Two Misfolding Routes for the Prion Protein around pH 4.5

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Abstract

Using molecular dynamics simulations, we show that the prion protein (PrP) exhibits a dual behavior, with two possible transition routes, upon protonation of H187 around pH 4.5, which mimics specific conditions encountered in endosomes. Our results suggest a picture in which the protonated imidazole ring of H187 experiences an electrostatic repulsion with the nearby guanidinium group of R136, to which the system responds by pushing either H187 or R136 sidechains away from their native cavities. The regions to which H187 and R136 are linked, namely the C-terminal part of H2 and the loop connecting S1 to H1, respectively, are affected in a different manner depending on which pathway is taken. Specific in vivo or in vitro conditions, such as the presence of molecular chaperones or a particular experimental setup, may favor one transition pathway over the other, which can result in very different PrPSc monomers. This has some possible connections with the observation of various fibril morphologies and the outcome of prion strains. In addition, the finding that the interaction of H187 with R136 is a weak point in mammalian PrP is supported by the absence of the {H187,R136} residue pair in non-mammalian species that are known to be resistant to prion diseases.

Introduction

The misfolding of the prion protein (PrP), which is a key aspect of transmissible spongiform encephalopathies (TSE), has been the subject of intense research during the past decades. Nonetheless, little is known about the underlying molecular mechanism. One serious hurdle remains the determination of the structure of the resulting misfolded isoform (PrPSc) [1]. As a consequence, various PrPSc models have been suggested with substantially different packing arrangements and monomer structures, and a consensus about the structure of PrPSc is far from being reached [1]. A particular subject of controversy is about the actual region of PrP that undergoes a deep refolding during the PrP → PrPSc conversion. According to the so-called “spiral” [2] and “β-helix” [3,4] models, extended β-sheets are formed in the N-terminal region and at the beginning of the C-terminal domain up to H1 (H1 is kept intact in the former and is refolded in the latter model). However, it has been recently shown that the H2/H3 core is also highly fibrillogenic by itself [5,6]. Finally, it has also been suggested that PrPSc could be entirely refolded in an irregular extended β-sheet [7].

Many in vitro [5,8–11] and computational [2,12–16] studies have tackled this issue using acidic conditions. They have consistently shown that low pH destabilizes PrP and favors its misfolding. This represents biologically relevant conditions insofar as endosomal organelles, whose typical pH is about 5 but can be as low as 4.3 [17], have been highlighted as possible locations for PrPSc growth [18–20]. Importantly, mammalian PrP contain one slightly buried residue, H107, that titrates right in the range of endosomal pH [11,13]. Several lines of evidence indicate that its protonation [13], or more generally the addition of a positive charge at site 187 [11], destabilizes the protein fold.

Whereas many theoretical studies have been performed on the globular C-terminal domain (residues 121–231 using the numbering of the human sequence) of mouse PrP (mPrP, Fig. 1-A), it is worth noting that the cellular form of PrP (PrPC) also contains a long unstructured N-terminal tail (residues 23–120) [21–29], a glycosylphosphatidylinositol (GPI) anchor [30–32] and can be mono or diglycosylated [27,33]. Nevertheless, previous MD simulations have suggested that the structure and dynamics of the globular domain of PrPC is rather independent of the anchoring to the membrane and the glycosylation [34]. In addition, our previous study of the misfolding propensity of mPrP using extensive REMD simulations [16] has revealed that various β-rich monomers can be formed from the C-terminal domain alone, which is also consistent with the results of Ref. [5,6].

Here, we have performed microsecond MD simulations of the structured C-terminal domain of mPrP at pH 4.5, which corresponds approximately to the lowest pH value observed in endosomes [17]. To this end, we assigned the protonation state of all titrable residues with the program PROPKA [35] (see also Materials and Methods section). The only buried residue for which the protonation state cannot be uniquely assigned is H187. The protonation/deprotonation of a buried residue usually affects the protein structure drastically [36,37]. Nevertheless, several semi-
Transmissible spongiform encephalopathies, which include the “mad cow” disease and the Creutzfeldt-Jakob disease, are related to the abnormal folding of a host protein termed the prion protein (PrP). Many aspects of the underlying molecular mechanism still remain elusive. Among the hypotheses that have been put forward in the past few years, it has been suggested that PrP could be destabilized by the protonation of a specific residue, H187, when the protein passes through acidic cell organelles. We have modeled PrP at the atomistic level, with the neutral and protonated forms of H187. Our simulations show that the destabilization process can follow two alternative pathways that could lead to different final structures. This discovery may shed some light on one of the most puzzling aspect of prion diseases, the fact that they exhibit various strains encoded in the structure of misfolded PrP. In addition, the atomistic details provided by our model highlight a key interactions partner in the destabilization process, R136. The {H187,R136} residue pair is not present in non-mammalian species that do not develop prion diseases.

Author Summary

Conformational changes of the backbone

Fig. 2 shows the effect of protonating H187 on the backbone of mPrP. The structure is very stable and remains close to the NMR structure when H187 is neutral, whereas simulations with the protonated H187 exhibit important backbone fluctuations and reorganizations. As depicted in Fig. 2-C, these enhanced fluctuations are mainly located in two specific regions of the protein, namely H2(Cter), which hosts H187, and [S1−H1].

Fig. 2-E shows that the protonation of H187 induces a drastic change in the free energy surface. The projection of the free energy on the Cα−RMSDs of H2(Cter) and [S1−H1] shows a single minimum when H187 is neutral, which corresponds to the native structure of PrP, and a complicated multiple minima landscape when H187 is protonated. The new free energy basins are located ~3−6 Å away from the native basin, thus corresponding to substantial conformational changes.

The two example snapshots provided in Fig. 2-B,D show that this reorganization is accompanied by a significant modification of the secondary structure of the protein. We will provide a more detailed analysis of the secondary structure changes later in the following sections. For the time being, it is interesting to rationalize how the perturbation that is introduced at one side of the protein (the protonation of H187 located in H2(Cter)) is transmitted through the macromolecule and affects strongly the structure at the opposite side ([S1−H1]).

Reorganization of charged residues around H187

In order to understand the mechanism by which the protonation of H187 induces the reorganization of the protein structure, it is necessary to have a closer look to the environment of H187 in PrP. It is particularly interesting to focus on nearby charged residues because they are expected to play a major role in the reorganization of the protein when H187 gets a positive charge upon protonation. In the NMR structure of mouse PrP, the closest charged residues are R136, R156, K194, E196 and D202 (Fig. S5-A). R136 is somewhat isolated in terms of proximity with charged residues other than H187 (when protonated), whereas K194, E196, R156, and D202 form a network of salt-bridge interactions. These four latter residues have been pointed out as possible key residues in the misfolding of PrP [13,39]. As shown in Fig. S5-B, our simulation provide a consistent picture with that of Ref. [13], because the protonation of H187 leads to the disruption of the salt bridge between E196 and R156 and the transient formation of a
new salt bridge between the protonated E196 and H187, while a
small salt bridge is maintained between R156 and D202 (K194 is
highly solvated, independently of the protonation state of H187,
and never interact strongly with E196). Nevertheless, the fact that
R136 does not have any close alternative partner makes it more
sensitive to the positive electric field created by the protonated
H187, as we shall see in the next section.

Dual response of PrP, 187\textsubscript{m}/136\textsubscript{out} vs 187\textsubscript{out}/136\textsubscript{m}

The observation of structural rearrangements in [S1–H1], which is
located far from H187, has motivated us to perform a thorough
analysis of the mobility of each residue in this region. It
turns out that R136 is a key partner of H187 in the destabilization
of mammalian PrP upon protonation of H187. In the NMR
structure of mPrP (Fig. 1), Im\textsubscript{H187} and Gu\textsubscript{R136} are about 8 A apart
and loop \{H1 – S2\} (residues 154–158) is located in between.
Gu\textsubscript{R136} is stabilized by a series of dipole-charge interactions
with four peptide bonds while Im\textsubscript{H187} is H-bonded to one carbonyl
group and establishes van der Waals contacts with the ring of P158
(Fig. 1-B).

Because of the proximity of Im\textsubscript{H187} and Gu\textsubscript{R136} in the native
structure of mPrP, the protonation of the former should induce an
electrostatic repulsion between the two groups. A discussion of the
corresponding energetics is provided in Text S1. Fig. 3 shows the
effect of the protonation of H187 on the position of Im\textsubscript{H187} (or
Im\textsubscript{H187}H\textsuperscript{+}) and Gu\textsubscript{R136}. When H187 is neutral, Im\textsubscript{H187} and
Gu\textsubscript{R136} are mostly located in their respective native cavities,
wheras they cover a much wider portion of conformational space
upon protonation of H187. We define four conformational states
according to the position of Im\textsubscript{H187} (or Im\textsubscript{H187}H\textsuperscript{+}) and Gu\textsubscript{R136}
inside or outside their respective native cavities. To do so we
consider the bivariate histogram of the distances between Im\textsubscript{H187}
(or Im\textsubscript{H187}H\textsuperscript{+}) and Gu\textsubscript{R136} from their respective cavities (Fig. 3-
C). The conformational state in which both groups stay close to
their original location will be termed 187\textsubscript{m}/136\textsubscript{m}, and we define states
187\textsubscript{m}/136\textsubscript{out} and 187\textsubscript{out}/136\textsubscript{m} according to the departure of
Im\textsubscript{H187}H\textsuperscript{+} or Gu\textsubscript{R136}+, respectively. Interestingly, the
187\textsubscript{out}/136\textsubscript{out} state is almost not populated. The picture that is
the most consistent with these data is that PrP exhibits a dual
response to the protonation of H187, by pushing away either
Im\textsubscript{H187}H\textsuperscript{+} or Gu\textsubscript{R136}+ (but not both at the same time), thus
decreasing the electrostatic repulsion between them. Because
H187 and R136 are attached to H2(Cter) and [S1–H1],
respectively (Fig. 1-A,B), the local reorganization of either
Im\textsubscript{H187}H\textsuperscript{+} or Gu\textsubscript{R136}+ affects the global structure of these two
regions (Fig. 2). We stress that, once H187 is protonated, the
dynamics of the system proceeds smoothly through a series of
locally thermalized states giving rise, in a reproducible way, to
either the 187\textsubscript{m}/136\textsubscript{out} or 187\textsubscript{out}/136\textsubscript{m} state. Fig. S6 and S7 show
that Im\textsubscript{H187}H\textsuperscript{+} and Gu\textsubscript{R136}+ remain in their native pockets during
at least 100 ns before one of the two moves out.

A similar electrostatic repulsion can be expected for the H187R
mutation, for which the positive charge of the introduced arginine
has been suggested to destabilize the overall fold of human PrP
[11,40,41]. An interesting aspect of this finding is that none of the
non-mammalian PrP exhibit this specific H2(Cter)–
Im\textsubscript{H187}H\textsuperscript{+}⋯[H1–S2]⋯Gu\textsubscript{R136}−[S1–H1] spatial arrange
ment (Fig. S4). In other words, these non-mammalian proteins
do not have this pH-sensitive “weak point” in there structure and
this probably explains the fact that non-mammalian species do not
exhibit TSEs.

Due to the buried character of H187 and the fact that its
protonation induces a substantial modification of the protein
structure, the quantitative evaluation of its
corresponding contributions of other residues) during the misfold-
ing is challenging [36,37]. Nevertheless, PROPKA calculations
[35] provide physically sound estimates that can help to rationalize
the underlying physics. Such calculations for representative

Figure 2. Effect of protonating H187 on the backbone conformation. (A,B) and (C,D) Simulation frames extracted every 50 ns from simulations with a neutral and protonated H187, respectively. The Cα atoms of H187 and R136 are represented with cyan and magenta spheres, respectively. The two regions of high fluctuations when H187 is protonated, H2(Cter) and [S1–H1], are represented by dashed red arcs in panel C. In panels A,C frames were extracted from two independent simulations of 1 \mu s with a neutral H187 and two independent simulations of 1 \mu s with a protonated H187, respectively, and colored according to the Cα–RMSF. Note that this corresponds to a subset of all our simulation, aimed at providing comparable data between panels A and C. We provide the same representations for each individual simulation in Figure S2. (E) Bivariate distribution of the Cα–RMSD in H2(Cter) and [S1–H1]. The contour plots are constructed from all the simulations with a neutral (blue) or protonated (red) H187. The reference structure used to compute the RMSD is the average structure calculated from all our simulations with a neutral H187.

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snapshots of our simulations are provided in Fig. S8. The pK_a of H187 is systematically shifted up as soon as the protein starts to misfold, independently of the pathway (187_in/136_out vs 187_out/136_in) that is taken. This is in agreement with the fact that the proximity with the positive charge of Gu^3136 in the native structure of mPrP induces a down-shift of the pK_a of H187 (this is supported by the fact that our PROPKA calculations report R136 as a key residue in the electrostatic environment of H187, see the corresponding PROPKA output file for a representative 187_in/136_out structure in Dataset S1). As soon as Gu^3136 moves out of its cavity (187_in/136_out state) the electrostatic repulsion between ImH187H^+ and Gu^3136 decreases and the protonated form of H187 becomes much more stable (pK_a shifted up). When the protein adopts a 187_out/136_in state, ImH187H^+ is much more solvated by water and the pK_a of H187 approaches the corresponding value in water (≈6).

S1, S2 elongation

The positioning of ImH187 (or ImH187H^+) and Gu^3136 has a strong influence on the length of the S1, S2 β-sheet, as shown in Table 1. Typically, both 187_in/136_in and 187_out/136_in states correspond to structures with a short native β-sheet, while the 187_in/136_out state is characterized by the preference of an elongated β-sheet. This is illustrated by the simulation depicted in Fig. 4. At the beginning of the simulation, the protein is in its native conformation. As depicted in the insets of Fig. 4, the native location of Gu^3136 at t~0 is a key aspect of the protein fold because it forms a sort of “clip” that forces the |S1−H1| backbone to remain packed against the rest of the protein (Fig. 1-A) in a specific conformation. The permanent departure of Gu^3136 out of its cavity at t~350 ns induces an important release of |S1−H1| backbone constraint and the system is consequently more prone to reorganize in this region. Then the system relaxes during about 400 ns, and |S1−H1| and |H1−S2| come close together. The number of hydrogen bonds between the two strands increases concomitantly and the β-sheet elongates (Fig. 4-A, B).

H2 unraveling

As shown in Fig. 5, both 187_out/136_in and 187_in/136_out states are characterized by an unraveling of H2(C ter). However, the underlying mechanisms (and the corresponding transition pathways) differ substantially. The portion of the helix that undergoes an unraveling is represented by a dashed purple arrow in Fig. 1-A (see also the example snapshot depicted in Fig. 2-D). The departure of ImH187H^+ (187_out/136_in conformation) out of its cavity obviously destabilizes H2 because the helix looses a key tertiary contact with loop |H1−S2| (Fig. 1-A). The unraveling of H2(C ter) in the 187_in/136_out state has its roots in the polar interactions of ImH187H^+ with the nearby residues. A closer look to the shape of the ImH187 cavity (Fig. 1) reveals that it is a narrow groove at the bottom of which lies the carbonyl group of T183. The contact analysis shown in Fig. 6 reveals that the neutral ImH187 is H-bonded to R156 only, consistent with the NMR structure of mPrP [21], whereas new contacts are formed with the CO group of T183 when H187 is protonated. A key aspect of these extra contacts is that they involve not only the N_H group of H187, but also the C_H group. They reflect dipole-charge interactions between the extra positive charge of ImH187H^+ and the dipole moments of the 156–157 and 182–183 peptide bonds. In other words, the imidazole ring can take two conformations around the C_p–C_y bond and still maintain a
significant interaction with one of the two nearby backbone CO groups, which results in four stable conformations inside the pocket.

The formation of new contacts between ImH187H and T183(CO) has two effects that explain the loss of helical character in H2(Cter). First of all, it weakens the tertiary contact between H2(Cter) and C138H1{S2}. Second, the native intra-helix H-bond between T183(CO) and H187(NH) is lost. The tighter the interaction between ImH187H and T183(CO) the weaker the local stability of H2.

Concluding remarks

In this paper we have shown that the protonation of H187 in mPrP at pH 4.5, which corresponds approximately to the lowest pH observed in endosomes [17], leads to extensive conformational changes on the microsecond time scale. The picture that emerges

Table 1. Number of short/long β-sheets.

|                | 187in/136in | 187in/136out | 187out/136in |
|----------------|-------------|-------------|-------------|
| Population of short β-sheets [%] | 93.2 | 65.3 | 99.0 |
| Population of long β-sheets [%] | 6.8 | 34.7 | 1.0 |

*The number of residues in a β conformation in the structures of mammalian PrP taken in the PDB is either 4 or 6. Hence we define a short and a long β-sheet as a β-sheet with a number of residues ≤ 6 or > 6, respectively. The populations are in % of the corresponding cluster.

*Cluster extracted from the simulations with a neutral H187.

*Cluster extracted from the simulations with a protonated H187.

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Figure 4. Link between the motion of GuR136 toward bulk water and the elongation of S1,S2. The data are taken from a simulation in which a 187in/136out state is formed permanently. (A) Time evolution of the number of residues in a β conformation. (B) Time evolution of the number of backbone hydrogen bond between S1–H1[1] and H1–S2[1] (the backbone atoms of these two loops are represented with sticks in the two MD snapshots shown in panel C). (C) Time series of d(rU136cavity) (same definition as in Fig. 3) and the distance between the extremities of the two strands (represented with a thick green line in the two MD snapshots).

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Figure 5. Effect of the 187in/136out and 187out/136in conformations on the number of helical residues (Nα). (A) Distribution of Nα in the 187in/136out state extracted from simulations with a protonated H187 (red) compared to the 187in/136out cluster extracted from simulations with a neutral H187 (blue). (B) Distribution of Nα in the 187out/136in state extracted from simulations with a protonated H187 (red) compared to the 187in/136in state extracted from simulations with a neutral H187 (blue). The populations are in % of the corresponding cluster.

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Figure 6. Polar interactions of ImH187H+ in its cavity when the system is in the 187in/136out state. (A) Number of contacts (N) between ImH187 (or ImH187H+) and the CO groups of R156 and T183 in the 187in/136out cluster extracted from simulations with a protonated H187 (red) compared to the 187in/136out cluster extracted from simulations with a neutral H187 (blue). (B) Example snapshot showing ImH187H+ interacting with both CO groups when the systems is in the 187in/136out state. Note that ImH187H+ can interact with R156(CO) alone, T183(CO) alone, or both.

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Two Misfolding Routes for PrP

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from our simulations is that the protonation of H187 leads to an electrostatic repulsion between the positive charges of ImH187H⁺ and Guᵦ₁₃₆-, which results in conformational transitions in the regions to which H187 and R136 are linked, namely H2(Cter) and [S1 – H1] respectively.

Our findings hence highlight two possible routes for PrP misfolding with either the unraveling of H2(Cter) alone (187out/136in route) or the unraveling of H2(Cter) with simultaneous elongation of S1,S2 (187in/136out route). This dual behavior seems to reconcile the various observations and proposals that have been made regarding the actual PrP region that undergoes a deep reconfiguration upon conversion to PrPSc [2–5]. It is indeed possible that a particular computational or experimental setup favors one of the 187in/136out or 187out/136in substrates at the beginning of the misfolding process. Such conformational shift could be assisted in vivo by molecular chaperones such as polyanionic molecules [1,42]. This variability in misfolding pathways may also be connected to the fact that prion exhibits a variety of conformations, because it is believed that changes in polyanionic molecules [1,42]. This variability in misfolding pathways may also be connected to the fact that prion exhibits a variety of conformations, because it is believed that changes in polyanionic molecules [1,42].

Finally, it is interesting to note that the H2(Cter) – ImH187H⁺ · · · [H1 – S2] · · · Guᵦ₁₃₆⁻ · · · [S1 – H1] pattern is not present in those non-mammalian species who are known to be resistant to TSEs. This is a possible explanation for the observed resistance to TSEs in these species.

Materials and Methods

Initial structure and protonation state

All simulations were started from the NMR structure of mPrP published by Rick et al. (PDB code 1AG2). We aimed at modeling mPrP with a neutral or protonated H187 at pH 4.5.

The protonation state of titrable residues apart from H187 was first estimated from PROPKA [35] calculations. The protonation state of most of them can be determined without ambiguity. All buried or semi-buried residues other than H187 are all aspartic or glutamic acids whose side chains are hydrogen-bonded to other groups in the protein. This has the effect to shift up their pKₐ above the typical values of ~4 that they adopt in water, i.e. significantly above the pH we want to model. Hence they are expected to be protonated. The solvent-exposed histidines are expected to exhibit a pKₐ of ~6 so they can be considered protonated at a pH of 4.5. The remaining solvent-exposed aspartic or glutamic acids are more ambiguous because their pKₐ is close to the pH we want to model. Nevertheless, their solvent-exposed character makes them much less important for the global fold of the protein. We chose their protonation state according to the pH we want to model. Nevertheless, their solvent-exposed histidines are expected to be protonated. The solvent-exposed histidines are expected to exhibit a pKₐ of ~6 so they can be considered protonated at a pH of 4.5. The remaining solvent-exposed aspartic or glutamic acids are more ambiguous because their pKₐ is close to the pH we want to model. Nevertheless, their solvent-exposed character makes them much less important for the global fold of the protein. We chose their protonation state according to the pH we want to model.

Simulation setup

Two topologies (one with H187 neutral and one with H187 protonated) were built with the GROMACS 4.0.7 [43–47] suite of programs. For each of them, the protein was immersed in a rhombic dodecahedral water box. The size of the box was chosen so that the distance between the protein and the edge of the box was ≥ 15 Å. The system was neutralized by adding 2 or 3 chloride counterions (depending on the protonation state of H187). The resulting system contained about 30000 atoms.

The AMBER99SB force field [48] was used to describe the protein and the TIP3P model [49] was employed for the water molecules. The force field was included in GROMACS thanks to the ports provided by Sorin and coworkers [50,51]. The particle mesh Ewald method [52] together with a Fourier grid spacing of 1 Å and a cutoff of 12 Å was used to treat long-range electrostatic interactions. A cutoff of 12 Å was used for van der Waals interactions. The water box was first relaxed by means of NpT simulations with restraints applied to the positions of the heavy atoms of the protein. Then the system was optimized in a series of energy minimization runs in which the restraints on the protein were progressively removed. Finally, we run eight simulations with a time step of 2 fs. Three and five of them were conducted with a neutral or protonated H187, respectively.

Each simulation was initiated with a set of velocities taken at random from a Maxwell-Boltzmann distribution corresponding to a temperature of 10 K. Then the system was heated up to 300 K in 300 ps using two Berendsen thermostats [53] (one for the protein and one for the solvent) with a relaxation time of 0.1 ps each. The simulation was prolonged for 100 ps and the Berendsen barostat with a relaxation time of 2 ps was switched on during 100 ps. Finally, we switched to production phase using a Nose-Hoover [54,55] thermostat and a Parrinello-Rahman barostat [56] with relaxation times of 0.5 and 10.0 ps, respectively. The total simulation lengths were 1.9, 1.3 and 1.6 μs for simulations with a neutral H187, and 1.9, 1.5, 1.6, 1.2 and 1.2 μs for simulations with a protonated H187. The C₄₋–RMSD plot of each simulation is provided in Figure S1.

Molecular visualization and analysis

All the representations were done with the program VMD [57]. Secondary structure assignments were done using the STRIDE algorithm [58].

Supporting Information

Text S1 Energetics of the ImH187H⁺ - Guᵦ₁₃₆⁻ ion pair in the native structure of mPrP. Estimation of the electrostatic interaction between ImH187H⁺ and Guᵦ₁₃₆⁻ using a Coulomb-type expression [59–61] and the typical dielectric constant inside a protein [59,61,62]. Discussion of the strength of this interaction and its implication on the protein stability.

Dataset S1 PROPKA [35] output file obtained from a representative structure of a 187in/136in state (same structure as Fig. S8-A).

Dataset S2 PROPKA [35] output file obtained from a representative structure of a 187out/136in state (same structure as Fig. S8-B).

Dataset S3 PROPKA [35] output file obtained from a representative structure of a 187out/136out state (same structure as Fig. S8-C).

Figure S1 C₄₋–RMSD. Each panel corresponds to one individual simulation, differing by the initial velocities extracted at random from a Maxwell-Boltzmann distribution. (A–C) Three individual simulations in which H187 is neutral. (D–H) Five individual simulations in which H187 is protonated.

Figure S2 Backbone fluctuations. Each panel corresponds to one individual simulation, differing by the initial velocities extracted at random from a Maxwell-Boltzmann distribution. (A–C) Three individual simulations in which H187 is neutral. (D–H)
Five individual simulations in which H187 is protonated. Simulation frames are extracted every 50 ns. The C$_z$ atoms of H187 and R136 are represented with cyan and magenta spheres, respectively. The backbone is colored according to the C$_z$-RMSF (same scale as Fig. 2).

**Figure S3 Position of Im$_{H187}$ (or Im$_{H187}H^+$) and Gu$_{R136}$.**

The positions of the H187(C$_z$) and R136(C$_z$) atoms are represented by cyan and magenta spheres, respectively. Each panel corresponds to one individual simulation, differing by the initial velocities extracted at random from a Maxwell-Boltzmann distribution. (A–C) Three individual simulations in which H187 is neutral. (D–H) Five individual simulations in which H187 is protonated. Simulation frames are extracted every 400 ps.

**Figure S4 Mammalian VS non-mammalian species.**

The upper panel of the figure shows the sequence alignment in the H2(Cter) region. H187 is represented in cyan in the sequence and is conserved throughout all mammalian PrP. The lower panel represents the charged residues in examples of mammalian (mouse) and non-mammalian (chicken) PrP. The geometric center of positively and negatively charged groups are represented by blue and red opaque spheres, respectively. The two transparent spheres in cyan and magenta correspond to the position of Im$_{H187}$ and Gu$_{R136}$ in mPrP, respectively. Sequence and structural alignments were done with the MultiSeq plugin [63] implemented in VMD [57].

**Figure S5 Charged residues around Im$_{H187}$.**

(A) Relative positioning of the residues. All charged groups around Im$_{H187}$ fall approximately within the same range of distance, which is represented by a transparent sphere of 8 Å diameter centered on the geometric center of Im$_{H187}H^+$. (B) Population of salt bridges in our simulations with a neutral (blue) or protonated (red) H187.

**Figure S6 Distance of Im$_{H187}$ (or Im$_{H187}H^+$) from its cavity as a function of time.**

(A–C) Three individual simulations in which H187 is neutral. (D–H) Five individual simulations in which H187 is protonated. The distance of Im$_{H187}$ from its cavity is defined as in Fig. 3-C. The magenta box represents the cutoffs used in Fig. 3-C to define 187in/136in, 187out/136out, 187out/136m and 187out/136out states.

**Figure S7 Distance of Gu$_{R136}$ from its cavity as a function of time.**

(A–C) Three individual simulations in which H187 is neutral. (D–H) Five individual simulations in which H187 is protonated. The distance of Gu$_{R136}$ from its cavity is defined as in Fig. 3-C. The magenta box represents the cutoffs used in Fig. 3-C to define 187in/136in, 187out/136out, 187out/136m and 187out/136out states.

**Figure S8 pK$_a$ of H187 as a function of the relative positioning of Im$_{H187}H^+$ and Gu$_{R136}$.**

Representative snapshots of (A) a 187in/136m state (equilibrated structure before Im$_{H187}H^+$ or Gu$_{R136}$ moves out of its cavity), (B) a 187out/136m state, and (C) a 187in/136out state. Water molecules that are within 3 Å of Im$_{H187}H^+$ or Gu$_{R136}$ are represented in cyan and magenta, respectively. The number close to H187 in each panel indicates the pK$_a$ of this residue, as estimated by PROPKA [35] from the corresponding structure. The calculations were performed using the PDB2PQR software [64,65]. We also provide the PROPKA output files corresponding to panels (A), (B) and (C) in Dataset S1, S2 and S3, respectively.

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**Author Contributions**

Conceived and designed the experiments: JG IT UR. Performed the experiments: JG. Analyzed the data: JG. Contributed reagents/materials/analysis tools: JG. Wrote the paper: JG.

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