

**Original article:**

**CONFORMATIONAL INSTABILITY OF HUMAN PRION PROTEIN  
UPON RESIDUE MODIFICATION:  
A MOLECULAR DYNAMICS SIMULATION STUDY**

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**ABSTRACT**

Technical strategies like amino acid substitution and residue modification have been widely used to characterize the importance of key amino acids and the role that each residue plays in the structural and functional properties of protein molecules. However, there is no systematic approach to assess the impact of the substituted/modified amino acids on the conformational dynamics of proteins. In this investigation to clarify the effects of residue modifications on the structural dynamics of human prion protein (PrP), a comparative molecular dynamics simulation study on the native and the amino acid-substituted analog at position 208 of PrP has been performed. It is believed that Arginine to Histidine mutation at position 208 is responsible for the structural transition of the native form of human prion protein to the pathogenic isoform causing Creutzfeldt-Jakob disease (CJD).

So, three 10 ns molecular dynamics simulations on three model constructs have been performed. Simulation results indicated considerable differences of conformational fluctuations for Alanine substituted construct (PrP<sub>ALA</sub>) and the analog form (PrP<sub>SB</sub>) comprising the neutralized state of the Arginine residue at position 208 of the human prion protein.

According to our data, substitution of the Arginine residue by the uncharged state of this residue induces some reversible structural alterations in the intrinsically flexible loop area including residues 167–171 of PrP. Thus, deprotonation of Arg<sup>208</sup> is a weak perturbation to the structural fluctuations of the protein backbone and the resulting construct behaves almost identical as its native form. Otherwise, Alanine substitution at position 208 imposed an irreversible impact on the secondary and tertiary structure of the protein, which leads to conformational instabilities in the remote hot region comprising residues 190–195 of the C-terminal part of helix 2.

Based on the results, it could be deduced that the observed conformational transitions upon Arg<sup>208</sup> to His point mutation, which is the main reason for CJD, may be mainly related to the structural instabilities due to the induced-conformational changes that caused alterations in local/spatial arrangements of the force distributions in the backbone of the human prion protein.

**Keywords:** Amino acid substitution, electrostatic interaction, molecular dynamics simulation, human prion protein

## INTRODUCTION

Amino acid substitution is known as an important strategy to detect, and to analyze key residues in the structural and functional properties of proteins, and in the protein-ligand interactions (Yang et al., 1997; Chiaoto et al., 2007; Bogan and Thorn, 1998; Lo Conte et al., 1999; Hu et al., 2000). It has also been used in a number of studies with the aim of improving rational designs of drugs and of engineered peptides for various aspects (Susankova et al., 2007; Wells, 1996). Furthermore, this strategy has employed as a powerful tool to clarify the structure-activity relationships of those proteins that are involved in the neurodegenerative amyloid diseases (Williams et al., 2006; Lund et al., 2007; Gu et al., 2003). In more detail, this method is based on the substitution of a certain residue in the protein backbone by another one in order to identify the role of the eliminated residue in the physicochemical properties of the wild type structure (Grant et al., 1999, 2001). It is generally accepted that Alanine amino acid plays a neutral role in the physicochemical properties of proteins. Consequently, this has made it feasible for researchers to probe the role each residue plays in the protein backbone simply by substituting the desired residue with an Alanine amino acid (Prilla et al., 2006; Conner et al., 2006; Beezhold et al., 2001). While such replacements may modify the size, acidity, hydrogen-bonding character, hydrophobic properties, nucleophilicity of an amino acid side chain or the protein backbone itself (Mendel et al., 1995), on the other hand, there is no systematic strategy to evaluate the impact of the newly inserted amino acid on the physicochemical properties of proteins. Frequently, circular dichroism spectroscopy is used to prove that the observed physicochemical properties of the mutant protein are not side effects of the structural alterations due to the amino acid replacements (Renauld-Mongénie et al., 2004). However, circular dichroism analyses of the native and mutat-

ed mammalian metallothionein revealed no changes in the conformational properties of these structures, while NMR studies detected significant differences in the chemical shifts between wild type and the mutant protein (Pan et al., 1994).

In this study, using molecular dynamics simulations as a powerful computational tool to investigate the dynamical behavior of proteins (Pikkemaat et al., 2002; de-Marco and Daggett, 2004), we tried to obtain an assessment of the contributions of newly substituted amino acids to the structural fluctuations of the protein backbone of the human prion protein PrP as our model structure.

According to previous results, three highly occupied salt bridges in the human prion protein have been identified. Two of them ( $\text{Asp}^{144} \leftrightarrow \text{Arg}^{208}$  and  $\text{Arg}^{164} \leftrightarrow \text{Asp}^{178}$ ) are supposed to be responsible for the early stages of conformational changes in the disease-associated mutations ( $\text{Arg}^{208} \rightarrow \text{His}$  and  $\text{Asp}^{178} \rightarrow \text{Asn}$ ) (Zuegg and Gready, 1999). Other lines of evidence have led to the opposite interpretation on the role of such electrostatic interactions in the structural stability of human PrP (Speare et al., 2003; Bamdad and Naderimanesh, 2007). In the present work, we tried to elucidate this conflicting situation by studying the role of  $\text{Asp}^{144} \leftrightarrow \text{Arg}^{208}$  in the structural stability of human PrP. In one simulation, similar to the amino acid substitution approach, the Arginine residue at position 208 was substituted by Alanine (hereafter PrP<sub>ALA</sub>), which removes the salt bridge between  $\text{Asp}^{144}$  and  $\text{Arg}^{208}$ . This construct served to identify the consequences of amino acid substitution on the structural changes of the protein. A second situation was considered, where the deprotonated state of  $\text{Arg}^{208}$  (hereafter PrP<sub>SB</sub>) was employed in order to break the salt bridge. The latter situation had been used to impose weak steric perturbations as it is the case by amino acid replacement. The conformational changes of these two model structures have been compared to the native prion protein (here-

after PrP<sub>N</sub>). In the present work, for the first time, a comparative molecular dynamics study on the human prion protein has been performed to assess the contribution of two types of elimination of a salt bridge mediated by Arg<sup>208</sup>.

## METHODS

The NMR structure of the C-terminal domain (125–228) of human prion protein in solution (Protein Data Bank accession code: 1hjm) was chosen as the initial structure for the wild-type protein (Calzolari and Zahn, 2003). Based on this structure we have built a model structure for PrP<sub>ALA</sub> by means of the Swiss-Pdb Viewer (Guex and Peitsch, 1997), which introduced the corresponding mutation into the wild-type structure. In order to break the salt bridge connecting Asp<sup>144</sup> and Arg<sup>208</sup>, we have designed the deprotonated state of Arg<sup>208</sup> using the interactive feature of the package. In order to obtain a locally minimized conformation of the new structure, the imposed local constraints were relaxed by energy minimization. Each model structure was solvated in a cubic box containing ~10,000 of SPC (Berendsen et al., 1981) water molecules and simulated employing periodic boundary conditions. To obtain an electro-neutralized simulation system, randomly selected water molecules were replaced by appropriate numbers of Na<sup>+</sup> and Cl<sup>-</sup> ions. Using the Berendsen algorithm (Berendsen et al., 1984), temperature and pressure were kept constant at 300 K and 1 bar, respectively. Bond lengths were constrained to their equilibrium values by the SETTLE algorithm (Miyamoto and Kollman, 1992) for water and the LINCS algorithm (Hess et al., 1997) for protein molecules. Electrostatic interactions were computed using the particle-mesh Ewald method (Darden et al., 1993). Non-bonded interactions were truncated at a cut-off radius of 1.0 nm. All systems were initially equilibrated with an energy minimization of 1200 steps using the steepest descent method and 10 ps of position restraint MD on the protein structure

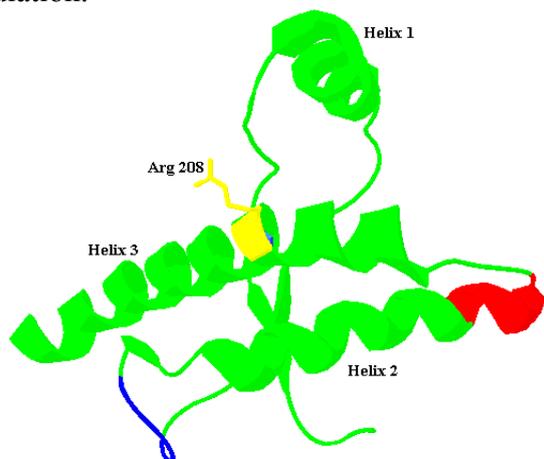
before the start of the simulation run. The integration time step was set at 2.0 fs and the energies and atomic coordinates were saved every 2 ps for analysis. All calculations and data analysis were performed using GROMACS 3.3.1 package (van der Spoel et al., 2005), with the united-atom protein force field for MD simulations (Schuler et al., 2001). Analysis of the secondary structure was done with the DSSP program (Kabsch and Sander, 1983).

## RESULTS AND DISCUSSION

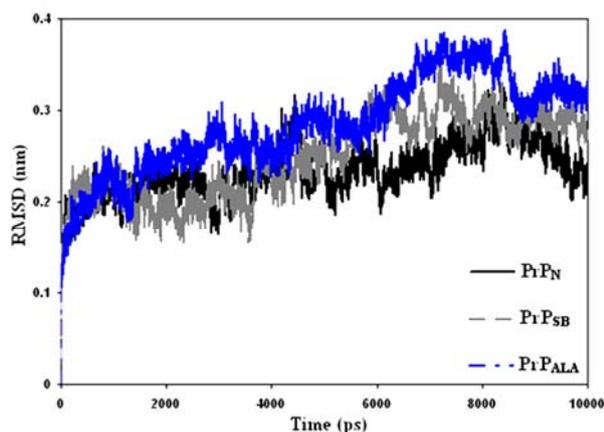
### *Structural stability measurements*

Average molecular structure of the native human prion protein after 10 ns molecular dynamics simulation is presented in Figure 1. Furthermore, to analyze the flexibility of the model constructs during the simulation time, root mean square deviations (RMSDs) of the C $\alpha$ -positions for three analog structures including the native protein (PrP<sub>N</sub>), the Alanine substituted model (PrP<sub>ALA</sub>) at position 208, and the analog form comprising the neutralized state of Arginine residue (PrP<sub>SB</sub>) at the same position have been extracted (Figure 2). Based on these data RMSD values increased sharply from 0.10 nm to around 0.20 nm for the first 0.5 ns. Between 0.5 and 2 ns, generally, the same pattern of RMSD values (~0.21 nm) for three model structures has been detected. Between 2 and 6 ns, the RMSD values of the model structures gradually increased with different patterns for each construct. During this period, the average RMSD for PrP<sub>ALA</sub> is approximately 0.27 nm, while a lower average value of 0.23 nm achieved for both of PrP<sub>SB</sub> and PrP<sub>N</sub>. The RMSD pattern from 6 ns until the end of the run is clearly different for each structure. The RMSD of PrP<sub>ALA</sub> exhibits a maximum of 0.35 nm around 8 ns with a sharp increase between 6 and 8 ns, while the RMSD for PrP<sub>SB</sub> and PrP<sub>N</sub> have maxima of about 0.29 nm and 0.27 nm, respectively. These results indicated that PrP<sub>ALA</sub> exhibits large conformational flexibility due to the amino acid substitution, which is not ob-

served in PrP<sub>SB</sub> and PrP<sub>N</sub> during 10 ns simulation.



**Figure 1:** Average structure of 1hjm after 10 ns molecular dynamics simulation. Arginine at position 208, flexible loop area including residues 167–171, and the hot region (190–195) at the C-terminal part of the helix 2 are depicted in yellow, blue, and red, respectively. Alanine substitution at position 208 induces significant dynamical instabilities in the hot segment of the backbone, while introducing the neutralized Arginine residue at the same position imposes some reversible structural changes to the flexible loop area.



**Figure 2:** Pattern of the dynamical instabilities for three model constructs based on root mean square deviations (RMSDs) of the C $\alpha$ -positions for 10 ns simulation, which indicates that protein models have been relatively reached to the stability. However, PrP<sub>ALA</sub> is experiencing higher dynamical flexibility in comparison to PrP<sub>SB</sub> and PrP<sub>N</sub> during the simulation time.

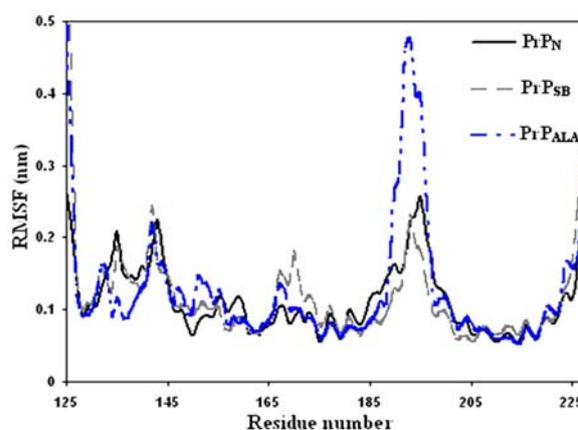
Pattern of the structural stability for three constructs indicates that the conformational instability of the protein backbone due to the charge neutralization of Arg<sup>208</sup> is less significant to the dynamical properties of the protein backbone. However, substitution of Alanine residue at position 208 imposed higher instabilities to the conformational dynamics of PrP<sub>ALA</sub> model structure. Although, based on these data, it is not possible to ascertain the contribution of different regions of the backbone to the observed structural flexibility. The high RMSD values of PrP<sub>ALA</sub> (Figure 2) cannot be a result of the elimination of the putative salt bridge between Asp<sup>144</sup> and Arg<sup>208</sup>, but might rather be related to the imposed local steric constraints due to the insertion of the Ala residue instead of Arg at position 208. Thus, the replacement of Arg by Ala at position 208 causes an increased dynamical flexibility of the protein backbone, which may lead to the high structural instability triggering the initial steps of conformational rearrangements. However, the elimination of the salt bridge, which is supposed to be the main reason of the Creutzfeldt-Jakob disease in Arg<sup>208</sup>→His mutation, has no significant effect on the dynamical behaviors of the protein backbone. Therefore, our results seem to disagree with the proposed role of this salt bridge as a putative disease-associated electrostatic interaction in human prion protein (Zuegg and Greedy, 1999). On the other hand, our findings are in good agreement with other theoretical and experimental investigations, which showed that the contribution of these types of solvent-exposed salt bridges to the overall stability of prion proteins is negligible (Speare et al., 2003; Bamdad and Naderimanesh, 2007). In summary, data indicates that the substitution of Arginine by the neutralized state of this residue at position 208 in the human prion protein induced some reversible structural dynamics to the backbone that is comparable to the recorded pattern of the native protein construct. However, substitution of Arginine

residue by Alanine at the same position imposed higher dynamical perturbation to the protein backbone, which is mainly related to the steric constraints due to the residue replacement. It could be supposed that mutation of Arg<sup>208</sup> to His residue in prion protein, which is the main reason for CJD in human, is mainly related to the imposed spatial constraints to the protein backbone because of the amino acid substitution rather than the elimination of the salt bridge between Asp<sup>144</sup> and Arg<sup>208</sup>.

### Dynamical fluctuations

In order to have a better insight to the most sensitive regions of the protein backbone to the residue modifications, root mean square fluctuations (RMSFs) of three model constructs have been analyzed and presented in Figure 3. According to the results, the difference between the fluctuations of PrP<sub>SB</sub> and PrP<sub>N</sub> is generally very small, except in a small part of the loop area including residues 167–171, where PrP<sub>SB</sub> experiencing higher fluctuations in comparison to PrP<sub>N</sub>. The recorded fluctuations are mainly due to the deprotonation of Arginine at position 208. According to the average pK<sub>a</sub> (around 12.5) value for this residue, the neutralized Arginine in PrP<sub>SB</sub> construct can be considered as a significant change to the pH value in the microenvironment of this residue, which can promote conformational fluctuations. It may be concluded that breakage of the salt bridge between Asp<sup>144</sup> and Arg<sup>208</sup> due to the neutralization of the Arg<sup>208</sup>, alternates some geometrical constraints in the protein backbone that induced fluctuations on the loop region including residues 167–171. Since loops are generally considered to be potential reservoirs of structural entropy, the release of the salt bridge leads to the recorded fluctuations in that area. These data from root mean square fluctuations (Figure 3) also indicates that the detected conformational changes of PrP<sub>SB</sub> (Figure 2) mainly originate from the entropic fluctuations of the loop region comprising residues 167–171.

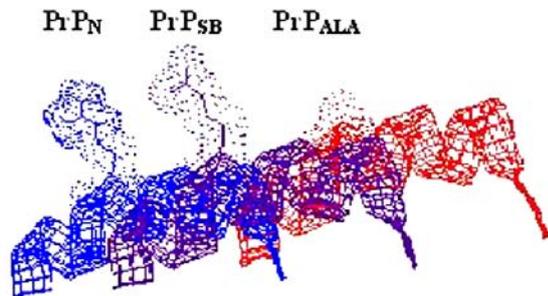
In order to demonstrate the relevant geometrical shapes and orientations of the modified residue at position 208, a small part of the helix 3 comprising the native Arginine, neutralized state of this residue, and the Alanine substituted amino acid, have been presented in Figure 4. As it is depicted in this figure, these spatial and geometrical alterations are the main reason for the observed patterns in the structural fluctuations of the protein backbone (Figures 2, and 3).



**Figure 3:** In order to specify sensitive regions of the backbone to the structural fluctuations, root mean square fluctuations (RMSFs) of the C $\alpha$ -positions for three model constructs are presented. Based on the data, a short segment comprising residues 190–195, which is placed in the C-terminal part of the helix 2 is experiencing the highest fluctuations. Blue, gray and black lines indicate RMSF values for PrP<sub>ALA</sub>, PrP<sub>SB</sub> and PrP<sub>N</sub> structures, respectively.

As Figure 3 shows, PrP<sub>ALA</sub> exhibits large dynamical fluctuations during the simulation time. The most significant fluctuations are localized around residues 190–195. These fluctuations are particularly large after 6 ns (Figure 2) and reach to a maximum value of ~0.47 nm (Figure 3). The large fluctuations of residues 190–195 are the main contribution to the observed RMSD of PrP<sub>ALA</sub> after 6 ns (Figure 2). Thus it can be concluded that this segment is the most flexible region of the backbone to the structural modifications. These data

are also in good agreement with the results of other theoretical and experimental investigations (Bamdad and Naderimanesh, 2007; Zahn et al., 2000).



**Figure 4:** A small segment of helix 3 comprising normal Arginine (left), deprotonated one (middle), and the Alanine substituted structure (right) has been presented to show the geometrical shape and orientation of the modified residue at position 208 of analog constructs.

In summary, the conformational fluctuations of three model constructs show three different patterns with the increasing order of magnitude:  $\text{PrP}_N < \text{PrP}_{SB} < \text{PrP}_{ALA}$ . It means that a single residue substitution may play as a molecular switch that consequently can alter the structural and functional properties of the protein. The experimental studies are also in good agreement with this conjecture (Solano et al., 1997). Our data also indicate that the breakage of the salt bridge alone does not lead to the significant structural and dynamical changes as compared to the native form of the human prion protein. Based on the data from structural fluctuations of three model constructs (Figure 3), the origin of the recorded conformational instabilities (Figure 2) could be analyzed. Accordingly, the most portion of the structural instabilities of the Alanine substituted analog (Figure 2) have to be mainly related to a short segment (Figure 3) of the protein backbone including residues 190–195. As Figure 3 shows, the area around position 208 is experiencing the lowest structural fluctuations during the time period of simulation, which implies that the

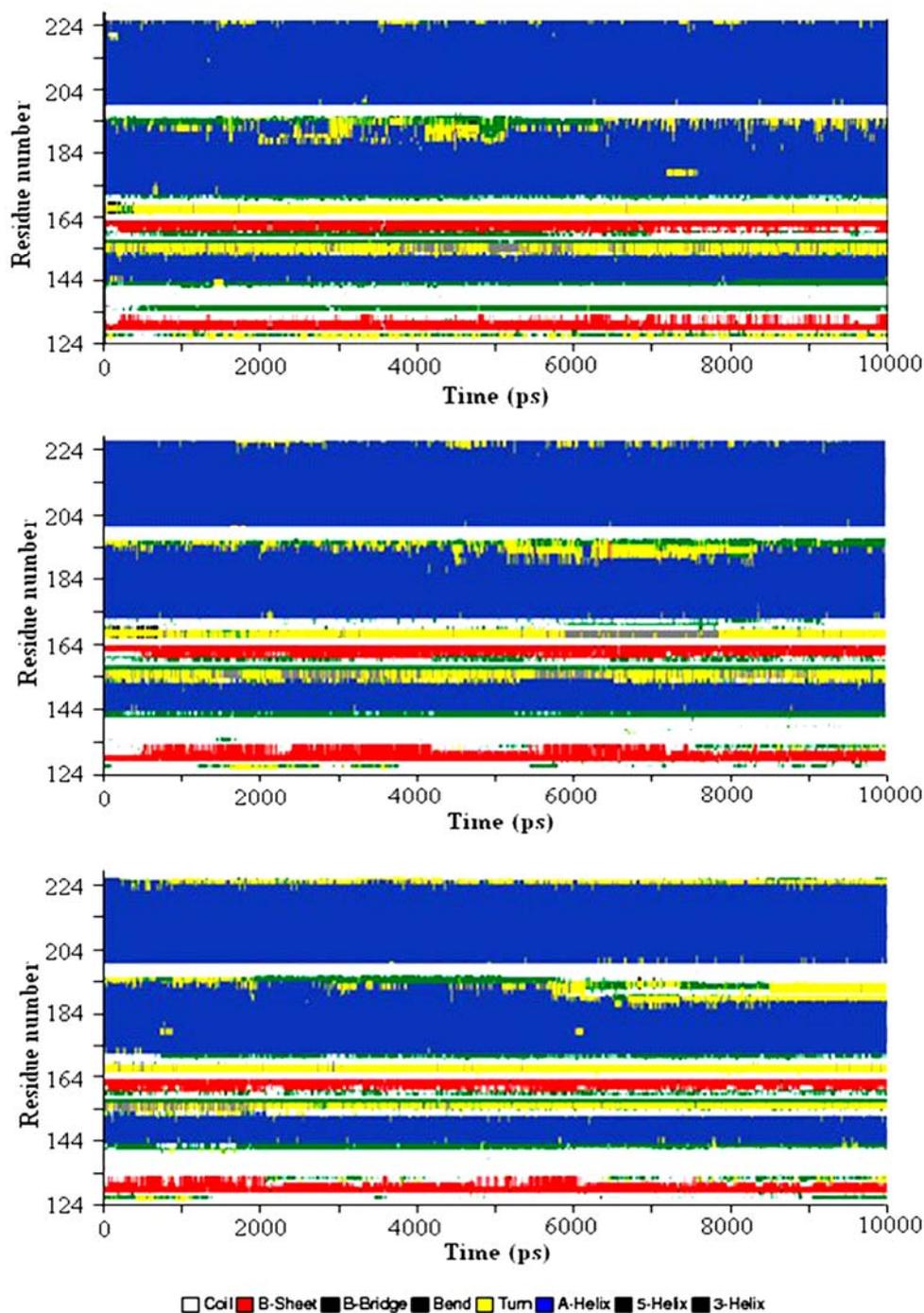
modification of Arg residue has no effects to the protein backbone at this position. However, another area of the backbone at the C-terminal part of helix 2 with at least thirteen residues distance has been mainly affected. Thus, it could be concluded that spatial interactions must be responsible for the structural fluctuations, which are localized in a short hot segment of the protein backbone including residues 190–195 due to the residue substitution at position 208.

### Secondary structure analysis

In order to assign the contribution of different parts of the backbone to the structural rearrangements, secondary structure fluctuations as a function of time for three model structures are shown in Figure 5. Comparison of the results shows that there is no significant difference in the secondary structure rearrangements of  $\text{PrP}_{SB}$  and  $\text{PrP}_N$ , whereas these fluctuations are significant in  $\text{PrP}_{ALA}$ . The only part of  $\text{PrP}_{SB}$  with observable changes in the secondary structure is located at around residues 167–171. Although these structural rearrangements started at around 6 ns, they returned to the previous structure at around 8 ns. During this period, the secondary structure of the relevant area had changed from turn to bend/3-helix, and from 8 ns until the end of the simulation returned to the previously turn structure. These data also indicate that the observed structural instabilities of  $\text{PrP}_{SB}$  (Figure 2) are related to the dynamical fluctuations of the loop area (Figure 3), which originate from the reversible secondary structure changes during 6–8 ns (Figure 5). Based on the results,  $\text{PrP}_{ALA}$  is experiencing higher alterations for the secondary structure, which cannot be observed in  $\text{PrP}_{SB}$  and  $\text{PrP}_N$  during a 10 ns simulation. These structural changes are localized at around the C-terminal part of helix 2 and helix 3. Unlike  $\text{PrP}_{SB}$ , the observed structural rearrangements of  $\text{PrP}_{ALA}$  were stable and did not return to the original structure during the simulation. Right at the start of the simulation, the C-terminal end of helix 3

changed to a turn structure. Subsequent alterations in the C-terminal part of helix 2 started at around 6 ns and induced a helix-to-turn/coil transition. This implies that the structural instability of PrP<sub>ALA</sub>, particularly after 6 ns (Figure 2), is mostly associated

with the transition of the secondary structure elements at the C-terminal part of helix 2 (Figure 5), which is accompanied by dynamical fluctuations of ~0.47 nm (Figure 3).



**Figure 5:** Transition pattern for secondary structural elements of three model constructs as a function of time. Upper, middle, and lower panel indicate alterations of the elements of secondary structures for PrP<sub>N</sub>, PrP<sub>SB</sub> and PrP<sub>ALA</sub> structures, respectively during the time period of the molecular dynamics simulation.

Our results show that under the same conditions, slight alterations to the protein backbone such as Arg<sup>208</sup> neutralization (PrP<sub>SB</sub>) do not impose significant structural perturbations, whereas the amino acid replacement (PrP<sub>ALA</sub>) causes an irreversible impact on the secondary and tertiary structure of the protein. The substitution of Arginine by Alanine at position 208 leads to the significant dynamical instabilities and a permanent structural change in the remote hot region (190–195) of the backbone. The replacement of the Arginine residue by its uncharged state, however, induces only reversible structural changes in the intrinsically flexible loop area (167–171). Based on these data, the origin of the observed structural instabilities (Figure 2) due to the Arg replacement by Ala at position 208 could be dedicated to a short segment of the protein backbone comprising residues 190–195 (Figure 3) in the C-terminal part of the helix 2 (Figure 1). It may be concluded that this residue modification imposed structural alterations to a specific sensitive segment of the human prion protein that is sequentially far from the position 208. In addition, it could be supposed that the detected structural changes (Figure 5) due to the residue alterations (Figure 4) are the main reason for triggering of the initial steps of the subsequent structural transitions. It seems that these structural changes arise because of the steric perturbations due to the residue replacement rather than the elimination of the electrostatic charge between Asp<sup>144</sup> and Arg<sup>208</sup>.

## CONCLUSIONS

Amino acid substitution is a powerful strategy, which has been widely used for probing the forces that govern protein structure and folding, biorecognition, catalysis, and structure-activity relationships of disease-associated proteins as well (Knowles, 1987). Such replacements may modify the size, acidity, hydrogen-bonding character, nucleophilicity, or hydrophobic properties of the amino acid side chain or the protein

backbone itself (Mendel et al., 1995). Hence, it is of considerable importance to identify the impact of modifications on the structural and functional properties of protein (Taverna and Goldstein, 2002; Wang and Chou, 2009, 2012). However, there is no systematic approach to monitor or to predict consequences of amino acid substitution. The main aim of this study is to investigate the effects of the amino acid modification at position 208 of the human prion protein that a certain electrostatic interaction is also mediating by Arg<sup>208</sup> (Asp<sup>144</sup> ↔ Arg<sup>208</sup>). This position was studied because it is supposed that Arginine 208 to Histidine mutation is the main reason for the conformational change of the native form of human prion protein to the pathogenic isoform causing Creutzfeldt-Jakob disease (CJD). We tried to show the significant differences between dynamical features of the native and the amino acid-substituted analog of this protein at the above mentioned position. In order to remove the salt bridge between Aspartate<sup>144</sup> and Arginine<sup>208</sup>, which is considered to be a weaker perturbation than an amino acid substitution, a computationally neutralized form of Arginine at position 208 (PrP<sub>SB</sub>) was employed. In addition, we have also considered the case of the amino acid substitution, where Arginine was replaced by Alanine residue (PrP<sub>ALA</sub>) at the same position. Comparison of the results from the molecular dynamics simulations on two modified protein constructs including PrP<sub>SB</sub> and PrP<sub>ALA</sub>, and the native form (PrP<sub>N</sub>) revealed that dramatic structural alterations to the native structure can be expected if Arginine is substituted by Alanine residue at position 208 of the protein backbone, especially in the C-terminal part of helix 2 comprising residues 190–195. These data are also in agreement with the experimental investigation carried out by Hosszu and coworkers (Hosszu et al., 2010) indicating that region 186–194 from the C-terminal part of helix 2 is under structural alterations due to the substitution of Histidine by Argi-

nine residue at position 187 (H187R) of the human prion protein. We think that they have employed this mutation strategy in order to impose permanent positive charge at position 187 of the backbone, while eliminating the unpredictable effects of acidic pH on physiochemical properties of the system under study to simulate the native physiological situation of the mutated protein. However, to have a better insight on the importance of the positive charge on the oligomerization process of the human prion protein due to the H187R mutation, a control construct including Histidine to Alanine substituted analog (H187A) at this position could also take into account. Otherwise, H187R mutation could generally be considered as a perturbing factor in the protein backbone, which increases the propensity to oligomerization that is not necessarily related to the direct effects of the positive charge imposition at position 187 of the human prion protein. This idea is also in agreement with the view of marginal stability of proteins (Taverna and Goldstein, 2002), in that, induced perturbations to short segments of the protein backbone may trigger large-scale alterations to the native state of proteins, which could start relevant conformational transitions in the protein structure. Furthermore, if we hold it true that each amino acid in the sequence of the protein backbone has been selected for that position by an evolutionary background; it is not surprising that the observed structural changes due to the residue replacements may also be related to the orientation/geometrical/spatial constraints of the substituted residue at that place. The authors (Hosszu et al., 2010) have also mentioned that pH induced protonation of key Histidine residues 155 and 187 induced conformational flexibility especially in the C-terminal part of helix 2, while, other regions of the backbone remain almost unchanged. This is in agreement with our data that the maximum flexibility of the structure was localized around region 190–195 at the end part of helix 2 due to the residue

replacement at position 208. It seems that this region is intrinsically sensitive to the induced perturbations, which candidates this part of the human prion protein as a hot structural segment to trigger conformational transitions. However, introducing the neutralized state of Arginine residue at position 208 caused only some reversible structural changes in the intrinsically flexible loop area comprising residues 167–171 of the human prion protein.

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