β-structure content of the mutations formed by the variant can be related to a higher propensity to aggregate in larger assemblies.

Conclusions

Our results clearly indicate that the central regions of the prion peptides 125–136 and 158–169 are endowed with a
significant propensity to adopt β-structures thus supporting that the β-sheet, once formed upon prion folding, likely retains its structure during the unfolding (Gerum, Silvers, Wirmer-Bartoschek, & Schwalbe, 2009). This implies that this region can play an active role in aggregation and pathogenesis. On the basis of the present results, it can be suggested that peptides designed on the sequences of the fragments here considered may interfere with the aggregation process of PrP. In this framework, conformationally restraint variants of these peptide, as the macrocycles characterized by Eisenberg et al. (Liu et al., 2011), may be particularly effective.

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Supplementary material

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References

Aguzzi, A., Baumann, F., & Bremer, J. (2008). The prion’s elusive reason for being. Annual Review of Neuroscience, 31, 439–477.
Antonyuk, S.V., Trevitt, C.R., Strange, R.W., Jackson, G.S., Sangar, D., Batchelor, M., ... Collinge, J. (2009). Crystal structure of human prion protein bound to a therapeutic antibody. Proceedings of the National Academy of Sciences USA, 106, 2554–2558.
Apostol, M.I., Sawaya, M.R., Cascio, D., & Eisenberg, D. (2010). Crystallographic studies of prion protein (PrP) segments suggest how structural changes encoded by polymorphism at residue 129 modulate susceptibility to human prion disease. The Journal of Biological Chemistry, 285, 29671–29675.
Apostol, M.I., Wittzius, J.J., Sawaya, M.R., Cascio, D., & Eisenberg, D. (2011). Atomic structures suggest determinants of transmission barriers in mammalian prion disease. Biochemistry, 50, 2456–2463.
Baskakov, I., Distler, P., Breydo, L., Shaw, M., Gill, A., James, W., & Tahiri-Alaoui, A. (2005). The presence of valine at residue 129 in human prion protein accelerates amyloid formation. FEBS Letters, 579, 2589–2596.
Behmard, E., Abdolmaleki, P., Asadabadi, E.B., & Jahan-dideh, S. (2011). Prevalent mutations of human prion protein: A molecular modeling and molecular dynamics study. Journal of Biomolecular Structure & Dynamics, 29, 379–389.
Blinov, N., Berjanskii, M., Wishart, D.S., & Stepanova, M. (2009). Structural domains and main-chain flexibility in prion proteins. Biochemistry, 48, 1488–1497.
Brown, P., Cervenakova, L., Goldfarb, L.G., McCombie, W.R., Rubenstein, R., Will, R.G., ... Gajdusek, D.C. (1994). Iatrogenic Creutzfeldt-Jakob disease: An example of the interplay between ancient genes and modern medicine[Raise last author name query]. Neurology, 44, 291–293.
Calzolai, L., & Zahn, R. (2003). Influence of pH on NMR structure and stability of the human prion protein globular domain. The Journal of Biological Chemistry, 278, 35592–35596.
Camilloni, C., Schaal, D., Schweimer, K., Schwarzinger, S., & De Simone, A. (2012). Energy landscape of the prion protein helix 1 probed by metadynamics and NMR. Biophysical Journal, 102, 158–167.
Chen, W., van der Kamp, M.W., & Daggett, V. (2010). Diverse effects on the native beta-sheet of the human prion protein due to disease-associated mutations. Biochemistry, 49, 9874–9881.
Colacino, S., Tiana, G., Broglia, R.A., & Colombo, G. (2006). The determinants of stability in the human prion protein: insights into folding and misfolding from the analysis of the change in the stabilization energy distribution in different conditions. Proteins, 62, 698–707.
Collinge, J. (2001). Prion diseases of humans and animals: their causes and molecular basis. Annual Review of Neuroscience, 24, 519–550.
Collinge, J., & Clarke, A.R. (2007). A general model of prion strains and their pathogenicity. Science, 318, 930–936.
Cordeiro, Y., Kraineva, J., Ravindra, R., Lima, L.M., Gomes, M.P., Foguel, D., ... Silva, J.L. (2004). Hydration effects on prion folding and beta-sheet conversion. High pressure spectroscopy and pressure perturbation calorimetry studies. The Journal of Biological Chemistry, 279, 32354–32359.
Costantini, S., & Facchiano, A.M. (2009). Human prion protein helices: Studying their stability by molecular dynamics simulations. Protein and Peptide Letters, 16, 1057–1062.
Darden, T., York, D., & Pedersen, L. (1993). Part Mesh Ewald: An N-log(N) method for Ewald sums in large systems. Journal of Chemical Physics, 98, 10089–10092.
De Simone, A., Dodson, G.G., Fraternali, F., & Zagari, A. (2006). Water molecules as structural determinants among prions of low sequence identity. FEBs Letters, 580, 2488–2494.
De Simone, A., Dodson, G.G., Verma, C.S., Zagari, A., & Fraternali, F. (2005). Prion and water: Tight and dynamical hydration sites have a key role in structural stability. Proceedings of the National Academy of Sciences USA, 102, 7535–7540.
De Simone, A., Esposito, L., Pedone, C., & Vitagliano, L. (2008). Insights into stability and toxicity of amyloid-like oligomers by replica exchange molecular dynamics analyses. Biophysical Journal, 95, 1965–1973.
De Simone, A., Zagari, A., & Derreumaux, P. (2007). Structural and hydration properties of the partially unfolded states of the prion protein. Biophysical Journal, 93, 1284–1292.
Dima, R.I., & Thirumalai, D. (2004). Probing the instabilities in the dynamics of helical fragments from mouse PrPC. Proceedings of the National Academy of Sciences USA, 101, 15335–15340.
Eghiaian, F., Daubenfeld, T., Quenet, Y., van Audenhaege, M., Bouin, A.P., van der Rest, G., ... Rezaei, H. (2007). Diversity in prion protein oligomerization pathways results from domain expansion as revealed by hydrogen/deuterium exchange and deuterium linkage. Proceedings of the National Academy of Sciences USA, 104, 7414–7419.
Eichner, T., & Radford, S.E. (2011). A diversity of assembly mechanisms of a generic amyloid fold. Molecular Cell, 43, 8–18.
Esposito, L., Paladino, A., Pedone, C., & Vitagliano, L. (2008). Insights into structure, stability, and toxicity of monomeric and aggregated polyglutamine models from molecular dynamics simulations. Biophysical Journal, 94, 4031–4040.
Intrinsic stability of the human prion β-sheet region

Ma, B., & Nussinov, R. (2006). Simulations as analytical tools to understand protein aggregation and predict amyloid conformation. *Current Opinion in Chemical Biology, 10*, 445–452.

Glocker, R., Voitchovsky, K., Mitchell, C., Tahiri-Alaoui, A., Ryan, J.F., Hore, P.J., & James, W. (2008). Inter-oligomer interactions of the human prion protein are modulated by the polymorphism at codon 129. *Journal of Molecular Biology, 381*, 212–220.

Gerum, C., Silvers, R., Wirmer-Bartoschek, J., & Schwalbe, H. (2009). Unfolded-state structure and dynamics influence the fibril formation of human prion protein. *Angewandte Chemie, 48*, 9452–9456.

Glaebe, C.G. (2008). Structural classification of toxic amyloid oligomers. *The Journal of Biological Chemistry, 283*, 29639–29643.

Goldfarb, L.G., Petersen, R.B., Tabaton, M., Brown, P., LeBlanc, A.C., Montagna, P., ... Pendlebury, W.W., et al. (1992). Fatal familial insomnia and familial Creutzfeldt–Jakob disease: disease phenotype determined by a DNA polymorphism. *Science, 258*, 806–808.

Goyaerts, C., Wille, H., Prusiner, S.B., & Cohen, F.E. (2004). Evidence for assembly of prions with left-handed beta-helices into trimers. *Proceedings of the National Academy of Sciences USA, 101*, 8342–8347.

Haire, L.F., Whyte, S.M., Vasisht, N., Gill, A.C., Verma, C., ... Bayley, P.M. (2004). The crystal structure of the globular domain of sheep prion protein. *Journal of Molecular Biology, 336*, 1175–1183.

Hauw, J.J., Sazdovitch, V., Laplanchue, J.L., Peoc'h, K., Kopp, N., Kemeny, J., ... Alperovich, A. (2000). Neuropathologic variants of sporadic Creutzfeldt–Jakob disease and codon 129 of PrP gene. *Neurology, 54*, 1641–1646.

Hosszu, L.L., Baxter, N.J., Jackson, G.S., Power, A., Clarke, A.R., Waltho, J.P., ... Collinge, J. (1999). Structural mobility of the human prion protein probed by backbone hydrogen exchange. *Nature Structural & Molecular Biology, 6*, 740–743.

Hosszu, L.L., Jackson, G.S., Trevitt, C.R., Jones, S., Batchelor, M., Bhetl, D., ... Collinge, J. (2004). The residue 129 polymorphism in human prion protein does not confer susceptibility to Creutzfeldt–Jakob disease by altering the structure or global stability of PrPC. *The Journal of Biological Chemistry, 279*, 28515–28521.

Humphrey, W., Dalke, A., & Schulten, K. (1996). VMD: Visual molecular dynamics. *Journal of Molecular Graphics, 14*, 33–38.

Ji, H.F., Zhang, H.Y., & Shen, L. (2005). The role of electrostatic interactions in triggering the unzipping of the beta-prion helix 1 in normal prion protein. A molecular dynamics simulation investigation. *Journal of Biomolecular Structure & Dynamics, 22*, 563–570.

Jorgensen, W.L., Maxwell, D.S., & Tirado-Rives, J. (1996). Development and testing of the OPLS all-atom force field on conformational energetics and properties of organic liquids. *Journal of the American Chemical Society, 118*, 11225–11236.

Kuwata, K., Li, H., Yamada, H., Legname, G., Prusiner, S.B., Akasaka, K., & James, T.L. (2002). Locally disordered conformer of the hamster prion protein: a crucial intermediate to PrPSc? *Biochemistry, 41*, 12277–12283.

Lee, S., Antony, L., Hartmann, R., Knaus, K.J., Szwierzcz, K., Szwierzcz, W.K., & Yee, V.C. (2010). Conformational diversity in prion protein variants influences intermolecular beta-sheet formation. *The EMBO journal, 29*, 251–262.

Liu, C., Sawaya, M.R., Cheng, P.N., Zheng, J., Nowick, J.S., & Eisenberg, D. (2011). Characteristics of amyloid-related oligomers revealed by crystal structures of macrocyclic beta-sheet mimics. *Journal of the American Chemical Society, 133*, 6736–6744.
Tcherkasskaya, O., Sanders, W., Chynwat, V., Davidson, E.A., & Orser, C.S. (2003). The role of hydrophobic interactions in amyloidogenesis: Example of prion-related polypeptides. *Journal of Biomolecular Structure & Dynamics, 21*, 353–365.

Van Der Spoel, D., Lindahl, E., Hess, B., Groenhof, G., Mark, A.E., & Berendsen, H.J. (2005). GROMACS: Fast, flexible, and free. *Journal of Computational Chemistry, 26*, 1701–1718.

Viles, J.H., Donne, D., Kroon, G., Prusiner, S.B., Cohen, F.E., Dyson, H.J., & Wright, P.E. (2001). Local structural plasticity of the prion protein. Analysis of NMR relaxation dynamics. *Biochemistry, 40*, 2743–2753.

Xue, W.F., Hellewell, A.L., Hewitt, E.W., & Radford, S.E. (2010). Fibril fragmentation in amyloid assembly and cytotoxicity: When size matters. *Prion, 4*, 20–25.

Zhang, J. (2009). Studies on the structural stability of rabbit prion probed by molecular dynamics simulations. *Journal of Biomolecular Structure & Dynamics, 27*, 159–162.

Zhang, J. (2011). The structural stability of wild-type horse prion protein. *Journal of Biomolecular Structure & Dynamics, 29*, 369–377.

Zhang, J., & Liu, D.D. (2011). Molecular dynamics studies on the structural stability of wild-type dog prion protein. *Journal of Biomolecular Structure & Dynamics, 28*, 861–869.

Zhong, L., & Xie, J. (2009). Investigation of the effect of glycosylation on human prion protein by molecular dynamics. *Journal of Biomolecular Structure & Dynamics, 26*, 525–533.

Ziegler, J., Viehrig, C., Geimer, S., Rosch, P., & Schwarzinger, S. (2006). Putative aggregation initiation sites in prion protein. *FEBS Letters, 580*, 2033–2040.