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Structural mechanisms of oligomer and amyloid fibril formation by the prion protein

Ishita Sengupta 📴 and Jayant B. Udgaonkar 📴 *^b

Misfolding and aggregation of the prion protein is responsible for multiple neurodegenerative diseases. Works from several laboratories on folding of both the WT and multiple pathogenic mutant variants of the prion protein have identified several structurally dissimilar intermediates, which might be potential precursors to misfolding and aggregation. The misfolded aggregates themselves are morphologically distinct, critically dependent on the solution conditions under which they are prepared, but always β -sheet rich. Despite the lack of an atomic resolution structure of the infectious pathogenic agent in prion diseases, several low resolution models have identified the β -sheet rich core of the aggregates formed *in vitro*, to lie in the $\alpha 2-\alpha 3$ subdomain of the prion protein, albeit with local stabilities that vary with the type of aggregate. This feature article describes recent advances in the investigation of *in vitro* prion protein aggregation using multiple spectroscopic probes, with particular focus on (1) identifying aggregation-prone conformations of the monomeric protein, (2) conditions which trigger misfolding and oligomerization, (3) the mechanism of misfolding and aggregation, and (4) the structure of the misfolded intermediates and final aggregates.

^a National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bengaluru 560065, India

^b Indian Institute of Science Education and Research Pune, Dr Homi Bhabha Road, Pashan, Pune 411008, India. E-mail: jayant@iiserpune.ac.in; Tel: +91 20 25908008

1. Introduction

The prion protein can exist in two distinct structural isoforms: PrP^{C} and PrP^{Sc} . Misfolding and aggregation of the monomeric α -helix rich PrP^{C} into infectious β -sheet rich, aggregated PrP^{Sc} is responsible for the deadly neurodegenerative diseases collectively known as transmissible spongiform encephalopathies (TSEs).



Ishita Sengupta

Ishita Sengupta studied at Presidency College, Kolkata, and obtained her BSc degree in Chemistry from the University of Kolkata. After obtaining her MSc degree in Chemistry from the University of Calcutta, she went to obtain her PhD in Chemistry (under the supervision of Prof. C. P. Jaroniec) from The Ohio State University in late 2012. She then joined Prof. Jayant Udgaonkar's laboratory as a post-doctoral fellow, at the National Centre

for Biological Sciences, Tata Institute of Fundamental Research in early 2013.



Jayant B. Udgaonkar

Department of Biochemistry at Stanford University, working with Prof. R. L. Baldwin. He joined the National Centre for Biological Sciences, Tata Institute of Fundamental Research, in 1990. In late 2017, he joined the Indian Institute of Science Education and Research as its Director.

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Jayant Udgaonkar studied at St

Xavier's College Mumbai, and

obtained his BSc degree in

Chemistry from the University of

Mumbai. After obtaining his MSc

degree in Chemistry from the

Indian Institute of Technology,

Chennai, he went on to obtain

his PhD in Biochemistry (under

the supervision of Prof. G. P.

Hess) from Cornell University in

1986, with a minor in Applied

Physics. From 1986-1989, he

was a postdoctoral fellow in the



Fig. 1 NMR structure of the CTD of the moPrP protein (PDB ID 1AG2) showing the locations of a subset of pathogenic mutations in the CTD (purple spheres), with the exception of pathogenic mutations in the disordered NTR, stop mutations and protective mutations. The sole disulfide between cysteine residues C178 and C213 is shown as pink sticks. The N and C termini and secondary structural elements are marked.

Prion diseases can be sporadic, inherited or infectious in nature.¹ Inherited prion diseases are a consequence of familial mutations in the protein, whereas infectious prion diseases spread when normal cellular protein (PrP^C) comes into contact with its pathogenic counterpart (PrP^{Sc}). It is not clear how sporadic prion diseases emerge, or how the interaction between PrP^C and PrP^{Sc} leads to the spread of infectious prion diseases. Consequently, the occurrence, spread and eradication of this unusual disease is a much investigated research topic worldwide.

Mature PrP^{C} is a monomeric 208 residue α -helix rich, GPIanchored protein, with two distinct domains: an intrinsically disordered N-terminal region (NTR) and a globular C-terminal domain (CTD) (Fig. 1). The NTR has between 4 and 5 octarepeats (depending on the species of prion), which can bind a variety of transition metal ions.^{2,3} A highly conserved hydrophobic stretch of amino acid residues has also been identified in the NTR, which is believed to aid in interactions with PrP^{Sc} and lipids.⁴⁻⁶ The globular CTD is composed of three α -helices and a short β-sheet. A single disulfide bond, between cysteine residues 178 and 213 (mouse numbering), holds the $\alpha 2-\alpha 3$ subdomain together.7 Reduction of the disulfide bond accelerates misfolding and aggregation, pointing to its role in stabilization and maintenance of the native fold.8 Interestingly, both the CTD as well as just the $\alpha 2-\alpha 3$ subdomain can independently adopt structures, which are identical to that in the full length protein.9,10 The atomic resolution structure of the globular CTD is conserved across species.^{11–15} In contrast, the high resolution structure of the misfolded PrPSc isoform has still not been determined, due to multiple technical challenges.¹⁶

The exact function of the prion protein still remains unknown. However, putative functions like copper homeostasis, protection from stress and neuronal damage, regulation of cell differentiation and adhesion, among others, have been suggested.¹⁷ Remarkably, PrP^C null mice do not suffer from drastic abnormalities, suggesting that it might have a redundant function.¹⁸

This review focuses on the structural aspects of prion misfolding and aggregation, with particular emphasis on structural perturbations in the monomeric protein, which promote misfolding, as well as on the mechanisms of folding, unfolding and aggregation of the prion protein. To address these questions, our laboratory has used a combination of tools, including but not limited to circular dichroism (CD), fluorescence, light scattering, size-exclusion chromatography, atomic force microscopy (AFM) along with high-resolution probes like hydrogen-exchange (HX) coupled to mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy to probe structural perturbations in the monomer prior to, during and after completion of aggregation, for both the WT moPrP as well as several pathogenic mutant variants.

2. Identification of misfolding and aggregation-prone precursor conformations

It is believed that the spontaneous conversion of the α -helix rich monomeric prion protein into misfolded β-sheet rich aggregates is likely to be initiated by aggregation-prone intermediate conformations sampled transiently during its folding or unfolding, or by local fluctuations in the native state itself. Indeed, we have shown that the specific heat capacity of the native protein is unusually high compared to that of other proteins of similar size, suggesting that it undergoes large structural fluctuations.¹⁹ Therefore, the search for amyloidogenic intermediate(s) has been widely pursued, with reasonable success. The diversity of structurally distinct intermediate species points to multiple folding and misfolding pathways of the native protein, dictated largely by the initial solvent conditions. In this context, it has been shown that diffusion across the misfolding landscape for the prion protein is significantly slower in comparison to that across the folding landscape.²⁰

HX coupled to MS and NMR has been particularly informative in not only identifying misfolding-prone conformations of the prion protein, but also in locating the β -sheet rich core of prion aggregates.

2.1 HX to study protein folding, misfolding and aggregation reactions

HX relies on the exchange of exchangeable protons (*e.g.* amide protons of the protein backbone) with the solvent. It is an acid or base catalysed process, the rate of which critically depends on the pH of the buffer in which the reaction takes place.^{21–23} Proteins are dynamic entities which constantly undergo fluctuations, which can range from local unfolding of certain parts to the global unfolding of the entire protein (Fig. 2a). Dynamic and/or unstructured parts of the protein undergo exchange very rapidly, whereas rigid and/or buried parts do so relatively slowly. Typically, the opening rates (k_{op}) are much slower compared to both the closing (k_{cl}) and intrinsic exchange rates (k_{ex}) and can



Fig. 2 HX methods to obtain structural insights into the folding/misfolding and aggregation of proteins. (a) A natively folded protein undergoes partial unfolding where the α -helix (blue) unfolds and subsequently exchanges with the solvent (red), but the β -sheet (violet) does not. (b) A misfolded aggregate has a protected β -sheet rich core (light violet) which does not exchange with the solvent, whereas unstructured parts (red) do so readily.

be ignored (eqn (1)). k_{ex} is dependent upon the local amino acid sequence, which affects the p K_a as well as the solvent accessibility of the exchanging proton.²⁴

When $k_{\rm cl} \gg k_{\rm ex}$, the protein must undergo a number of closing and opening events before it can exchange with the solvent. In such a situation, exchange is said to occur in the EX2 limit (eqn (2)). In the EX2 limit, the free energy of opening or $\Delta G_{\rm op}$ can be measured. On the other hand, when $k_{\rm cl} \ll k_{\rm ex}$, exchange takes place every time the protein undergoes an opening event. Under these conditions, exchange is said to occur in the EX1 limit (eqn (3)). In the EX1 limit, the rate constant of opening $k_{\rm op}$ can be measured.

$$k_{\rm obs} = \frac{k_{\rm op} \cdot k_{\rm ex}}{k_{\rm ex} + k_{\rm cl} + k_{\rm op}} \approx \frac{k_{\rm op} \cdot k_{\rm ex}}{k_{\rm ex} + k_{\rm cl}} \tag{1}$$

$$k_{obs} = \frac{k_{op}}{k_{cl}} \cdot k_{ex} = K_{eq} \cdot k_{ex} \text{ (EX2 limit)}$$

$$\Rightarrow \Delta G_{op} = -RT \ln K_{eq} = -RT \ln \frac{k_{obs}}{k_{ex}}$$
(2)

$$k_{\rm obs} = k_{\rm op}$$
(EX1 limit) (3)

When coupled to high-resolution detection tools like MS or NMR, this powerful technique can report on the thermodynamics or kinetics of local and global protein folding/unfolding at high resolution, depending upon whether HX is in the EX2 or EX1 limit respectively. After exchange with deuterium, signals from amide protons disappear and can no longer be detected by NMR, thereby allowing them to be identified unambiguously. In addition, proteins also undergo an increase in mass following deuterium uptake, which can be directly analysed by MS.

In a typical HX experiment, exchange into the protein is allowed to take place for increasing durations of time, following which the reaction is quenched by a lowering of pH at low temperatures. Depending upon the dynamics and hydrogen-bonded structure in different parts of the protein, this freezes the protein in a state wherein only certain parts have exchanged with the surrounding solvent. The structure of this state is then directly visualized by NMR experiments or by fragmenting the protein into smaller peptide segments before analysis by MS.²⁵ In this manner, valuable structural insights into folded proteins and misfolded aggregates have been obtained^{26,27} (Fig. 2b).

2.2 Presence of folding/unfolding intermediates

In contrast to the idea that a folding intermediate could be a precursor for scrapie, early urea-induced unfolding of CTD of the mouse prion protein (moPrP) 121-231 at 4 °C was found to be extremely fast (sub-millisecond regime), with intermediates not being detected.²⁸ This was indirectly supported by HX-MS experiments which proposed that complete unfolding was required for misfolding and aggregation to proceed.²⁹ However, kinetic intermediates populated on the folding pathway were missed in earlier studies, and hence, could not be characterized, due to the fast folding kinetics of the prion protein in the submillisecond regime, beyond the temporal resolution of traditional stopped-flow instrumentation.³⁰ Recently, rapid mixing techniques allowing measurements on the sub-millisecond time scale have enabled the detection of folding intermediates for the human prion protein. The populations of these intermediates are elevated at acidic pH, and in the presence of pathogenic mutations like F197S, making them likely precursors for aggregation.³¹ Similar results have also been observed for mutant variants of the ovine prion protein, where the population of an intermediate was directly correlated with the susceptibility to disease.³² In contrast, the resistant variant Q167R of the ovine prion protein was destabilized compared to the WT protein, but did not populate an unfolding intermediate, suggesting different folding pathways for the two protein variants.³³ Folding and unfolding intermediates have also been captured in pressure-induced folding and unfolding studies of the prion protein.34,35 Force-spectroscopy experiments at single molecule resolution have further shown that binding of the pharmacological chaperone Fe-TMPyP to the native state inhibited misfolding and aggregation by stabilizing it and disrupting intermolecular interactions favouring multimerization.³⁶ In contrast to these results, it has been shown that misfolding is initiated by off-pathway intermediates, whereas folding to the native state is primarily two-state.37

2.3 β-Sheet rich intermediate

If misfolding is a pre-requisite for aggregation, then the assembly-competent precursor is also likely to be misfolded and β -sheet rich. Indeed, early experiments had identified a β -sheet rich intermediate in equilibrium unfolding studies at low pH.^{38,39} In contrast, in the absence of denaturants, but upon reduction of the disulfide bond, a monomeric β -rich conformation was found to be reversibly populated at neutral pH. This state was partially-protease resistant, akin to PrP^{Sc}, and capable of forming amyloidogenic aggregates.⁴⁰ In addition, highly susceptible VRQ and ARQ mutant variants of the ovine prion protein were shown to unfold *via* β -sheet rich intermediates, whereas resistant ARR and AHQ mutant variants unfolded *via* random-coil intermediates.⁴¹ A β -sheet rich intermediate was also

identified in constant pH⁴² and mechanical unfolding simulations.⁴³ Moreover, stabilizing the monomer by an engineered disulfide bond (similar to its counterpart, the doppel protein) led to the disappearance of the α to β switch during folding.⁴⁴

This drastic conformational switch could be triggered by a change in temperature, pH and oxidising conditions or a combination thereof.²⁹ Owing to the high thermodynamic stability of the β -sheet rich isoform, folding into the α -helical native fold has been proposed to be under kinetic rather than under thermodynamic control. It has been suggested that denaturing and slightly acidic conditions reduce the free energy barrier and facilitate the formation of the β -sheet rich form.⁴⁵

Despite it being a promising hypothesis, the existence of a monomeric β -sheet rich intermediate is currently debatable, owing to the ready formation of misfolded β -rich oligomeric species, at low pH, high ionic strength, in the presence of a denaturant or a reducing agent.

2.4 Molten globule intermediates

The hydrophobic core of the monomeric prion protein is situated in and around the sole disulfide bond between cysteine residues 178 and 213. Temperature jump kinetics and ϕ -value analysis have revealed that the folding nucleus is located between $\alpha 2$ and $\alpha 3.^{46}$ Not surprisingly, monomeric variants with cysteines substituted by alanines, or with the disulfide bond reduced, were molten-globular and capable of misfolding into β-sheet rich aggregates in the presence of salt.8 Molten globule-like folding intermediates have also been identified in equilibrium studies of single tryptophan mutants of Syrian hamster prion protein.47 Under high pressure, slow motions of $\alpha 2$ and $\alpha 3$ have been shown to lead to the population of a metastable intermediate PrP*, with a disordered $\alpha 2-\alpha 3$ subdomain.^{48,49} In contrast, at pH 2, a molten globular A-state with an unfolded $\beta 1-\alpha 1-\beta 2$ subdomain has been identified. The population of the A-state, similar to many pathogenic mutant variants, was directly correlated with the rate of oligomerization.⁵⁰ Interestingly, the A-state was later established to be a late-folding intermediate, located at the branching point of the folding and aggregation landscapes.⁵¹ Remarkably, our experiments on the highly aggregation-prone hydrophobic core mutant variant T182A have shown that it is molten globular in the monomeric form itself.⁵²

2.5 Partially unfolded intermediates

We have identified at least two partially unfolded intermediates (PUFs), populated in equilibrium with the native state at pH 4 using native state HX experiments of the prion protein coupled to MS and NMR (Fig. 3a and b). These were not detected in earlier experiments carried out at pH 5.5, which had accordingly reported the absence of intermediate species.^{29,53} One of the high energy intermediates, PUF2, with a stability of ~3 kcal mol⁻¹, possessed a disordered β 1, β 1- α 1 loop and C terminus of α 3, with α 1 and β 2 detached from the α 2- α 3 subdomain.⁵⁴

We were further able to show in an engineered mutant variant W144F/F174W of the CTD, that introduction of a tryptophan residue in the hydrophobic core of the CTD led to the population of a monomeric intermediate I, with remarkable



Fig. 3 Detection of an aggregation-prone intermediate, populated at equilibrium. (a) Residue-specific ΔG_{op} values for moPrP 23–231 obtained from HX-NMR measurements at pH 4, 25 °C, compared to the free energy of global unfolding (dashed line). (b) Partially unfolded forms PUF1 and PUF2, populated in equilibrium with the native state. (c) Non-overlapping CD and fluorescence-monitored unfolding curves indicate the presence of an unfolding intermediate. (d) The population of the intermediate has a bell-shaped dependence on urea concentration, with the highest concentration at ~4 M urea. (e) Misfolding rate constant is higher at about 5 M (red squares) compared to at 6 M (green diamonds) and 2 M (black circles) urea. (f) The dependence of the apparent rate constants of CD-monitored misfolding on urea concentration. Adapted from Moulick *et al.*, 2015 and Moulick and Udgaonkar, 2017, with permission from The American Society for Biochemistry and Molecular Biology and Elsevier respectively.

structural similarity to PUF2. The population of I was dependent on pH and urea concentration, and correlated with the rate constant of misfolding, similar to multiple pathogenic mutant variants (Fig. 3c–f). These features made I a likely candidate for initiating misfolding and aggregation.⁵⁵

3. Pathogenic mutations

Familial mutations in the prion protein are responsible for the inherited form of the disease. While most pathogenic mutations are concentrated in the $\alpha 2-\alpha 3$ region of the globular domain (Fig. 1), a significant number are also localized in the intrinsically disordered NTR. Since conversion to the pathogenic PrP^{Sc} counterpart involves

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both misfolding and multimerization, the effect of these mutations on both processes must be considered together, while analysing their overall effect. In addition, studying the effect of these mutations on the structure, dynamics, stability and folding of the prion protein might help uncover details about the mechanism of pathogenic conversion, not directly apparent by studying the WT PrP alone.^{56–60}

3.1 Pathogenic mutations in the CTD

If the precursor to PrP^{Sc} is a completely unfolded or partially structured intermediate, then any mutation which increases the population of such an intermediate under aggregation-promoting conditions should in turn increase the rate constant of misfolding and aggregation. Indeed, the GSS associated mutant variant F197S (among others) has been shown to possess an elevated population of partially structured intermediates on its refolding pathway, low thermodynamic stability and faster aggregation rate constants, even in the absence of denaturants.^{30,59,61}

In agreement with these studies, we have shown that a set of pathogenic mutant variants D177N, F197S, D201N, R207H, and Q216R have reduced thermodynamic stabilities and faster misfolding and oligomerization rate constants, compared to the WT protein at pH 4. Remarkably, our native state HX-MS experiments on these pathogenic mutant variants have revealed that the extent of global destabilization and acceleration in the misfolding/oligomerization rate constants is highly correlated with the extent of destabilization of $\alpha 1$. This suggested that the unfolding of $\alpha 1$ or its detachment from the $\alpha 2-\alpha 3$ subdomain might initiate misfolding, at least at low pH.62 In an independent study, the mutations D177N and E199K were shown to dramatically reduce the stability of $\alpha 1$, due to changes in charge distribution, implying a role for $\alpha 1$ in the PrP^C to PrP^{Sc} transition.⁶³ The M204S/R mutation on α 3 has also been shown to disrupt the α 1- α 3 interaction. Other experiments have suggested that the mutations H186R/E195K promote subdomain separation.⁶⁴ The introduction of R186 causes repulsion between R186 and R155, driving the two side chains away and exposing the hydrophobic core, which makes the protein susceptible to misfolding.65 These interactions are important for the maintenance of the native fold and stability of the monomer.⁶⁶

In contrast, at pH 2, the pathogenic mutant variants E195K, F197S, V202I and R207H have thermal stabilities comparable to that of the WT protein, but aggregate with different rate constants. These results imply that the unfolded state, and not an intermediate may be the precursor for aggregation.⁶⁷

Simulations of the hydrophobic core mutants, V179I, F197S, V202I, T182A and V209I, have shown that both dynamics and thermodynamic stability are affected upon introduction of these mutations. Mutant variants F197S and T182A cause enhanced flexibility, and separation of the β -sheet from the rest of the protein, whereas mutant variants V179I, V202I and V209I cause changes in the native protein, similar to those that take place upon a lowering of pH.⁶⁸ In particular, we have demonstrated that the T182A mutation disrupts the core packing and converts the folded PrP into a MG, with reduced co-operativity and enthalpy of unfolding.⁵² MD simulations have further confirmed

that in the T182A mutant variant, under denaturing conditions, α 1 remains fixed, but the α 2– α 3 subdomain is destabilized with a higher propensity to form β -sheets.⁶⁹

The mutation V209I introduces the bulky amino acid isoleucine in the hydrophobic core, disrupting interactions between $\alpha 2$ and $\alpha 3$ and promoting PrP^C–PrP^{Sc} interactions.⁷⁰ It also increases the flexibility of the protein by disrupting multiple π – π interactions in the $\beta 2$ – $\alpha 2$ loop, predisposing it to misfolding and aggregation.⁷¹ Indeed, in the NMR structure of the V209I mutant variant, several side chains are seen to be re-oriented, exposing the hydrophobic core and altering contacts in the $\beta 2$ – $\alpha 2$ loop and $\alpha 2$ – $\alpha 3$ domain.⁷² In contrast, the V209M mutation stabilizes the monomeric protein by eliminating a cavity, thereby preventing the *in vivo* spread of prion disease.⁷³

Interestingly, the loss of salt bridges in the $\alpha 2-\alpha 3$ subdomain and $\pi-\pi$ interactions in the $\beta 2-\alpha 2$ loop appears to be a common structural perturbation caused by other pathogenic mutant variants such as E220K and Q211P, which cause the prion diseases fCJD and GSS respectively.⁷⁴ Such local structural instabilities might cause partial denaturation, making them prone to aggregation. The E220K mutation also changes the charge distribution on the protein surface (without changing its structure), which might facilitate interactions with PrP^{Sc} and promote aggregation.^{75,76} A similar mechanism has been proposed for the mutations D201N, E210Q and Q216R,⁷⁷ with an additional destabilization of $\alpha 3$ by the capping mutation D201N. Other mutations which have also been shown to change the thermodynamic stability, but not the structure, are T187R/A/K.⁷⁸

In marked contrast to these results, the backbone dynamics of the WT protein, inherited pathogenic mutant variants P101L and H186R, as well as protective mutants Q167R and Q218K at pH 3.5 and 5.5 have been shown to be very similar using solution NMR. In addition, all mutations other than H186R have been shown to induce small perturbations in the structure. At low pH, H186 gets protonated, and has its greatest structural effects on the C terminal end of $\alpha 2$, indicating that PrP^{Sc} formation might be initiated in the folded region rather than in the NTR.⁷⁹

3.2 Pathogenic mutations in the NTR

Unlike pathogenic mutations in the CTD, which affect either thermodynamic stability, dynamics, or in a few cases, structure of the monomeric protein, pathogenic mutations in the NTR do not appear to affect any of these properties of the native protein. On the other hand, pathogenic mutations in the NTR seem to influence association and function, by influencing the interaction with other cellular factors and/or co-translational folding.⁸⁰

The conserved palindromic sequence AGAAAAGA in the middle hydrophobic region of the unstructured NTR has been studied extensively due to its unique amino acid composition and association with prion disease. The AGAAAAGA palindrome is required for PrP^{Sc} formation and PrP^C–PrP^{Sc} interaction.⁴ The pathogenic mutation A116V populates a transmembrane form of the protein, and is associated with GSS syndrome, but without detectable amounts of PrP^{Sc.⁸¹} We have shown that this

mutation increases the ability of the full length moPrP to form channels in both artificial and living cell membranes (Fig. 4a and b). Moreover, our HX-MS experiments in the presence of lipids have suggested that for the pathogenic mutant variant A116V, the region 109–132 binds to the lipid and shows enhanced protection to exchange (Fig. 4c). These channels are permeable to K^+ and Ca^{2+} ,

but not to Na⁺ and Cl⁻ ions. The enhanced binding to the lipid, along with the formation of channels, for the pathogenic mutant variant points to a possible mechanism by which it exerts its toxicity.⁸²

We have further shown that along with A116V, another pathogenic mutation G113V in the same sequence stretch



Fig. 4 NTR pathogenic mutant variant A116V forms channels in (a) artificial lipid membranes and in (b) HEK 293T cells. (c) Segment 109–132 shows enhanced protection to exchange in the presence of liposomes for the pathogenic mutant variant A116V. (d) The rate constant of oligomerization (open diamonds) is faster than the misfolding rate constant (closed circles) for the pathogenic mutant variants G113V and A116V. This is in sharp contrast to WT moPrP, which misfolds and oligomerizes with comparable rate constants. Adapted from Sabareesan *et al.*, 2016, and Sabareesan and Udgaonkar, 2016b, with permission from Cell Press and Elsevier respectively.

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accelerates misfolding and oligomerization without affecting the structure, stability or dynamics in the native state. Interestingly, in contrast to the WT protein, which undergoes oligomerization and misfolding simultaneously, the rate constant of oligomerization for these mutant variants was faster than their misfolding rate constant (Fig. 4d). In addition, in our experiments the sequence segment 89-132 containing these mutation sites showed enhanced protection to HX in the oligomeric state, implying that these mutations might be responsible for the accelerated oligomerization of these pathogenic mutant variants. These results suggested that this region might be responsible for the interaction of monomers during oligomerization.83 Replica-exchange MD simulations have further revealed that the pathogenic A116V mutation exposes more hydrophobic surface and induces β-hairpin like structures in the peptide fragment 106-126, whereas the mutation H110S reduces the exposure of the hydrophobic surface and induces a helical structure in it.84

While the function of the prion protein has still not been established conclusively, it has been implicated consistently in copper homeostasis. Wild type octarepeat (OR) domains in the NTR interact as a reversible copper-switch depending on copper concentrations. OR expansion makes this irreversible, facilitating multimer formation and selective binding to PrP^{Sc} in the absence of denaturants, or by resulting in a probable loss of function.⁸⁵ These effects are consistently brought about not by changing the folding pathway, but by enhancing specific misfolding pathways.⁸⁶

It has also been shown that pathogenic mutant variants interact more with N-terminal specific antibodies. They bind more efficiently to GAGs, which promotes their aggregation. In fact, mutations in the 109–136 region have shown to result in the exposure of a second GAG-binding motif. A pathogenic mutant variant with nine extra ORs has been shown to bind more strongly to GAGs, hinting towards a possible cause of pathogenesis in inherited prion diseases.⁸⁷ Interestingly, for the variants with the M129 polymorphism in the fragment 23–144, mutations P101L, P104L and A116V affected the fibril conformation, without any change in amyloidogenicity or barriers to cross-seeding. These results suggest that only certain parts of the protein are responsible for cross-seeding and transmission barriers.⁶⁰

4. Aggregation mechanism and aggregate structure

Upon fractionation, PrP^{Sc} is found to be highly heterogeneous in size and toxicity. The relatively small amounts of PrP^{Sc} that can be isolated have been resistant to structure determination efforts. Due to the inherent heterogeneity, insolubility and noncrystalline nature of prion aggregates, their structure still remains to be solved to atomic resolution. However, a number of models have been built based on structural information obtained from EPR,⁸⁸ HX,⁸⁹ solution and solid-state NMR,⁹⁰ cryo-EM⁹¹ and X-ray diffraction⁹² of smaller peptides derived from the full length protein. The length and exact location of the β -sheet rich core of the aggregates vary between the structures, but an overall agreement is present between many of the proposed structures. The presence of a helical structure in these aggregates is debatable. Some structural models have suggested that the $\alpha 2$ and $\alpha 3$ helices retain their helical structure and the β -sheet rich core of the oligomer is built from the β -sheet present in the monomeric native form of the protein.⁹³ In contrast, HX-MS experiments using brain-derived PrP^{Sc} have shown that the β -sheet core extends into the NTR.⁹⁴ However, FTIR measurements from our laboratory suggest the absence of a helical structure in the misfolded β -rich oligomers formed at low pH.⁸³ Clearly, the conditions under which the aggregates have been generated seem to play an important role in determining their final form.

4.1 β-Sheet rich oligomer formation at low pH

Conversion to the pathogenic PrP^{Sc} form is believed to initiate in the endocytic pathway, when the prion monomer encounters acidic pH.^{95–97} In addition, the propensity to form misfolded β -rich oligomers has been shown to be correlated very well with the susceptibility to disease.⁹⁸ Oligomers of different sizes have been shown to form *via* parallel misfolding pathways.^{99,100} In fact, the most infectious prion particles are small oligomers composed of 14–28 monomers, and not amyloid fibrils.¹⁰¹ The rigidity of the β 2– α 2 loop, controlled primarily by the conserved Y168 residue, is believed to be important in determining priondisease susceptibility.^{102–104} Hexameric β -sheet rich oligomers formed from disulfide bonded peptides derived from the α 2– α 3 subdomain have been crystallized.¹⁰⁵

We have shown that both oligomerization and misfolding are pH dependent in vitro, with a mid-point of the transition at pH 4.7, resulting from the titration of residues H186 and D201 in this pH range (Fig. 5a and b).¹⁰⁶ We believe that the pathogenic mutant variants H186R and D201N mimic the effect of protonation of these residues at low pH, by reversing and neutralizing the charge respectively (Fig. 5c).¹⁰⁷ Surprisingly, we find that even at low pH, the presence of salt is necessary to trigger both misfolding and oligomerization, and accelerate oligomerization into an experimentally tractable timescale. We have shown that this is facilitated by the specific binding of anions to the monomer, which not only screen the high positive charge on the monomers to drive oligomerization, but also disrupt the K193-E195 salt bridge, which is part of a crucial network of electrostatic interactions holding the two subdomains together (Fig. 5d-f).¹⁰⁸ These results are in agreement with previous measurements which have shown that locking the two subdomains by an artificial disulfide bond abolishes oligomerization, whereas facilitating subdomain separation by the introduction of pathogenic mutations accelerates it.64,100 Surprisingly, we find that despite this structural requirement, oligomerization and misfolding at low pH are rate-limited by association, with an average residuespecific reaction order of 2.6.108

A conserved stretch of amino acid residues, TVTTTT in the C terminus of $\alpha 2$ has been identified as a potential site for the initiation of misfolding and oligomerization.¹⁰⁹ This stretch of amino acid residues is highly frustrated with an enhanced propensity for β -sheet formation. We have shown that substitution of this stretch with the helix-favouring amino acid alanine,



Fig. 5 Salt-mediated misfolding and oligomerization of moPrP at low pH. (a) Far-UV CD spectra after 24 h of oligomerization at different pH values. (b) Misfolding is pH-dependent with a mid-point at pH \sim 4.7. (c) The protonation of residues H186 and D201 is responsible for the pH dependence of misfolding and oligomerization. (d) Anions bind specifically to the positively charged moPrP at low pH. (e) Anions bind and cause chemical shift perturbations (CSPs) all over the protein. (f) The highest CSPs are located in and around the K193-E195 salt-bridge located in the loop between $\alpha 2$ and $\alpha 3$. (g) Substituting the TVTTTT stretch in the C terminus of $\alpha 2$ with an increasing number of alanine residues increases the overall thermodynamic stability as well as the (h) local stability of the 182–196 sequence segment in which it is located. (i) The local and global stability difference (in comparison to the WT protein) is well correlated with each other as well as to the (j) rate of oligomerization and misfolding. Adapted from Singh *et al.*, 2014, Singh and Udgaonkar, 2016c, and Sengupta *et al.*, 2017, with permission from American Chemical Society and Elsevier respectively.

resulting in the rationally designed variant A6, completely abolishes misfolding and oligomerization. We have further shown that this effect is due to the overall thermodynamic stabilization of the mutant variants in comparison to the WT protein, along with decreased structural dynamics and increased stability of the 182–196 segment, which houses this unique stretch of amino acid residues (Fig. 5g–j).¹⁰⁶

Other studies have shown that the NTR of the prion protein is important for the formation of the β -rich oligomer.¹¹⁰ Copper binding to the NTR has been shown to induce a structural change and facilitate oligomerization by enhancing its affinity for other monomers.¹¹¹ Oligomers have also been shown to be formed by domain swapping¹¹²⁻¹¹⁴ as well, by reduction and oxidation of the disulfide bond.¹¹⁵ A molten globule intermediate has been identified as the precursor of the β -rich oligomer.¹¹⁶ These oligomers, despite being β -sheet rich are not on-pathway to amyloid fibrils.^{52,117}

4.2 WLF formation at pH 2

Early work from our laboratory has established that recombinant moPrP forms soluble worm-like fibrils (WLFs), which resemble curvilinear protofibrils, at pH 2 (Fig. 6a), in a process that is accelerated at higher temperature. WLFs are distinct from insoluble straight long amyloid fibrils which are formed at neutral pH, in the presence of denaturants. WLFs are formed directly from β -rich soluble oligomers of a critical size, which are readily formed at pH 2, in the presence of 150 mM NaCl at 25 °C. At low protein concentration, the reaction is rate-limited by aggregate growth (longer and fewer WLFs are seen), whereas at high protein concentration, it is limited by conformational change (shorter and more WLFs are seen). After WLFs are formed, they associate laterally.¹¹⁸ The β -rich structure, and the rate constant of formation of the oligomers could be tuned by NaCl concentration and the nature of anions (Fig. 6b). At low NaCl concentrations, WLF growth occurs before conformational



Fig. 6 Kinetics of formation and structural characterization of worm-like and straight fibrils at pH 2 and pH 7 respectively. (a) AFM image of WLFs formed at pH 2. (b) Extent of WLF formed at different NaCl concentrations monitored by ThT fluorescence. (c) Separation of β-rich oligomers (solid black line) into large (dotted line) and small (dashed line) oligomers. (d) Far-UV CD spectra of the corresponding oligomers. (e) Oligomer L is on-pathway to WLF formation as shown by the linear correlation of rate constant with fractional population of oligomer L. (f) Rate constant of WLF formation is different for different pathogenic mutant variants. (g) Extent of deuterium incorporation into oligomers S, L and WLF measured using HX-MS. (h) Kinetics of amyloid fibril formation at pH 7 monitored by ThT fluorescence and light scattering. (i) AFM image of straight fibrils formed at pH 7. (j) Extent of deuterium incorporation into monomers compared to fibrils, measured using HX-MS. Adapted from Jain and Udgaonkar, 2008, Jain and Udgaonkar, 2010, Jain and Udgaonkar, 2011, Singh *et al.*, 2012, and Singh and Udgaonkar, 2013, with permission from American Chemical Society and Elsevier respectively.

change, whereas at high NaCl concentrations, the opposite is true. $^{\rm 119}$

In these experiments, we detected two sub-populations of the β -rich oligomer: oligomers L and S, the relative populations of which could be tuned by NaCl concentration or pathogenic mutations in the protein.¹²⁰ Oligomers S and L were distinct in their size and β -sheet content, with oligomer L being on-pathway to WLF formation, as evidenced by the direct correlation of the rate constant of WLF formation with its concentration (Fig. 6c–f). We have further shown from HX-MS experiments that the highly protected β -sheet rich core of the WLF was located in the $\alpha 2-\alpha 3$ subdomain. The extent and pattern of protection to deuterium incorporation into oligomers S, L and WLF were, however, distinct. It is interesting to note that oligomer S, which is off-pathway to the formation of WLF, has an intact $\alpha 1$, in comparison to oligomer L and WLF, in both of which $\alpha 1$ is unfolded (Fig. 6g).

4.3 Amyloid fibril formation at neutral pH

Under mildly denaturing conditions, at neutral pH, the prion protein forms bona-fide amyloid fibrils by a nucleation-dependent polymerization mechanism (Fig. 6h and i).¹²¹ In contrast to β -oligomer formation, the autocatalytic nature of seeded amyloid

formation of prion proteins has made it a suitable model for studying the propagation of infectious prion diseases.¹²²

We have established that amyloid fibrils grow by the addition of monomers, following which conformational conversion in the monomer ensues in two steps: β -sheet formation in the $\alpha 2-\alpha 3$ region, followed by the unfolding of $\alpha 1$ (Fig. 6j).¹²³ The rate-limiting step in these polymerization reactions is the formation of a high-energy intermediate: the nucleus. We have further shown that in the case of the prion protein, the nucleus is a monomer, for both the WT and the protective mutant variant G126V.¹²⁴ Surprisingly, in contrast to earlier suggestions that β -rich oligomers and amyloid fibrils are formed by distinct misfolding pathways,¹¹⁷ amyloid fibrils have been shown to form at pH 4, in the absence of denaturants.¹²⁵

Amyloid fibril formation of the prion protein has been studied under a number of different conditions. It has been shown that disordered oligomers are on-pathway to amyloid fibrils, whereas ordered β -rich oligomers are not, when amyloid fibril formation is studied in the presence of the anionic detergent, SDS.¹²⁶ Low concentrations of SDS and NaCl are believed to promote amyloid formation by the partial denaturation of the monomer.¹²⁷ Disordered aggregates, as precursors of

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amyloid fibril formation have also been identified under neutral pH and vigorous shaking conditions.¹²⁸ At low pH, amyloid fibrils have been shown to dissociate into fragments, as a result of the protonation of histidine residues and repulsion in the charged NTR.¹²⁹ On the other hand, macromolecular crowding agents appear to have contrasting effects on fibril formation, depending upon the species of prion being studied.¹³⁰ In addition, amyloid formation by the prion protein has been shown to be promoted at low heparin/protein ratios and inhibited at high ratios.¹³¹

4.4 Fibril structure

High-resolution structures of amyloid fibrils formed from short peptides derived from the full length prion protein have been solved, primarily by solid-state NMR^{90,132} or X-ray diffraction.^{92,133–136}

A steric-zipper arrangement of β -sheets^{133,137-139} is found in amyloid fibrils formed by peptide 106–126 from the NTR of PrP.⁹⁰ Non-fibrillar oligomers formed from the same peptide are more dynamic than the corresponding amyloid fibril, but with similar packing interactions and local structure in their core.¹⁴⁰ In fact, a structural model of amyloid fibrils formed from the peptide 106–126, built using experimental HX-NMR data and MD simulations, proposes a four-stranded β -sheet structure stabilized by interactions between methyl side chains in the palindromic region.⁸⁹ In agreement with these observations, a similar steric-zipper arrangement was adopted by amyloid fibrils formed from a smaller fragment of this peptide, encompassing the palindromic AGAAAAGA sequence alone. This arrangement of β -sheets is found in amyloid fibrils formed by many other proteins, and appears to be a common structural motif.^{132,141}

HX-MS experiments on amyloid fibrils formed by the 89–143 peptide with the P101L mutation have identified the β -rich core to be located between residues 102–109 and 117–136.¹⁴² In contrast, solid-state NMR of amyloid fibrils formed by the 23–144 peptide of the human prion protein have revealed a short two-stranded core composed of residues ~113–125 and ~130–140 in a parallel β -sheet arrangement.^{143–145} The β -rich core extends up to residue 106 in amyloid fibrils formed from a deletion variant of this peptide with residues 113–120 removed.¹⁴⁶ These results are in agreement with MD simulations on amyloid fibrils formed from the 120–144 peptide, which also report an in-register parallel β -sheet arrangement.¹⁴⁷

Despite the emergence of multiple high-resolution structures of amyloid fibrils, amyloid fibrils formed from truncated versions of PrP are not necessarily good structural mimics of those formed by the full length protein.¹⁴⁸

Indeed, SSNMR and MD simulations of fibrils formed by the full length 23–231 variant of the Syrian Hamster PrP have found the core to be composed of residues 173–224 in a parallel arrangement of β -sheets.¹⁴⁹ This is in agreement with the arrangement and location of the β -sheet rich core modelled using EPR data on 50 single cysteine mutants of the 90–231 variant of the human prion protein.¹⁵⁰ Seeded preparations of fibrils also adopt a parallel β -sheet architecture, but with the core located between residues 124 and 227¹⁵¹ whereas the amyloid fibrils of the full length moPrP protein, grown under mildly denaturing conditions at neutral pH, studied in our lab

possess a core composed of residues ~159 to 225 (Fig. 6j).¹²³ In contrast to the parallel β -sheet architecture, PrP27–30 amyloid fibrils devoid of the GPI anchor assemble into a β -solenoid structure composed of 4 rungs, with each molecule about 17.7 Å in height.¹⁵² Interestingly, HX-MS experiments have revealed that the β -sheet rich core of brain derived PrP^{Sc} is not identical to that seen in the diverse range of synthetic amyloid fibres.⁹⁴ The former have an extended β -sheet rich core up to residues 80–90 in the intrinsically disordered NTR, with residues 81 to 167 definitively within the core. Interestingly, we find that in a fraction of the WLF molecules formed at pH 2, the β -sheet rich core is longer, similar to brain-derived PrP^{Sc} (Fig. 6g).¹⁵³

5. Conclusion and outlook

The search for the amyloidogenic precursor(s) for prion misfolding and aggregation, using multiple probes, while undoubtedly informative, has also resulted in a number of conflicting results, as demonstrated above. The tuning of the already complex folding/ aggregation landscape of the prion protein by mutations, solvent conditions, temperature and a variety of other co-factors have further complicated our understanding of how misfolding and aggregation might be triggered. The prion protein has been shown to populate multiple intermediates during its unfolding and folding, many of which have been shown to have high propensity for misfolding and aggregation.

The β -sheet rich core of the aggregates appears to be located in the $\alpha 2-\alpha 3$ subdomain in most structural models. The current understanding of prion protein aggregation and aggregate structure appears to suggest that anti-prion drugs interfering with sub-domain separation can inhibit oligomerization, whereas drugs which interfere with the binding of monomers to pre-formed fibrils can inhibit amyloid fibril formation (Fig. 7). Not surprisingly, the highly protected β -sheet rich core of the prion aggregates formed *in vitro* is sensitive to not only the length of the peptide and its amino acid sequence, but also the conditions under which it is grown. With such a heterogeneous ensemble of aggregated structures, solving the high resolution structure of PrP^{Sc} becomes additionally challenging.

Several important questions about the aggregation of the prion protein persist, and need to be addressed in the near future. (1) Although the effects of several pathogenic as well as protective mutations on the kinetics and thermodynamics of aggregation have been identified, there is little molecular understanding of how these mutations exert their effect. For example, multiple pathogenic mutations dispersed in the structure of CTD independently have the same effect on the stability of $\alpha 1$, but the dynamic coupling of short range and long range interactions that lead to this effect is poorly understood. The effects of mutations in the disordered NTR are even less understood. (2) It is critical to characterize the temporal sequence of structural events during oligomer formation, starting from the initial perturbation of the native monomeric structure, and to identify the sequence segments of the protein that first interact during oligomer formation, and to determine whether the same



Seeded Amyloid Fibril Formation

Fig. 7 The misfolding pathways for oligomer and amyloid fibril formation are distinct for the prion protein. At low pH, the subdomains in the CTD must separate before misfolding and oligomerization can proceed. At neutral pH, seeded growth of amyloid formation occurs by addition of monomers, which is followed by conformational conversion. β -Sheet formation is followed by the unfolding of α 1, after binding. The NTR is not shown for clarity.

sequence segments play a role in template-driven fibril formation. (3) Little is known about the mechanism of template-driven conformational conversion of PrP^C to PrP^{Sc}. Comprehending how autocatalysis of the conversion is effected, and whether conformational conversion occurs prior to, or after monomer addition, are issues that need to be addressed soon because it is these processes that have to be inhibited by drug-based therapies for prion diseases. (4) Nothing is known about the structure of the monomeric nucleus whose formation drives spontaneous amyloid fibril formation. Knowledge of the structure of this nucleus will help in the development of therapies targeted towards sporadic prion disease. (5) While it is clear now that monomeric PrP can form channels in the lipid membrane, which are specific to calcium and potassium ions, the downstream effects, particularly on cellular signaling pathways, of activating these ionic currents in the cell membrane, are not known. It will be necessary to determine how these effects are related to prion pathogenicity.

An important challenge is to generate infectious prion particles *in vitro*, whose infectivity titres are as high as brain-derived PrP^{Sc} . The preparation of homogeneous aggregated samples, whether oligomers or fibrils, will have a direct influence on the successful structure determination of these multimers, as well as in the testing for anti-prion molecules with the ability to either abolish misfolding and aggregation or disaggregate pre-formed toxic/infectious aggregates in to more benign forms. Another important challenge is to elucidate the exact function of the prion protein. A third important challenge is not only to elucidate the kinetic mechanism of aggregation, but also to structurally characterize each of the intermediate species that are populated, albeit transiently, during the misfolding and aggregation of the prion protein. Some clear thinking is required to develop strategies to meet these challenges.

Conflicts of interest

There are no conflicts of interest to declare.

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