Investigation on salt bridge interactions of mammalian prion proteins by molecular dynamics simulation

Memeli prion proteinlerinin moleküler dinamik simulasyon ile tuz köprüleri etkileşimlerinin araştırılması

Abstract: Objective: Salt bridge interaction is one of the most important electrostatic interactions to stabilize the secondary and tertiary structures of protein. To obtain more insight into the molecular basis of prion proteins, the salt bridge networks in two animal prion proteins are studied in this work.

Methods: Molecular dynamics (MD) and Flow MD (FMD) simulations are employed to investigate the salt bridges interactions of rabbit prion protein (rPrPc), Syrian hamster prion protein (syPrPc) and the variants of the two prion proteins.

Results: The dynamic behaviors of salt bridges are characterized, and the relation between salt bridge interactions and local structures are also discussed. The type of salt bridges in the two prion proteins is divided into the helix-loop, intra-helix and inter-helix salt bridges. It is found that the helix-loop salt bridges is more important for the stability of prion proteins than the other two kinds of salt bridge.

Conclusion: The Asp201-Arg155 (rS1), Asp177-Arg163 (rS3) and Asp178-Arg164 (syS1) are the important salt bridges to stabilize the structures of rPrPc and syPrPc, respectively. The structural stability is partly depended on the number of helix-loop salt bridge.

Keywords: salt bridge; stability; prion; molecular dynamics (MD); mutation

Özet: Amaç: Proteinlerin ikcincil ve üçüncü (terciy) yapılarının stabilizasyonunda tuz köprüleriinin elektrostatik etkileşimleri çok önemlidir. Bu çalışmada, prion proteinlerin moleküler yapalarını kavramak için, iki hayvan türünün prion proteinlerinde tuz köprüsü örütleri (network) çalışılmıştır.

Metod: Tavşan prion protein (rPrPc), Syrian hamster prion protein (syPrPc) ve bu iki prion proteininin varyantlarında moleküler dinamik (MD) ve akış MD (FMD) simulasyonları kullanılarak tuz köprüleri etkileşimleri çalışılmıştır.

Bulgular: Tuz köprülerinin dinamik davranışları karakterize edilmiş ve ayrıca tuz köprüleri ile yerel yapılar arası ilişkiler de tartışılmaktadır. İki prion proteininde tuz köprüleri yapışal olarak helix-loop, intra-helix ve inter-helix tuz köprüleri olarak ayrılmaktadır. Bunlardan helix-loop yapışında olan tuz köprüleri prion proteinlerin yapışmasını stabilitesi diger ikisine nazaran açısından daha önemli olarak bulunmuştur.

Sonuç: rPrPc ve syPrPc yaplarının stabilizasyonunda sırası ile Asp201-Arg155 (rS1), Asp177-Arg163 (rS3) ve Asp178-Arg164 (syS1) önemli tuz köprülerini oluşturmakta. Yapışal stabilite ise kusma helix-loop yapışının sayısına bağlıdır.

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**Anahtar Kelimeler:** tuz köprüsü, stabilite, prion, moleküler dinamik(MD), mutasyon

**Introduction**

Prion diseases, also called transmissible spongiform encephalopathies (TSEs), are fatal neurodegenerative disorders that have attracted enormous attention not only for their unique biological features, but also for their impact on public health. This group of diseases includes scrapie and bovine spongiform encephalopathy (BSE) of mammalian animals, Creutzfeldt-Jakob disease (CJD) and kuru in humans [1–3]. It has been found that the key pathogenic mechanism of prion diseases is the misfolding and conversion of the normal prion protein (PrPc) into the scrapie isoform of prion protein (PrPsc). The primary structure of PrPc is same as PrPsc, however, experimental results show that the secondary structure and biofunctions of the two isomers are rather different [4,5]. The molecular mechanisms by which PrPc is converted into PrPsc have not been established. Some studies show that there are intermediates during the transition process, however, some experiment results do not support it. One putative form of PrPc which is named PrP* is considered to coexist with the native PrPc under physiological conditions [6]. Kuwata et al. also found this intermediate conformer with disordered helices 2 and 3 under roughly physiological conditions by high-pressure nuclear magnetic resonance (NMR) experiments [7]. Hosszu et al. use NMR to examine the unfolding transitions of PrPc at equilibrium, and they found five metastable forms of the protein [8]. Recently, a three-state mechanism with a highly enthalpically stable state is characterized in the unfolding process of some prion-susceptible species, such as mouse, cattle and sheep variants [9]. The single-molecule force spectroscopy has been applied by Yu et al. to unfold the PrPc [10, 11]. However, they found that the native folding pathway involves only two states and no evidence for partially folded intermediates is found, which is contrary to the finding of Kuwata et al. Hoffmann’s work also indicates that the native folding pathway of PrPc does not include any intermediate state [12].

Recently, some researches show that both the disulfide bridge and salt bridge are important for stabilizing the PrPc. NMR analysis has revealed that a disulfide bridge (Cys178-Cys213) and two salt bridges (Asp143-Arg147, Asp146-Arg150) in helix 1 of mouse PrPc play key roles on maintaining the tertiary structure of protein. Yet the contribution of the salt bridges is still controversial [13]. Morrissey and Shakhnovich propose in the paper that the conversion mechanism of PrPc to PrPsc involves critical interactions at helix 1 and this helix is stabilized by two intra-helix salt bridges (Asp144-Arg148, Asp147-Arg151) [14]. Many studies also showed that the partial or the complete loss of the salt bridge network in the α2–α3 region in all diseased-linked mutations may lead to a destabilization of PrPc fold in those regions [15–18].

The α2–α3 region is proposed to constitute the main seeding element for the conformational transition and the tendency of PrPc to form fibrils. High-pressure NMR studies and isotope exchange experiments point to relatively large conformational flexibility of this region [7, 19–21]. Recently, the β2-α2 loop has also been found to play an important role in the structural stability of rabbit and horse prion proteins [22,23]. Particularly, a strong salt bridge between Asp177 and Arg163 (like a taut bow string) in horse and rabbit prion proteins links the β2-α2 loop. Moreover, four salt bridges (Glu196-Arg156, Arg156-His187, Arg156-Asp202 and Glu211-His177) also contribute to the structural stability of horse prion protein [23]. For human prion protein and Syrian hamster prion protein, four key salt bridges (Glu146-Arg208, Asp144-Arg208, Arg164-Asp178, and Arg156-Glu196) have been reported by Zuegg and Gready [24]. In Speare’s work, Asp144 and/or Asp147 in hamster PrPc are replaced with Asn. These substitutions, designed to abolish one or two salt bridges in helix 1, result in a decreased thermodynamic stability of the protein. However, the magnitude of this effect is really small [25]. A molecular dynamics simulation on the intrinsic helical propensities of hamster PrP also indicates that the salt bridges do not stabilize helices [26]. But it should be noticed that the authors only studied two salt bridges (Asp144-Arg148, Asp147-Arg151) in helix1.

Considering the large amount of previous studies identifying specific salt bridges are important to the structural stability of PrPc, we performed molecular dynamics (MD) simulations on two typical animal prion proteins to investigate this kind of electrostatic interaction. MD simulations appear to be a promising way to further our understanding of the intra-molecular interactions of proteins. They can be used to probe into details at the atomistic level that may not be accessible to experimental techniques. They also provide dynamics information on the interactions within the protein in addition to the static equilibrium configuration and energy calculations [22–24, 26]. The Flow MD (FMD) method is a recommended technique to probe the mechanical stability of a protein under shear flow conditions. The flow was generated by directly applying force to the selected water molecules, with the pulling force is controlled easily. The force-unfolding dynamics of proteins can be studied by the FMD method. FMD has become one of the most effective supplementary means of in vitro
single molecule experiments [27,28]. Studies have shown that the susceptibility to prion disease varies depending on the donor species, strain of infectious prion, genotype of the recipient and the species. Rabbit prion protein (rPrPc) is one of several mammalian species reported to be resistant to infection from prion diseases [29]. Syrian hamster prion protein (syPrPc) appears to be susceptible to prions and it has often been used as an animal model [30]. The two proteins are selected to study the dynamic stability of mammal prion proteins. In this work, MD and FMD simulations are combined to study the salt bridge interactions of the wild type and the mutants of the two proteins.

Materials and Methods

System setup

The structural models are based on the globular domain NMR structures of rPrPc (residues 124-228, PDB code 2FJ3 first structure) and syPrPc (residues 125-228, PDB code 2LH8) [29,30]. The missing hydrogen atoms were added using the AUTOPSF plug-in of VMD [31]. The globular domain (Fig. 1a) of rPrPc contains three α-helices comprising the residues 143-153 (r-α1), 171-186 (r-α2), and 199-224 (r-α3) as well as a very short anti-parallel β-sheet comprising residues 129-132 (r-β1) and 159-161 (r-β2). The r-α2 and r-α3 are bridged by a disulfide bridge composed of Cys178 and Cys213 in rPrPc and it has been stated in this work. The globular domain (Fig. 1b) of syPrPc also contains three α-helices comprising the residues 144-154 (sy-α1), 172-193 (sy-α2), 200-227 (sy-α3) and a β-sheet comprising residues 129-133 (sy-β1) and 160-163 (sy-β2).

The proteins were immersed in a periodic water box with TIP3P water molecules [32]. The volumes of the water boxes for rPrPc and syPrPc were set to 52.39×65.96×48.52 Å³ and 69.66×60.05×55.19 Å³, respectively. The average distance left between the protein and the walls of water box is 13 Å, which is more than the cut-off of the long-range electrostatic interaction. Moreover, The water box was designed to be large enough to accommodate the structural evolution during MD and FMD processes. Sodium and chloride counterions were added to neutralize the charges in rPrPc and syPrPc boxes.

MD simulation details

All the simulations were performed using the program NAMD version 2.7 [33] in combination with the Charmm27 force field [34]. Here we performed three parallel 50 ns MD simulations for the relaxation of prion structure. The statistical accuracy of our results was investigated by performing three independent simulations for each system. A time step of 2 fs was selected and the coordinates were saved every 1 ps. The particle mesh Ewald (PME) method was employed to calculate the long-range electrostatic interactions with a cut-off of 12 Å for the separation of direct and reciprocal space summation. The van der Waals interactions were truncated at 10 Å. During MD runs, the Langevin method was turned on to control constant temperature at 310K and constant pressure at 101.3 kPa. Energy minimization was performed to optimize the
geometry of the protein and then MD simulation was conducted to equilibrate the systems for 50 ns.

**FMD simulation details**

The constant force pulling FMD simulations for 10 ns were conducted following the MD simulations. A constant external force was applied on one or more atoms, which are referred to as FMD atoms. In this work, a slab of water molecules with the interval [0, 3] Å are selected as the FMD atoms to produce the shear flow. The other water molecules and protein are free. Since the module of protein might be roughly described as a rectangular box, we considered six different starting orientations. The systems are named with the six faces of the water box along the Cartesian axis and they are donated as +x, -x, +y, -y, +z and -z, respectively. The equilibrium state of the MD simulation is used as the starting state in the FMD simulations. The snapshot of the system with the force applied in the direction +x is shown in Fig. 1a as an example. The final state of rPrPc and water molecules after FMD simulation in +x direction is shown in Figure s1. The pulling force was set to $4 \times 10^3$ pN for the two proteins, and it has been used successfully to investigate the dynamics properties of prion protein [35,36]. The trajectory analysis of protein was performed with VMD package.

**Mutants preparation**

In order to investigate the function of the selected salt bridges in this paper, we replaced the residues Arg of these salt bridges with Ala by using the VMD package based on the equilibrated structures. Three variants Arg155Ala, Arg150Ala and Arg163Ala of rPrPc are named as rM1, rM2 and rM3, respectively. The four mutants Arg164Ala, Arg148Ala, Arg208Ala and Arg151Ala of syPrPc are denoted as syM1, syM2, syM3 and syM4, respectively. For each mutant system, we performed energy minimization and three parallel 50ns-MD simulations without any restraining potential applied to relax the complex structure.

**Analytical methods**

Cα-RMSD is the root mean square distance of the Cα atom of protein between the two different frames. The first time step of the simulation is used as a reference. Cα-RMSF is the root mean square position fluctuation of Cα atom for each selected protein during the simulation. The secondary structure of proteins is calculated with STRIDE which is implemented in the VMD package. Here the H-bond was defined by the following criterion: given a heteroatom A attached to an H-atom and another heteroatom B not bonded to A, an H-bond is formed only if the distance between two heavy atoms is smaller than 3.5 Å and the A-H...B angle is smaller than 30°. A distance cutoff of 4 Å was used as a criterion for the presence of the salt bridges [37,38]. For the salt bridge interaction, we used the distance from the anionic carboxylate (RCOO⁻) of either aspartic (Asp) acid or glutamic (Glu) acid and the cationic ammonium (RNH₃⁺) from lysine (Lys) or the guanidinium (RNHC(NH₂)⁺₂) of arginine (Arg).

**Results and Discussion**

**Equilibrium states**

Fig. 2 shows the Cα-root mean square distances (Cα-RMSDs) of the proteins in the three parallel MD runs. All the three Cα-RMSD curves are similar in rPrPc system. Yet in the syPrPc system, the curve of Run1 is slightly different from the others. However, the average Cα-RMSD values of all the curves are about 3 Å, which shows that the proteins have been equilibrated.

**Structural flexibility**

We show per-residue Cα-RMSF of the rPrPc proteins in the three MD runs in Fig. 3. Moreover, we also compare
the simulation-derived Ca-RMSF plots to the RMS values obtained from the first NMR structure of rPrPc (PDB code 2FJ3). Our MD-generated Ca-RMSF of the structures of rPrPc agrees reasonably well with the published NMR data. Although there are differences among the three MD runs, the shapes of the three MD-curves are similar to the curve of experimental data. Except for the two terminal peaks in Fig. 3, there are also exhibiting three other notable peaks, which locate at about residues 140, 168 and 195, respectively. The three Ca-RMSF peaks are produced by the large fluctuation of loop and/or coil structure of rPrPc. The first one is the loop region that links the r-β1 (129-132) and the r-α1 (143-153). The second peak locates at the flexible region between r-α2 (171-186) and r-β2 (159-161). The last one is generated by the flexible residues between r-α2 and r-α3 (199-224). The rigid structures (α-helix and β-sheet) show small Ca-RMSF values. The Ca-RMSF information of syPrPc in the three MD runs is shown in Fig. s2.

**Structural evolution in relaxed state**

We compare the structural evolution of the two proteins during the MD simulations. Fig. s3 shows different conformational transitions appearing in the Run1 MD trajectory of rPrPc and syPrPc. The secondary structural evolution graphs in the other two MD trajectories of rPrPc and syPrPc are shown in Figs. s4 and s5, respectively. As shown in Fig. s3, the β-sheet (yellow belts) in rPrPc disappears at 1.1 ns, and it mainly transforms into β-bridge
As shown in Fig. s4, the disappearance of the β-sheet also occurs in the Run2 MD simulation. Yet in the third trajectory, the β-strands only disappear before 8.0 ns. For the syPrPc system, the β-sheet is kept intact in most of the MD simulation time. Another obvious difference between the two systems is the transition of the second α-helix. The r-α2 is the most unstable helix, and it is broken from the inner part of the helix column. Differently, the sy-α2 is beginning to unfold at about 4.0 ns from the Lys194-terminus and it transforms to π-helix and turn structures. Both the third α-helices of the two proteins are ready to unfold from the C-terminal of protein. With the analysis of the structure of the two proteins, it is found that there is a disulfide bridge (Cys178-Cys213) which links r-α2 and r-α3 in the rPrPc. The flexibility of the two helices r-α2 and r-α3 is limited by the disulfide bridge. Then the r-α2 is easily broken in the position close to the residue Cys178. However, there is no disulfide bond limits the motion of sy-α2 and sy-α3. So the sy-α2 is more stable than r-α2 during the MD simulation.

We also show the snapshots of rPrPc and syPrPc during the three MD trajectories in Figs. s6 and s7, respectively. Interestingly, the β-sheet of rPrPc is kept intact during all the three simulations, which is contrary to the result of secondary structure evolutions (Figs. s3 and s4). We also watch the cartoon of the MD simulations by VMD package. It is found that the β-sheet of rPrPc is rather unstable with the motions of twisting, unfolding and even extending. To further the investigation of the disappearance of β-sheet during the MD process, we study the evolution of the H-bond (Met128:HN-Tyr162:O) distance and the N-H...O angle in rPrPc during the Run1 MD simulation. As shown in Fig. 4, the distance of the H-bond keeps at less than 3.5 Å in most of the simulation time. However, the angle easily rises up to more than 30°, which shows that the interaction between the two strands is not completely lost. This finding is also supported by the snapshots of rPrPc during the MD simulations (Fig. s6). The graphs show that the β-sheet still exists during the simulation. Yet the shape of the β-sheet is twisting with the change of the related H-bond angle.

**Screening of key salt bridges**

The occupancy of salt bridges was calculated by the following formula:

\[
\text{Occupancy} = \frac{\text{occupancy time of salt bridge}}{\text{simulation time}}
\]  

(1)

The occupancies of all the salt bridges of rPrPc and syPrPc in the three MD runs are shown in Table 1. The numbers of the salt bridges of rPrPc and syPrPc are 9 and 12, respectively. The order of salt bridges depends on the occupancy, and they are named as S1, S2, S3... and Sn, respectively. Among these salt bridges, the S1 and S3 of rPrPc [39] and the S1, S2, S4, S8 of syPrPc [22,24] have been referred in the references. Particularly, the occupancies of the same salt bridge are not same in the three parallel MD runs. Then the salt bridges with the occupancy more than 50% in all the three runs are selected as the key salt bridges to be discussed. Three key salt bridges of Asp201:Arg155 (rS1), Asp146:Arg150 (rS2) and Asp177:Arg163 (rS3)
Arg163 (rS3) are selected for the rPrPc system. The Asp178-Arg164 (syS1), Glu152-Arg148 (syS2), Glu146-Arg208 (syS3), Asp147-Arg151 (syS4) are defined as key salt bridges for the syPrPc system.

The interaction pairs and the location of the salt bridges are illustrated in Fig. 5. Three kinds of salt bridge interactions are shown in the graph. The rS1, rS3, and syS1 are helix-loop salt bridges. The rS2, syS2, and syS4 are intra-helix salt bridges. The syS3 is the inter-helix salt bridge. The rS2 is formed between Asp146: OD2 and Arg150: HH21 in the r-α1 helix of rPrPc. Moreover, one helical H-bond of r-α1 is also formed between these two residues with Asp146: O and Arg150: HN. Previous experimental and theoretical studies have demonstrated that isolated α1 of mammalian PrPc is very stable [17,40,41]. Interestingly, both r-α1 and sy-α1 possess the intra-helix salt bridges, and it may result in the higher rigidity and stability of α1 in both the two mammalian prion proteins.

Both rS1 (Asp201:OD1-Arg155:HH11) and rS3 (Asp146-Arg147) link the helix and the loop structure, however, the distance curves of the two salt bridges are different. The rS1 plays the stepwise decrease motion during the MD process in Fig. 6. It keeps on about 4.5 Å before 5 ns, and then it drops to 3.3 Å. Finally, the distance is quite stable at about 1.8 Å from 14 to 50 ns. However, the fluctuation of rS3 curve is rather large, and it changes between 9.0 and 1.7 Å. In order to explain the different dynamic behaviors of rS1 and rS3, we study the local structure neighboring the salt bridge. Arg177, involves in the rS3, is next to the Cys178 which forms the disulfide bridge. Yet the Arg201 is far from the Cys213. The disulfide bridge provides extra stability of the residue, and the flexibility of the backbone atoms of Arg177 is restricted. Then it is hard for Arg177 to rearrange its location to maintain the salt bridge interaction. For rS1, the rigid r-α3 fixes the Arg201, and the loop structure moves randomly to regulate the salt bridge interaction. Simultaneously, the helical part where Arg201 is located can be bent to some extent to facilitate the existence of rS1. Thus the rS1 is more stable than rS3. As rS2 locates in r-α1, the backbone of the residues which forms rS2 is fixed by the helix. It fixes the two residues of rS2 and provides the meeting chance of the two side chains. At the same time, it also limits the backbone flexibility of the two residues to maintain the salt bridge. Then the fluctuation of the rS2 (Asp146:OD2-Arg150:HH12) curve is a result of the motion of side chains. Simultaneously, the H-bond formed by the same residues of rS2 works together with the salt bridge to stabilize the helix structure. The existence of rS2 and H-bond stabilizes the helix, and the rigidity of the helix also increases the occupancy of rS2.

As shown in Fig. 7, syS1 (Asp178:OD1-Arg164:HH11) distance possesses the smallest fluctuation in all the four curves. The occupancy of syS4 (Asp147:OD2-Arg151:HH11) is less than that of syS2 (Glu152:OE1-Arg148:HH21). Similar to the occupancy difference of rS1 and rS3, the difference between the two salt bridges is mainly caused by the flexibility of the side chains. The Arg147 in syS4 is located between rS2 and rS3 (Fig. 5b), and its flexibility is restricted by the two neighboring interactions. Then the formation of rS4 is hard to be regulated, and the distance curve is mainly directed by the thermal motion of the side chains of Arg147 and Glu151. Two α-helices are linked by syS3 (Glu146:OE2-Arg208:HH12), and the rigidity of the two helices restricts the flexibility of the salt bridge.

| Table 1: Salt bridge interactions and occupancies in the three parallel MD runs. |
|---------------------------------|---------------------------------|---------------------------------|
|                                | rPrPc (Occupancy (%))           | syPrPc (Occupancy (%))          |
|                                | Run 1  2  3                      | Run 1  2  3                      |
| Asp201-Arg155 (rS1)            | 90.8  97.0  92.1                | Asp202-Arg156                   | 95.8  8.0  50.3                 |
| Asp146-Arg150 (rS2)            | 88.2  63.1  89.0                | Asp178-Arg164 (syS1)            | 95.1  99.1  99.0                |
| Asp177-Arg163 (rS3)            | 83.6  60.7  85.7                | Glu152-Arg148 (syS2)            | 87.8  89.2  88.9                |
| Arg207-Glu210                  | 77.5  8.1  70.7                 | Glu146-Arg208 (syS3)            | 82.5  99.5  97.2                |
| Asp143-Arg147                  | 53.1  7.3  59.1                 | Asp147-Arg151 (syS4)            | 56.4  77.6  78.9                |
| Lys184-Glu206                  | 39.6  –  29.0                   | Glu221-Lys220                   | 55.2  19.5  14.3                |
| Glu145-Lys203                  | 19.5  23.6  26.7                | Lys194-Glu196                   | 41.8  44.1  40.7                |
| Glu199-Lys203                  | 7.8  9.0  8.0                   | Glu196-Arg156                  | 27.6  52.8  55.8                |
| Asp166-Arg227                  | 5.7  30.5  3.0                   | Lys204-Glu207                   | 23.2  32.9  35.7                |
|                               |                                 | Glu146-Lys204                   | 4.3  7.7  5.0                   |
|                               |                                 | Glu200-Lys204                   | 4.2  7.9  5.4                   |
|                               |                                 | Asp147-Arg136                   | 2.2  2.2  –                     |

The symbol “–” represents that there is no formation of salt bridge.
motion of the helix may greatly influence the formation of syS3, which results in the large and frequent fluctuation of the syS3 distance.

**Structural deformation under water perturbation**

The secondary structural evolution of rPrPc is shown in Fig. s8. In all the six graphs of Fig. s8, it is clearly found that the β-sheet is rather unstable in all the perturbation directions. There is almost no β-sheet left during the FMD simulations. Yet the changes of the α-helices vary from the directions. The r-α2 is unfolded from the His186-terminus in –x direction. The unfolding of r-α3 mostly begins from the Tyr225-terminus in the directions of +x, +y, -y and +z. The r-α1 is the most stable one.

Differently, the two β-strands in syPrPc are more stable under the water flow than that in rPrPc (Fig. s9). The sy-α1 is stabilized by two intra-helix salt bridges (syS2 and syS4) and one inter-helix salt bridge (syS3). Then the three salt bridges maintain the integrity of helix and the magenta belt of sy-α1 is kept intact during the whole simulation. The residue Arg164 of the β-sheet is linked with sy-α2 by salt bridge syS1, which stabilizes the conformation of the β-sheet. The unfolding of sy-α3 begins from Tyr226 and Asp227 and it is mainly caused by the flexibility of the terminus of syPrPc. By comparing the secondary structure evolutions in the MD and FMD simulations, it is found that the helices are not obviously unfolded by the external force. The helix-3 in some directions in the FMD process even becomes more stable. It might be a result of

![Figure 8](image_url)

**Figure 8:** (a) Time evolution of the salt bridges distance of rPrPc under the external force in direction +y. Snapshots of (b) the initial state and (c) the final state of rPrPc with the model salt bridges tagged. The distances of rS1, rS2 and rS3 are represented by the interaction pair of Asp201:OD1-Arg155:HH11, Asp146:OD2-Arg150:HH12 and Asp177:OD1-Arg163:HH21, respectively.

![Figure 9](image_url)

**Figure 9:** Distance evolution of rS3 (Asp177:OD1-Arg163:HH21) and the external force in the direction of (a) +x, (b) -x, (c) +y, (d) -y, (e) +z and (f) -z during the FMD simulation.
the forces due to water flow is too small compared to the thermal fluctuations.

**Dynamics of salt bridges**

The distance evolutions of the three key salt bridges in rPrPc under the water flow are displayed in Fig. 8. Among the three salt bridges in rPrPc, rS1 (black curve) is the most stable one. The distance of rS1 is kept on 1.85 Å during the whole FMD process. The stability of rS1 is maintained by the backbone of the salt bridge. The helix works as an anchor to fix rS1. And the loop is flexible enough to reduce the breaking due to water flow. Simultaneously, the existence of rS1 maintains the contact of secondary structures in rPrPc. Under the perturbation of water flow, the distance of Asp146:OD2 and Arg150:HH21 (red curve) fluctuates between 1.52 and 6.18Å, which shows that the salt bridge interaction of Asp146 and Arg150 (rS2) is unstable. The occupancy time of rS2 during the FMD simulation is 94.0%. The results indicate that the rS2 is perturbed by the water flow, however, the force is not big enough to completely break the salt bridge interaction in +y direction. The stability of rS2 is enhanced by the helical H-bond of Asp146 and Arg150 in r-α1. The distance of H-bond is also shown in Fig. 8. The H-bond distance is rather stable during the FMD simulation, which shows the superior stability of r-α1. Thus the occupancy time of rS2 is ensured by the integrity of α-helix. Similarly, it can also be proposed that the H-bond and salt bridge work together to stabilize the α-helix under the water perturbation. The distance of Asp177:OD1-Arg163:HH21 (rS3, blue curve) is kept about 3.46 Å before 4.2 ns and then it quickly increases to 18.36 Å in the last 5.0 ns. The breaking of the rS3 is a result of the separation of the loop from the helix at 4.86 ns. Moreover, it is also determined by the direction of the applied force. To obtain more insight into the dynamic stability of the salt bridge under different force directions, the distance evolution of rS3 in all the six directions is shown in Fig. 9. The various curves show the anisotropic properties of
rS3 in different directions. The interaction distances in the directions of +x, +y and +z rise up to high values, which shows that the rS3 is rather unstable with the perturbation from these directions. With the analysis of the conformation of rPrPc, it is discerned that the loop structure linked by rS3 is exposed to the surface in the directions of +x, +y and +z. The water flow can therefore directly push the loop away from the r-α3 helix, and rS3 is easily destroyed in these directions.

The dynamic details of the four model salt bridges in syPrPc are separately displayed in Fig. 10. With the applying force in +y direction, the syS1 is the most stable one among all the four salt bridges during the FMD simulation. The interaction distance between Glu152:OE1 and Arg148:HH21 (rS1) is stable at about 3 Å in most of the FMD time. The syS2 distance is sensitive to the perturbation of water flow. With the analysis of the cartoon of FMD trajectory, it is found that the sy-α1 is exposed on the xz plane. When the force is applied along the +y direction, the syS2 is easily broken. However, the force applied in this work is not big enough to unfold the helix during the simulation time. The H-bond (Asp148:O-Arg152:HN) still exists and keeps the intact of sy-α1. At the same time, the rigid helix structure provides a backbone to help the reforming of salt bridge. Similar dynamics motion appears in the syS4 (Fig. 10d). In Lu’s work, the intra-helical salt bridge syS4 is also studied. They conclude that the occurrence of salt bridges is not correlated with the helical propensity, indicating that salt bridges do not stabilize helices [26]. The dynamics analysis of syS4 in our paper also shows that the stability of the salt bridge is less than that of the H-bond, however, it can be stabilized by the related H-bond and the helix. During the FMD simulation, sy-α1 and sy-α3 are separated by the water flow at 3.5 ns, and gather again when the perturbations passed through. And a high platform in the syS3 curves is a result of the separation of the two helices. Simultaneously, the reformation of syS3 also stabilizes the connection of sy-α1 and sy-α3.

As discussed above, the salt bridge and the helix can stabilize each other. The intra-helix salt bridge syS2 is composed of the side chains of Glu152 and Arg148. A helical H-bond of sy-α1 is also formed by the same residues. The distances of the two kinds of interactions in syPrPc under the external force in the six different directions are displayed in Fig. 11. Caused by the flexibility of the side chains, syS2 distances in all six directions of the applied force fluctuate dramatically. The syS2 salt bridge is most stable under the force in the direction of +x, and the distance is kept about 4.0 Å. Large distance fluctuations appears in the directions of –x, –y and –z. As shown in Figs 11e and 11f, the H-bond distance increases from 1.7 to 4.1 and 4.3 Å, respectively. Similarly, the intra H-bond (green) is also disturbed by the shear flow of water in these directions. The trends of salt bridge and H-bond curves are similar, which shows the two interactions interrelate and interact with each other.

**Structural evolution of mutants**

As the key salt bridges selected in this paper were cancelled by the single amino acid mutation, the stability of the local structure close to the replaced residue might change. In order to show the relevance of the thermodynamics stability and the salt bridges in the two prion proteins, the secondary structure evolution of the proteins in the seven mutation systems are shown in Figs. s10 and s11. In the rM1 system, the yellow belts (β-sheet) become rather unstable in the MD Runs 2 and 3. As the cancelled salt bridge rS1 locates in one terminus of r-β2, the shortening of the yellow belt might be a result of the mutation of rS1. However, the β-sheet is stable in the first MD run of rM1 system. The rS2 is a intra-helical salt bridge, and it is shown in the three structural evolution graphs of rM2 that there is no obvious change for the r-α1 (the magenta belt, residues 143-153). For rM3, the β-sheets (yellow) become rather unstable in all the three MD runs. Especially, the r-α2 (the second magenta belt) is broken from the residue 177, which is just the mutation site. This result shows that the substitution of rS3 might lead to the rearrangement of local structure.
As shown in the Fig. s11, the sy-α2 (the second magenta belt) appears to be unfolded neighboring to the mutation site (residue 178) in the 2 and 3 MD runs of syM1 system. Furthermore, the sy-β2 (the second yellow belt) partly unfolds in all the three runs. However, the abolish of the intra-helical salt bridges of syS2 and syS4 do not show obvious change in the local structure, which is similar to the rM2 system. In the syM3 system, the salt bridge between sy-α1 and sy-α3 is broken. By comparing the graphs of syS3 in Figs. s3, s5 and s11, it is found that there are no significant structure changes near the mutation site between the wild-type and the mutation systems.

The above analysis discerns that the local structure in rM1, rM3 and syM1 systems is changed with the disappearing of the original salt bridges. However, the other mutation systems do not show obvious mutation-related structure changes. By analyzing the type of salt bridge, it is found that the helix-loop salt bridge is more important for the stabilization of the local structure of prion protein. For example, the unfolding of the helix appeared at the mutation site in rM3 and syM1 system. Furthermore, the β-sheet becomes unstable in rM1, rM3 and syM1 systems. Yet the abolish of intra-helix (rM2, syM2 and syM4) and the inter-helix (sySM3) salt bridges do not lead to obvious structural change in the mutation region, which is similar to the conclusions of Speare and Lu [25,26]. There are two helix-loop salt bridges in rPrPc and only one in syPrPc. Then the structure of rPrPc is easier to be disturbed by the breaking of salt bridges. With the analysis of the secondary structure evolution of rPrPc and syPrPc in the FMD process (Figs. s8 and s9), it is also found that the β-sheet is rather unstable in the rPrPc system. Both the mutation and FMD studies show that the helix-loop salt bridge is more important for the stability of prion protein.

Conclusion

The studies of the salt bridges in mammalian prion proteins are performed to obtain more insight into the molecular basis of the intra-molecular interactions and the stability of rPrPc and syPrPc. MD simulations and FMD simulations are carried out to produce the relaxed and disturbed states for the two prion proteins, respectively. The salt bridge networks of the two proteins are mapped. Among all the salt bridges, seven key salt bridges with the occupancy more than 50% in all the three parallel MD runs are discussed in this work. They are divided into helix-loop salt bridge, intra-helix salt bridge, and inter-helix salt bridge. It is found that the helix-loop salt bridges have the largest occupancy in both the two systems. The rigid helix works as an anchor to fix one end of the salt bridge, and the flexible loop can move randomly to protect the salt bridge from breaking. All the intra-helix salt bridges are associated with the helical H-bond interactions, and they work together to stabilize the helix, which demonstrates and supports the superior stability of α1 in experiments. The salt bridge, the intra H-bond and the helix worked together to stabilize the structure of prion protein. Furthermore, the single amino acid mutation on the seven salt bridges is also performed. MD simulations on the mutation systems are carried out to study the roles of salt bridge on the structural stability of prion proteins. The results of the mutation systems emphasize the finding that helix-loop salt bridge is more important to stabilize the local structure of the two prion proteins. The rS1(Asp201-Arg155), rS3(Asp177-Arg163) and syS1(Asp178-Arg164) are the key salt bridges to maintain the stability of rPrPc and syPrPc, respectively. The structural stability is partly depended on the number of helix-loop salt bridges, and it might be investigated by the NMR experiments in the future work. It should be noticed that the results in this work show that the number of helix-loop salt bridge in rPrPc is more than that in syPrPc. Then the structure of rPrPc is easier to be disturbed by the external force, which is not directly related with the superior resistance to the infection from prion diseases of rPrPc. The mechanism of the misfolding from PrPc to PrPsc is complicated and influenced by many factors. The studies in this paper provide a insight into the relevance between salt bridge and the structural stability of rPrPc and syPrPc.

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