

Understanding the molecular mechanism of mammalian prion protein misfolding

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उपाधि की अपेक्षाओं की आंशिक पूर्ति में प्रस्तुत शोध प्रबंध

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Dedicated to Maa and Baba

CERTIFICATE

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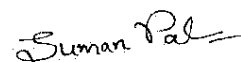
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List of abbreviations

AFM: Atomic force microscopy
ANS: 1-anilino-8-naphthalene sulfonate
CD: circular dichroism
CJD: Creutzfeldt-Jakob disease
 C_{sat} : Saturation concentration
CTD: C-terminal domain
DLS: dynamic light scattering
fCJD: familial Creutzfeldt–Jakob disease
FFI: Fatal Familial Insomnia
GdnHCl: guanidine hydrochloride
GPI: glycosylphosphatidylinositol
GSS: Gerstmann-Straussler-Scheinker
HDX: hydrogen-deuterium exchange
huPrP: human prion protein
IDP: Intrinsically disordered protein
IDR: Intrinsically disordered region
 k_{int} : Intrinsic exchange rate constant
 k_{obs} : Observed exchange rate constant
MALS: Multi angle light Scattering
MD: molecular dynamics
MG: Molten Globule
moPrP: mouse prion protein
MRE: mean residue ellipticity
MS: mass spectrometry
MWCO: molecular weight cut-off
N: Native state
NDP: nucleation-dependent polymerization
NMDA: N-Methyl-D-Aspartate
NMR: nuclear magnetic resonance
NTR: N-terminal REGION
PEG: polyethylene glycol

Pf: protection factor
PMCA: protein misfolding cyclic amplification
Prnp: the prion protein gene
PrP: recombinant prion protein
PrP^C: cellular prion protein
PrP^{Sc}: scrapie PrP
PUF: Partially unfolded form
RPC: reverse-phase chromatography
sCJD: sporadic CJD
SEC: size exclusion chromatography
Tg: transgenic
ThT: thioflavin T
TSEs: transmissible spongiform encephalopathies
TS: Transition state
U: Unfolded state
wt: wild-type
 α 1: helix-1
 α 2: helix-2
 α 3: helix-3
 β 1: β -strand-1
 β 2: β -strand-2

Synopsis

The mammalian prion protein (PrP^C) is a cell-surface glycosylphosphatidylinositol (GPI)-anchored protein, is synthesized from the *Prnp* gene located on the chromosome 20 (Carlson et al., 1986; Liao et al., 1986; Stahl et al., 1987b). The gene is highly conserved across all mammalian species and is expressed in most tissues, but the highest levels are found in the central nervous system (Mironov et al., 2003). It is expressed as a 253-residue immature protein, which undergoes multiple post-translational modifications to form the 208-residue monomeric, mainly α -helical PrP^C protein (Riek et al., 1996a; Zahn et al., 2000). The PrP^C protein has been implicated in many physiological processes, such as metal ion homeostasis (Hornshaw et al., 1995), myelin-sheath maintenance (Bremer et al., 2010), immune regulation (Chida et al., 2018), cell signaling (Um et al., 2012) and protection against oxidative stress (Bounhar et al., 2001). Transgenic mice in which the *Prnp* gene (*Prnp*^{0/0}) is knocked out, show sleep disturbance, altered circadian rhythms (Gasperini and Legname, 2014) as well as age-related cognitive decline (Steele et al., 2007a). However, the mice were able to survive (Büeler et al., 1992), suggesting that the mammalian prion protein might have redundant functions and not be an essential protein.

The sequence and structure of the mammalian PrP are highly conserved across species (Bernardi and Bruni, 2019a). It consists of two domains: an intrinsically unstructured N-terminal domain (NTD) and a structured C-terminal domain (CTD) (Billeter et al., 1997b; Zahn et al., 2000). The structured CTD includes three α -helices (α 1, α 2 and α 3) and two β -strands (β 1 and β 2), with a disulfide bond linking α 2 and α 3 (Billeter et al., 1997b). The misfolding of the cellular prion protein from its normal, α -helical form (PrP^C) into a misfolded, β -sheet-rich form (PrP^{Sc}) is linked to a group of fatal, infectious, mammalian neurodegenerative diseases, collectively termed as transmissible spongiform encephalopathies (TSEs) (Prusiner, 2017). *In vivo*, spongiform encephalopathies may occur due to genetic mutations in the *Prnp* gene (familial type), which enhances the misfolding and aggregation propensity of the protein or as a result of infection by diseased PrP^{Sc} (Prusiner, 2004). However, around 85% of the reported cases of spongiform encephalopathies are a result of de novo spontaneous misfolding of PrP^C to PrP aggregate forms (PrP^{Sc}) (Prusiner, 2004).

In vivo, during its trafficking through the endocytic pathway, PrP^C enters into lysosomes, where it encounters a low pH (Borchelt et al., 1992b). *In vitro* studies have shown that moPrP undergoes substantial structural fluctuations at acidic pH upon protonation of a critical residue, His186 (Singh and Udgaonkar, 2016f; Van der Kamp and Daggett, 2010b). It is, therefore, likely that the prion protein misfolds into its oligomeric form when it encounters the low pH in the lysosome. Importantly, oligomers formed *in vitro* at low pH are cytotoxic (Erlich et al., 2010; Huang et al., 2010; Simoneau et al., 2007) and able to disrupt lipid membranes (Caughey et al., 2009; Chich et al., 2010; Singh et al., 2014a), which suggests to a possible mechanism of their toxicity. Remarkably, highly infectious small non-fibrillar oligomeric prion assemblies have been found in diseased human brain extracts (Vanni et al., 2020). A better understanding of the disease and the development of therapeutic strategies against prion diseases will arise from knowledge of the initiation of misfolding in the prion protein, which results in oligomerization.

In this context, the questions of immediate interest are as follows:

What makes the prion protein highly susceptible to spontaneous conformational conversion from its native structure to misfolded forms? What sequence and structural features of the protein make it prone to such conversion? Which regions of the protein are most likely to initiate misfolding? Does misfolding start from the native state, or are there high-energy conformations that promote misfolding? What are the structural and energetic characteristics of these high-energy conformations? What are the molecular steps involved in the formation of misfolded oligomers?

The main objectives of this thesis have been to identify the mechanisms of the initiation of conformational conversion events involved in misfolded oligomer formation at low pH by the full-length mouse prion protein (moPrP). This study has used hydrogen-deuterium exchange coupled with mass spectrometry (HDX-MS) as a major tool along with several other probes to study the structural dynamics of both the native monomers and the misfolded oligomers. This study also utilizes site-directed mutagenesis to understand the molecular events involved in the misfolding of moPrP.

In this thesis, it is shown that two sets of evolutionarily conserved residues—(a) Pro136, Pro157, and Pro164, and (b) Tyr168, Phe174, and Tyr217—act as gatekeepers, preventing misfolding. Mutations of these residues to alanine accelerate misfolding by up to 1000-fold.

HDX-MS analysis reveals several partially unfolded forms (PUFs) in equilibrium with the native state, including PUF2*, where $\alpha 1$ and the adjacent N-terminal end of $\alpha 2$ become disordered, and PUF2**, where the $\beta 2$ - $\alpha 2$ loop and the C-terminal end of $\alpha 3$ lose their structure. These mutations lower the free energy of unfolding from the native state to PUF2* and PUF2**, enabling misfolding to initiate from structurally distinct precursor conformations. Increasing the rigidity of the $\beta 2$ - $\alpha 2$ loop significantly affects the dynamics and raises the free energy of unfolding to PUF2**. Additionally, filling cavities in the protein's hydrophobic core reduces the equilibrium population of PUFs and prevents misfolding. A mutant variant of moPrP adopts a molten globule (MG)-like conformation, which misfolds into a β -rich dimer in the absence of salt and rapidly forms oligomers upon salt addition. In the presence of salt, the 3A moPrP mutant misfolds into a β -rich oligomer several fold faster than wt moPrP. The misfolded dimer and salt-induced oligomer share similar structures, suggesting that misfolding into oligomers is initiated at the dimeric level and oligomerisation grows through the accumulation of misfolded dimers. Furthermore, full-length moPrP undergoes phase separation in PBS at neutral pH, driven by the unstructured N-terminal region (NTR) and modulated by the structured C-terminal domain (CTD). The condensates initially exhibit liquid-like properties, which transition to a solid-like state over time. HDX-MS reveals structural changes in both NTR and CTD during this aging process.

The main findings reported in this thesis are as follows:

1. The N state of PrP is in equilibrium with multiple aggregation-prone PUFs at pH 4 that can initiate misfolding, suggesting there are multiple misfolding pathways for moPrP leading to oligomerisation **(Pal and Udgaonkar, 2022, 2023)**.
2. Evolutionary conserved proline residues (Pro 136, 157 and 164) and aromatic residues (Tyr 168, Phe174 and Tyr 217) present at the structured C-terminal domain act as ‘gatekeepers’ against misfolding into toxic β -rich oligomers. Mutations of these residues destabilize the N state and lower the energy barrier to PUFs, accelerating misfolding by up to 1000-fold due to increased PUF populations **(Pal and Udgaonkar, 2022, 2023)**.
3. A MG-like conformation of the protein misfolds into a β -rich dimer, whose structure closely resembles that of oligomers, suggesting that misfolding into β -rich oligomers is initiated at the dimeric unit level through monomer-monomer association, with the oligomer grows by the accretion of misfolded dimers **(Pal and Udgaonkar, 2024a)**.

4. The rigidity of the $\beta 2$ - $\alpha 2$ loop significantly impacts the dynamics and misfolding pathways of PrP **(Pal and Udgaonkar, 2024b)**.
5. Removal of the packing imperfections in the hydrophobic core of the protein results in a significant reduction in the equilibrium population of PUFs, and impedes the misfolding **(Pal and Udgaonkar, in preparation)**.
6. Full-length moPrP undergoes instantaneous liquid-liquid phase separation (LLPS) at cytosolic pH (7.4), forming liquid-like condensates driven by the intrinsically disordered NTR through weak intermolecular interactions. Electrostatic screening of the NTR is essential for LLPS, while the structured CTD does not undergo LLPS independently, although its destabilization can induce phase separation. HDX-MS reveals that within the condensates, the NTR interacts with the CTD, and this interaction strengthens as the condensates mature, leading to structural organization in both domains, with the condensates eventually maturing into a solid-like state **(Pal and Udgaonkar, in preparation)**.

Brief descriptions of the results which led to the aforementioned findings are as follows:

Evolutionarily conserved proline residues impede the misfolding of the mouse Prion protein by destabilizing an aggregation-competent partially unfolded form

The misfolding of the prion protein has been linked to several neurodegenerative diseases. Despite extensive studies, the mechanism of the misfolding process remains poorly understood. The present study structurally delineates the role of the conserved proline residues present in the structured C-terminal domain of the mouse prion protein (moPrP) in the misfolding process. It is shown that mutation of these Pro residues to Ala leads to destabilization of the native (N) state, and also to rapid misfolding. Using hydrogen–deuterium exchange (HDX) studies coupled with mass spectrometry (MS), it has been shown that the N state of moPrP is in rapid equilibrium with a partially unfolded form (PUF2*) at pH 4. It has been shown that the Pro to Ala mutations make PUF2* energetically more accessible from the N state by stabilizing it relative to the unfolded (U) state. The apparent rate constant of misfolding is found to be linearly proportional to the extent to which PUF2* is populated in equilibrium with the N state, strongly indicating that misfolding commences from PUF2*. It has also been shown that the Pro residues restrict the boundary of the structural core of the misfolded oligomers. Overall, this study highlights how the conserved proline residues control

misfolding of the prion protein by modulating the stability of the partially unfolded form from which misfolding commences.

Mutations of evolutionarily conserved aromatic residues suggest that misfolding of the mouse prion protein may commence in multiple ways.

An understanding of the molecular mechanism of prion protein misfolding requires the identification of the regions that are critical for its misfolding. An identification of the regions critical for PrP misfolding will lead to a better understanding of the molecular basis of prion disease. It will help in the development of better therapeutics against prion diseases. In this study, it is shown that perturbation of the packing interface of the $\beta 2$ - $\alpha 2$ loop and the C-terminal end of $\alpha 3$ in the structured C-terminal domain of moPrP by mutation of three evolutionarily conserved aromatic residues, Tyr168, Phe174, and Tyr217 present at significantly destabilize the N state of the protein and reduce the free energy differences between the N state and two PUFs identified as PUF1 and PUF2**. It is shown that PUF2** in which the $\beta 2$ - $\alpha 2$ loop and the C-terminal end of $\alpha 3$ are disordered, has the same stability as the previously identified PUF2*, but to have a very different structure. Misfolding can commence from both PUF1 and PUF2**, as it can from PUF2*. Hence, misfolding can commence and proceed in multiple ways from structurally distinct precursor conformations. The increased extents to which PUF1 and PUF2** are populated at equilibrium in the case of the mutant variants, greatly accelerate their misfolding. The results suggest that the three aromatic residues may have been evolutionarily selected to impede the misfolding of moPrP.

Slow misfolding of a molten globule form of a mutant prion protein variant into a β -rich dimer

In this study, three conserved aromatic residues (Tyr168, Phe174, and Tyr217) located in the CTD of moPrP were simultaneously mutated to Ala. The resultant mutant protein, 3A moPrP, is shown to adopt a molten globule (MG)-like native conformation. Hydrogen-deuterium exchange studies coupled with mass spectrometry revealed that for 3A moPrP, the free energy gap between the MG-like native conformation and misfolding-prone partially unfolded forms is reduced. Consequently, 3A moPrP misfolds in native conditions even in the absence of salt, unlike wt moPrP, which requires the addition of salt to misfold. 3A moPrP misfolds to a β -rich dimer in the absence of salt, which can rapidly form an oligomer upon the addition of salt. In the presence of salt, 3A moPrP misfolds to a β -rich oligomer about a thousand-fold faster than wt moPrP. Importantly, the misfolded structure of the dimer is similar

to that of the salt-induced oligomer. Misfolding to oligomer seems to be induced at the level of the dimeric unit by monomer-monomer association, and the oligomer grows by accretion of misfolded dimeric units. Additionally, it is shown that the conserved aromatic residues collectively stabilize not only monomeric protein but also the structural core of the β -rich oligomers. Finally, it is also shown that 3A moPrP misfolds much faster to amyloid-fibrils than does the wt protein.

Rigidifying the $\beta 2$ - $\alpha 2$ Loop in the mouse prion protein slows down formation of misfolded oligomers.

Efficient transmission of PrP^{Sc} occurs within the same species, but a species barrier limits interspecies transmission. While PrP structure is largely conserved among mammals, variations at the $\beta 2$ - $\alpha 2$ loop are observed, and even minor changes in the amino acid sequence of the $\beta 2$ - $\alpha 2$ loop can significantly affect transmission efficiency. The present study shows that the introduction of the elk/deer-specific amino acid substitutions at positions 169 (Ser to Asn) and 173 (Asn to Thr) into the mouse prion protein, which are associated with the structural rigidity of the $\beta 2$ - $\alpha 2$ loop, has a substantial impact on protein dynamics as well as on the misfolding pathways of the protein. Native state hydrogen-deuterium exchange studies, coupled with mass spectrometry, show that the rigid loop substitutions stabilize not only the $\beta 2$ - $\alpha 2$ loop but also the C-terminal end of $\alpha 3$, suggesting that molecular interactions between these two segments are strengthened. Moreover, the energy difference between the native state and multiple misfolding-prone partially unfolded forms (PUFs) present at equilibrium is increased. The decreased accessibility of the PUFs from the native state leads to a slowing down of the misfolding of the protein. The results of this study provide important insights into the early events of conformational conversion of prion protein into β -rich oligomers, and add to the evidence that the $\beta 2$ - $\alpha 2$ loop is a key determinant in prion protein aggregation.

Removal of a packing imperfection in the hydrophobic core impedes the misfolding of the prion protein.

In this study, it is shown that cavities in moPrP influence its dynamics, affecting the formation of PUFs and the propensity for misfolding and oligomerization. The removal of packing imperfections in the hydrophobic core, particularly around residue Val 208, significantly increases the stability of the N state and raises the energy barrier between the N state and PUFs, reducing the sampling of PUFs and suppressing misfolding. Conversely, increasing the size of the cavities destabilizes the N state, lowers the energy difference between

the N state and PUFs, and leads to a 2.5-fold increase in the misfolding rate, promoting oligomer formation.

Phase separation induces structure in the intrinsically disordered region of the mammalian prion protein.

In this study, the phase separation properties of full-length moPrP were investigated to explore its role in condensate-mediated conformational change. It was observed that moPrP undergoes spontaneous phase separation in PBS (pH = 7.4), with the intrinsically disordered NTR driving phase separation and the structured C-terminal domain modulating the phase behaviour. PBS-induced PrP condensates initially exhibit liquid-like properties, which gradually transition to a solid-like state over 12 h. HDX-MS studies revealed significant structural rearrangements in the NTR as well as in specific regions of CTD as the condensates mature, highlighting the molecular factors regulating the formation and maturation of full-length PrP condensates.

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Chapter 1

Phase Transitions of mammalian Prion Protein: Molecular Mechanisms, Structural Insights, and Disease Relevance

Phase Transitions of Mammalian Prion Protein: Molecular Mechanisms, Structural Insights, and Disease Relevance

1.1 Introduction

1.1.1 Structural plasticity of proteins: *folding, unfolding, and misfolding*

Proteins are the building blocks of cells, and also have a wide range of biological activities. Their remarkable ability to fold precisely from an astronomically high number of possible conformations, within a fraction of a second, is one of the most captivating and paradoxical phenomena in biology. The inherent structural plasticity of protein molecules allows them to adopt a variety of conformations throughout their lifecycle, from synthesis to degradation. While this plasticity enables them to perform a vast range of biological functions, it also makes them susceptible to misfolding, where even minor conformational changes can lead to dysfunction and disease. While the development of current machine-learning approaches such as AlphaFold (Jumper et al., 2021), has revolutionized our ability to predict protein structures accurately from their sequences, predicting the consequences of protein misfolding remains a major challenge. A mechanistic understanding of how a protein misfolds will provide clues as to how to prevent misfolding and aggregation.

Folding: Structural gain

Protein folding is the process by which a polypeptide chain folds into its native structure, which is determined by its amino acid sequence (Peter et al., 1971; Dill and MacCallum, 2012). The sequence contains information that directs the protein to fold into its native, functional form, which is energetically the most stable state. The process of protein folding occurs through a series of intermediates that gradually stabilize to form the final conformation (Bhatia and Udgaonkar, 2022, 2024; Kim and Baldwin, 1982; Nath and Udgaonkar, 1997; Privalov, 1996). The pathways pass over a complex energy landscape on which various transient states exist (Gianni et al., 2021). The folding process is very dynamic, and intermediates can adopt flexible conformations before converging to the most stable native state (Bhatia and Udgaonkar, 2022). Chaperones, molecular machines that assist in folding, also play a critical role in ensuring that proteins fold correctly by preventing incorrect interactions and helping the protein reach its native conformation (Martin and Hartl, 1997; Walter and Buchner, 2002). In cellular environments, folding is finely tuned by factors such as pH, temperature, and the presence of chaperones or co-factors (Balchin et al., 2016; Hartl and Hayer-Hartl, 2009). The ability to adapt to these changes and yet maintain a functional conformation is a testament to the structural plasticity that underlies protein function (Henzler-Wildman and Kern, 2007).

Unfolding: Loss of Structure

Proteins can unfold due to environmental stresses such as high temperatures, alterations in pH, or the presence of denaturing agents (Dill and Shortle, 1991; Tanford, 1964). These factors disrupt the non-covalent interactions that stabilize the folded state, causing the protein to adopt the less stable, unfolded form. Unfolded proteins are more susceptible to misfolding, which leads to loss of cellular function (Hetz et al., 2020; Ron and Walter, 2007). Proteins that unfold under stress need to refold to regain their functional state. If the unfolding is not reversible, or if the protein fails to refold correctly, it can lead to a cascade of cellular events known as the unfolded protein response (UPR) (Acosta-Alvear et al., 2024; Hetz et al., 2020; Wang and Kaufman, 2012). Unfolded proteins, including partially unfolded ones, are also at risk of misfolding because they expose hydrophobic residues, which are usually buried in the folded state (de Oliveira and Silva, 2017; Dobson, 2003). Intermolecular hydrophobic interactions increase the likelihood of proteins forming nonfunctional aggregates that may disrupt normal cellular processes.

Misfolding: Structural Deviations

When a protein misfolds, it adopts a non-native conformation, distinct from its native functional structure. This can be the consequence of mutations, environmental stress, or errors during the folding process (Chiti and Dobson, 2006; Dobson, 2003). Misfolded proteins often aggregate into nonfunctional, toxic structures such as oligomers or amyloid fibrils, composed of β -rich structures, which are commonly associated with diseases (Dobson, 1999; Hartl, 2017).

Many proteins, particularly those involved in signaling, regulation, and molecular recognition, are intrinsically disordered in their native state (Oldfield and Dunker, 2014; Wright and Dyson, 1999). These intrinsically disordered proteins (IDPs) do not possess a stable, well-defined structure by themselves, but can transition to ordered structures upon binding to their targets (Malagrino et al., 2020; Mukhopadhyay, 2020). This ability to undergo conformational transitions allows IDPs to participate in diverse biological processes, offering a functional advantage that is dependent on their flexibility. For example, proteins such as α -synuclein (Bartels et al., 2010; Das and Eliezer, 2019), tau (Ait-Bouziad et al., 2017; Avila et al., 2004), are disordered in their unbound forms, but can adopt stable conformations when interacting with other molecules, enabling them to perform specific functions (Burré et al., 2014; Kadavath et al., 2015). The ability to transition between different structural states enables these proteins to interact with multiple partners, enhancing their functional versatility.

1.1.2 The role of misfolded proteins in diseases

Misfolded proteins are implicated in a wide array of diseases, collectively referred to as protein misfolding diseases (PMDs) or conformational diseases. These diseases can be categorized into two main groups: loss-of-function diseases and toxic gain-of-function diseases. In loss-of-function diseases, mutations in the protein-coding genes cause the proteins to become metastable or prone to misfolding, and lose their function due to misfolding (Sahni et al., 2015). A classic example of this is cystic fibrosis, where mutations in the CFTR gene result in a misfolded protein that is degraded before reaching its functional destination (Fraser-Pitt and O'Neil, 2015). On the other hand, toxic gain-of-function diseases are characterized by the misfolding of proteins into toxic structures, which are harmful to cells and tissues (Gregersen, 2006; Winklhofer et al., 2008). The most well-known examples of these diseases are neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and the prion diseases (PrDs). In these diseases, misfolded proteins not only fail to function properly, but also aggregate into structures that disrupt cellular processes, leading to toxicity (Kayed et al., 2003). For example, in AD, the amyloid-beta ($A\beta$) peptide misfolds and aggregates into amyloid plaques, which accumulate in the brain and impair neuronal function (Finder and Glockshuber, 2007). The misfolding of the tau protein results in neurofibrillary tangles, which are a hallmark of AD and other tauopathies (Lee et al., 2001; Wood et al., 1986). Similarly, α -synuclein (α -syn) forms aggregates called Lewy bodies in PD, leading to the degeneration of dopaminergic neurons and motor dysfunction (Recchia et al., 2004). The misfolding process typically involves the formation of β -rich higher-order structures, such as oligomers, protofibrils, and amyloid fibrils. These aggregates accumulate in tissues, either extracellularly (as in AD, and prion diseases) (Aguzzi and O'Connor, 2010; Jankovska et al., 2021) or intracellularly (as in Parkinson's disease and Huntington's disease) (Sweeney et al., 2017).

Protein misfolding is implicated not only in neurodegenerative diseases but also in other PMDs such as type 2 diabetes (T2D), heart disease, and some cancers. In T2D, misfolded islet amyloid polypeptide (IAPP) aggregates, damaging insulin-producing beta cells in the pancreas (Brender et al., 2012; Westermark et al., 2011). In cardiac amyloidosis, misfolded proteins accumulate in the heart, impairing its ability to pump blood effectively (Martinez-Naharro et al., 2018). Misfolding of p53, a tumor suppressor protein, is linked to cancer development and tumorigenesis (Li et al., 2022).

Initially, protein misfolding was seen as a consequence of disease (Gregersen et al., 2006), but genetic studies have led to a change in this view, by showing that mutations in genes encoding misfolding-prone proteins can trigger familial forms of PMDs. For instance, mutations in the huntingtin (Htt) gene lead to Huntington's disease (Saudou and Humbert, 2016), while mutations in presenilin 1 (PS1) (Kelleher III and Shen, 2017) and amyloid precursor protein (APP) (Hunter and Brayne, 2018) cause early-onset AD through A β misfolding. Transgenic animal models have further confirmed that protein misfolding and aggregation are not just incidental occurrences but are essential drivers of pathology. Mice expressing human APP mutations develop amyloid plaques and cognitive decline (Cheng et al., 2004; Galvan et al., 2006), while overexpression of mutant α -synuclein leads to neurodegeneration (Su et al., 2009), supporting the role of misfolded proteins in disease.

1.1.3 Driving forces for protein misfolding

Energy landscape for protein misfolding

The energy landscape of protein misfolding is a crucial framework for understanding the complex transitions that proteins undergo as they change conformation from their native folded states to misfolded or aggregated forms. The energy landscape represents the free energy of a protein as a function of its conformations, illustrating the relative stabilities of states such as the native folded, partially unfolded, and unfolded states, as well as misfolded aggregates (Dill and MacCallum, 2012; Vabulas et al., 2010). For properly folded proteins, the energy landscape is often depicted as a funneled landscape, along which high-energy, high-entropy unfolded states fold to a low-energy, low-entropy native state. This funnel-shaped landscape is characterized by a network of stabilizing intramolecular contacts that make the native state energetically favorable and relatively easy to reach (Bryngelson et al., 1995; Dill and Chan, 1997). The energy landscape for misfolding and aggregation is considerably complex and rugged (Brooks III et al., 2001; Dinner et al., 2000; Wolynes et al., 1995). While the funnel of folding may be relatively smooth, the landscape for misfolding is thought to feature numerous competing conformations separated by substantial kinetic barriers (Figure 1.1). Misfolding, like folding is typically characterized by the transient formation of metastable intermediates, which are highly dynamic and can sample a range of conformations before ultimately forming stable aggregates. These intermediate species could rapidly transform into a diverse ensemble of conformations, possessing varying levels of native and non-native interactions, as well as both intramolecular and intermolecular interactions (Bitan et al., 2003; Calamai et al., 2005; Dinner et al., 2000). The complex dynamics of misfolding make it challenging to predict the

exact pathways and intermediates involved in the process. The energy landscape for misfolding is further complicated by the behavior of intrinsically disordered proteins. Unlike globular proteins, IDPs do not need to unfold to expose misfolding-prone regions; they can directly aggregate without adopting a well-defined global three-dimensional structure. MD simulation studies have shown that the energy landscape of an IDP is shallower and contains a large number of low-energy structures than that in the case of globular proteins, making the former more prone to misfolding (Chong and Ham, 2019).

Importance of kinetic barriers in protein misfolding

A protein in its misfolded form, such as in amyloid fibrils, often appears to be thermodynamically more stable than its native folded form (Norrild et al., 2021). In the native state, most of the hydrogen bonds in the peptide are satisfied through intramolecular interactions, which reduces the likelihood of aggregation (Ricchiuto et al., 2012). While the misfolded form may be thermodynamically favored, the primary factor preventing spontaneous misfolding and aggregation of a protein is the presence of large kinetic barriers (Louros et al., 2023). Kinetic barriers arise from the competing effects of enthalpy and entropy during the process of misfolding, which involves structural changes. Structured states are typically favored enthalpically but are disfavored entropically. The mismatch between the changes in by enthalpy and entropy leads to the formation of free energy barriers (Oliveberg and Wolynes, 2005), that slows down misfolding (Johnson et al., 2005). The barriers separate the stable native state from the misfolded conformations, influencing the probability of misfolding events. The likelihood of misfolding depends on the height of the kinetic barriers, which can cause misfolding to occur over various timescales, ranging from microseconds to milliseconds (the earliest stages of misfolding) to minutes or hours (the formation of oligomers) and even weeks (the development of fibrils) (Dee and Woodside, 2016). Therapeutic strategies against diseases involving protein misfolding, often focus on modulating these kinetic barriers. For example, small molecules or ligands can be designed to stabilize the native conformation and prevent or delay misfolding by raising the energy barrier to misfolding (Johnson et al., 2005; Kuwata et al., 2007b).

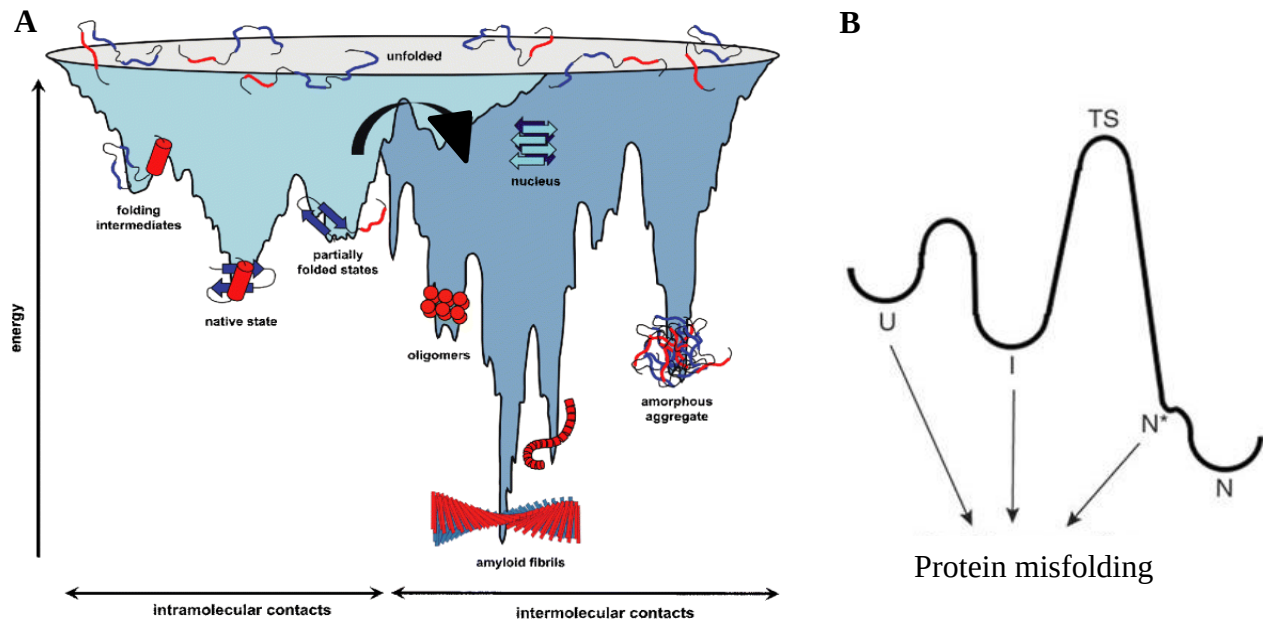


Figure 1.1. A. Comparison of the energy landscape for protein folding and misfolding.

The folding landscape is relatively smoother. The energy landscape for misfolding and aggregation is shown to be considerably more complex and rugged. The surface illustrates how various conformations funnel towards the N state through intramolecular contact formation, or towards aggregate formation through intermolecular contacts. (Reproduced from Jahn & Radford, 2005) B. Misfolding can occur from different protein conformations. Under physiological conditions, the N state is in equilibrium with other conformational ensembles (N*, one or more locally unfolded states; I, folding intermediate; U, unfolded state). These high-energy conformations are separated from N by energy barriers under physiological conditions and form transiently. These states populate rarely at equilibrium, and are transiently sampled from the N state due to thermal fluctuations. Both I and N* need not be on-pathway but could be off-pathway to folding. A protein in either in the U, I, or N* states could be prone to misfolding and can assemble to form aggregates. However, N* is more accessible from N than I and U, both thermodynamically and kinetically, making it a key precursor to misfolding (Reproduced from Chiti & Dobson, 2009).

1.2 Protein misfolding as a phase transition

Proteins are capable of undergoing both intramolecular and intermolecular phase transitions (Uversky and Finkelstein, 2019). The process of protein folding can be viewed as a spontaneous intramolecular phase transition, in which an unfolded polypeptide chain transitions into a well-defined structure. Similarly, protein misfolding can also be viewed as a phase transition. Protein aggregation, which either accompanies or leads to misfolding, can be viewed as an intermolecular phase transition. Depending on the degree of structural alteration, these transitions can result in the formation of amorphous aggregates, oligomers, fibrils, gels, or crystals (Dumetz et al., 2008). In all such cases, protein molecules must either attract each other or repel the solvent in such a way that the gain in interaction energy outweighs the entropic cost of forming an interface between phases. Aggregation and fibrillation are characterized by substantial conformational changes, unlike crystallization, which generally involves minimal structural distortion (George and Wilson, 1994). Another important phase transition process is liquid-liquid phase separation (LLPS), which leads to the formation of biomolecular condensates, and which plays a significant role in the formation of membrane-less organelles within cells (Banani et al., 2017; Toretzky and Wright, 2014).

Transition between the dilute solution phase and solid states

In supersaturated solutions, proteins tend to undergo self-association, leading to crystallization (Goto et al., 2022). Supersaturation occurs when protein molecules remain in solution for an extended period at concentrations exceeding their equilibrium solubility limit (So et al., 2016). Despite their ordered structure, protein crystals contain significant solvent (typically 27-65%) (Chruszcz et al., 2008; Matthews, 1968) and form due to changes in environmental conditions, such as temperature or salt concentration (Stahnke, 1979).

In contrast, fibrillization, often associated with major conformational changes, involves the transition from a soluble, folded protein to an insoluble β -rich amyloid fibril. From a physicochemical perspective, amyloid fibril formation is a phase transition from a soluble to a crystal-like state, occurring only when the solubility limit is surpassed and supersaturation exceed (Goto et al., 2022). It follows a nucleation and growth mechanism, with the formation of ordered fibrils being rate-limited by the nucleation step (Come et al., 1993). Fibrillization displays a characteristic sigmoidal curve, including a lag phase that reflects the energy barrier for nucleation (Dovidchenko et al., 2014; Frieden, 2007; Kumar and Udgaonkar, 2010). After nucleation, fibrils grow through the addition of monomers at the fibril end (Dovidchenko et al., 2014; Wetzel, 2006). Amyloid fibrils, which are implicated in neurodegenerative diseases, are

distinct from amorphous aggregates due to their characteristic filamentous, twisted morphology and cross- β -rich structure (Kuroda, 2018; Sunde et al., 1997). Notably, the ability to form amyloid fibrils is not restricted to disease-linked proteins but is a common trait of any polypeptide, with most proteins capable of fibril formation under suitable conditions (Chiti and Dobson, 2006; Rochet and Lansbury Jr, 2000).

On the other hand, aberrant phase transitions of proteins can lead to the formation of large, insoluble clusters known as amorphous aggregates (Yoshimura et al., 2012). These aggregates form instantaneously without the high energy barrier seen in nucleation and do not exhibit the ordered, β -rich structures seen in fibrillation (Boatz et al., 2017; Kuroda, 2018). Amorphous aggregation results in disordered clusters without a defined higher-order structure. Thus, the transition from the dilute solution phase to a solid phase, whether by crystallization, aggregation, or fibrillation, highlights the delicate balance between protein stability and instability, with misfolding events leading to the formation of diverse, often pathological, protein structures.

1.2.1 Transition between the dilute solution phase and liquid-like condensates

The phenomenon of LLPS in supersaturated protein solutions has long been known to protein crystallographers (Dumetz et al., 2008; Muschol and Rosenberger, 1997), but it has only recently gained significant attention in a range of scientific fields, including protein misfolding, cellular biology, biotechnology, and biomedicine. This growing interest stems from the recognition that LLPS plays a crucial role in the formation of biomolecular condensates or protein-rich liquid droplets, which form membrane-less organelles in cells, and are vital for cellular compartmentalization and organization (Hyman et al., 2014; Shin and Brangwynne, 2017). Although membrane-less compartments like the nucleolus were identified as early as the 1830s (Wagner, 1835), their biological importance was largely overlooked for many years due to their transient nature and challenges associated with isolating them for study. Unlike membrane-bound organelles, proteinaceous membrane-less organelles (PMLOs) are formed through protein-protein and protein-nucleic acid interactions, without requiring lipid bilayers (Toretsky and Wright, 2014). Found in the cytoplasm, nucleus, and mitochondria, PMLOs compartmentalize processes like gene regulation and metabolism, acting as microreactors to facilitate biochemical reactions and respond to stimuli (Forman-Kay et al., 2018; Li et al., 2024).

The LLPS process is influenced by several factors, including concentration, temperature, pH, and specific protein interactions (Dignon et al., 2019; Pintado-Grima et al., 2022; Xu et al., 2024). Recent studies have shown that proteins linked with neurodegenerative diseases and cancers, invariably undergo LLPS, forming dense, liquid-like protein-rich condensates that often transition to a more solid-like state (Alberti and Hyman, 2021; Babinchak and Surewicz, 2020; Zbinden et al., 2020). Studies have also shown that misfolded fibrillar species can emerge from these condensates when they undergo amyloidogenic solidification, suggesting that protein-rich condensates are involved in the nucleation of amyloid aggregation (Babinchak et al., 2019; Ray et al., 2020). Interestingly, factors that promote neurodegenerative diseases, such as familial mutations, DNA damage, metal ion exposure, and post-translational modifications, can also regulate LLPS and trigger the transition from a liquid-like to a solid-like state (Hofweber and Dormann, 2019; Pessina et al., 2021; Polymenidou, 2018; Tsang et al., 2020). The process of protein misfolding *via* LLPS is emerging as an alternative mechanism, alongside the canonical aggregation pathway discussed above.

1.3 The Prion protein and Prion diseases

1.3.1 Background and Importance

Prion diseases are a group of fatal neurodegenerative diseases associated with the misfolding of the mammalian prion protein (PrP) in humans and other mammals (Collinge, 2001; Prusiner, 1998, 2017). The conformational conversion of the cellular prion protein (PrP^C) into an insoluble, misfolded, multimeric, β -rich, protease-resistant isoform, PrP^{Sc}, is the fundamental pathological event in prion diseases (Pan et al., 1993b). The accumulation of PrP^{Sc} aggregates in the brains of diseased individuals is a hallmark feature of these diseases (Aguzzi et al., 2008b; Chesebro, 2003; Hope, 1994). These diseases include Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI), Gerstmann-Straussler-Scheinker syndrome (GSSS), and kuru in humans (Brown et al., 1986; Collinge, 2001); scrapie in sheep and goats (Acín et al., 2021); bovine spongiform encephalopathy (BSE) in cattle (Prusiner, 1997); and chronic wasting disease (CWD) in deer and elk (Schauber and Woolf, 2003). Prion diseases can be categorized into three primary groups: genetic, sporadic, and acquired. More than 30 mutations in the PRNP gene, encoding PrP^C, have been identified with human prion diseases (Collinge, 2001). Although 10-15% of prion diseases are genetic, about 85% are sporadic and arise from the spontaneous misfolding of PrP (Apetri et al., 2005; Prusiner, 1998). To date, there is no available therapy to arrest the progression of prion diseases in either humans or other animals.

Moreover, prion diseases progress to fatality within six months to a year from onset, unlike other neurodegenerative diseases which progress over decades.

Prion research has had a transformative impact on modern biology. In 1976, Carleton Gajdusek received the Nobel Prize for his discovery that CJD and kuru were transmissible (Gajdusek et al., 1966; Gibbs Jr et al., 1968). In 1996, Stanley B. Prusiner received the Nobel for his revolutionary work showing that prions are infectious because PrP^{Sc} can self-replicate, converting PrP^C into more PrP^{Sc}, a concept known as the 'Prion Principle' (Prusiner, 1982c). It has become evident over time that the progression of protein aggregates in a prion-like manner occurs in other neurodegenerative diseases too, including AD and PD (Jucker and Walker, 2013; Prusiner, 2012). Furthermore, distinct strains of PrP^{Sc} can induce different disease outcomes in seemingly identical hosts. This phenomenon, known as 'prion strain diversity', has been extended to other neurodegenerative diseases (Kaufman et al., 2016; Peelaerts et al., 2015; Sanders et al., 2014). The ability of PrP^{Sc} to self-replicate through a template-driven process has also led to the development of diagnostic methods such as protein misfolding cyclic amplification (PMCA) and real-time quaking-induced conversion (RT-QuIC), which are used to detect protein aggregates in biofluids, enabling early diagnosis of prion diseases, and also other neurodegenerative diseases (Saborio et al., 2001; Wilham et al., 2010). The prion principle, which involves the transfer of biological information *via* protein misfolding, has not only been helpful in understanding disease but also for understanding numerous physiological processes, including nitrogen metabolism (Wickner, 1994); transcriptional termination (Yuan and Hochschild, 2017), memory formation (Si et al., 2003), chromatin maintenance (Harvey et al., 2020) and innate immunity (Franklin et al., 2014), indicating the importance of fundamental research on prion biology.

1.3.2 Structural features of cellular PrP

The prion protein is ubiquitously expressed as a 250 amino acid residue long cell-surface glycoprotein in multiple tissues, but primarily in neuronal cells (Bendheim et al., 1992a). It undergoes multiple post-translational modifications to form the 208-residue, monomeric PrP^C, which is attached to the cell membrane by a C-terminal GPI anchor (Puig et al., 2014). PrP^C exhibits significant sequence identity across species, from amphibians to mammals (van Rheede et al., 2003a). The structures of monomeric PrP^C proteins from different species have been solved using techniques like NMR and X-ray crystallography and found to be similar (Knaus et al., 2001; Wüthrich and Riek, 2001; Zahn et al., 2000). All mammalian

prion proteins contain two distinct domains, an intrinsically disordered N-terminal domain (NTD) and a structured C-terminal domain (CTD) (Wüthrich and Riek, 2001). The NTD has four to five octapeptide repeats (Guadagno and Medina, 2023), which have a high affinity for divalent metal ions, such as Cu^{2+} (Hornshaw et al., 1995; Stöckel et al., 1998), and which become partially structured upon interaction with Cu^{2+} (Millhauser, 2007). It also has a highly amyloidogenic hydrophobic region spanning residue positions 112 to 120 [mouse numbering has been used] (Forloni et al., 1993). On the other hand, the CTD is well-structured (residues 125–231) and is predominantly composed of α -helical structure (42%) with a small fraction of β -sheet (3%) (Pan et al., 1993b). It contains three α -helices (α 1: 144–153, α 2: 172–193, α 3: 200–225) and two short antiparallel β -strands (β 1:127-130 and β 2:160–163) (Riek et al., 1996a). A disulfide bond between Cys 178 and Cys 213 tightly links α 2 and α 3, and is shown to be crucial for maintaining the native structure (Ning et al., 2014a).

1.3.3 Putative functions of cellular PrP

Even though the misfolded prion protein has long been associated with prion diseases, defining the physiological role of PrP^{C} is an area of active investigation. Investigating the function of PrP^{C} is important, as reducing or eliminating its expression presents a promising therapeutic approach for treating prion diseases (Minikel et al., 2020; White et al., 2008). Moreover, the fact that the sequence of the prion protein is highly conserved across different species (van Rheede et al., 2003a) suggests that it might have an important role that is evolutionarily beneficial.

The intrinsically disordered NTD of the prion protein has sequence similarity with the Zrt, Irt-like protein (ZIP) family of zinc transporter proteins (Ehsani et al., 2011; Schmitt-Ulms et al., 2009). The NTD can also interact with divalent metal ions like Ni^{2+} , Cu^{2+} (Jackson et al., 2001), implying that it might play a role in metal ion homeostasis. The prion protein can also interact with multiple proteins, such as the neural cell adhesion molecule (NCAM) (Santucci et al., 2005), and Fyn kinase (Um et al., 2012) involved in the neuronal signaling pathway promoting myelin maintenance, suggesting that it might play a role in cell adhesion and cell signaling. PrP^{C} also appears to possess neuroprotective activity, indicating that it might have a beneficial role under stress conditions (Bounhar et al., 2001; Kuwahara et al., 1999; McLennan et al., 2004). PrP^{C} has been shown to interact with oligomers of other proteins associated with neurodegeneration, including $\text{A}\beta$ and α -synuclein, and to facilitate cellular uptakes of the oligomers (Aulic et al., 2017; Laurén et al., 2009).

Prnp gene knocked out (*Prnp* ^{-/-}) studies show, however, only mild phenotypic defects in various organisms, including mice (Büeler et al., 1992), cattle (Richt et al., 2007b), and zebrafish (Leighton et al., 2018). These defects include demyelinating neuropathy (Bremer et al., 2010), sleep disturbance, altered circadian rhythm (Tobler et al., 2019), increased susceptibility to viral infection (Chida et al., 2018) as well as age-related cognitive decline (Steele et al., 2007a). Nevertheless, the organisms are able to survive. This has made it difficult to assign a highly specialized or essential function to PrP^C, as its absence does not lead to severe or lethal outcomes. It seems that the prion protein is not an essential protein and that it might have a redundant function, with moderate influence on several biological processes (Figure 1.2).

1.4 Defining PrP-mediated toxicity

1.4.1 PrP amyloid fibrils may be infectious, but not be responsible for neurodegeneration.

While it is widely accepted that misfolded PrP^C is associated with prion diseases and that accumulation of PrP^{Sc} in the brain is a major histopathological feature of the diseases, the exact misfolded conformation responsible for neurodegeneration remains elusive. This is because the misfolded forms purified from infected brains are highly heterogeneous. This heterogeneity in the sizes and conformations of aggregated forms makes it difficult to identify distinct neurotoxic and infective forms. The role of cellular PrP is still not clear, adding further complexity to the investigation. However, studies have shown that the deposition of protease-resistant PrP amyloid fibrils in the brain is not necessarily linked to neurodegeneration (Chesebro et al., 2005; Piccardo et al., 2007). In certain cases, deposits of PrP fibrils were found to be absent in individuals showing clinical symptoms of neurodegeneration (Lasmézas et al., 1997; Manuelidis et al., 1997). Similarly, several pathogenic mutations lead to neurodegeneration in humans without causing any detectable deposition of amyloid fibrils in the brain (Collins et al., 2000; Grasbon-Frodl et al., 2004; Nitrini et al., 1997), suggesting that prion infectivity and neurodegeneration may involve distinct mechanisms.

The cellular localization of PrP^C also plays an important role in determining its neurotoxicity. PrP^C is typically anchored to the cell membrane *via* a GPI anchor (Puig et al., 2014). In the case of transgenic mice expressing an anchor-less form of PrP, which is not tethered to the plasma membrane, PrP fibrils are detected, but the mice do not show symptoms

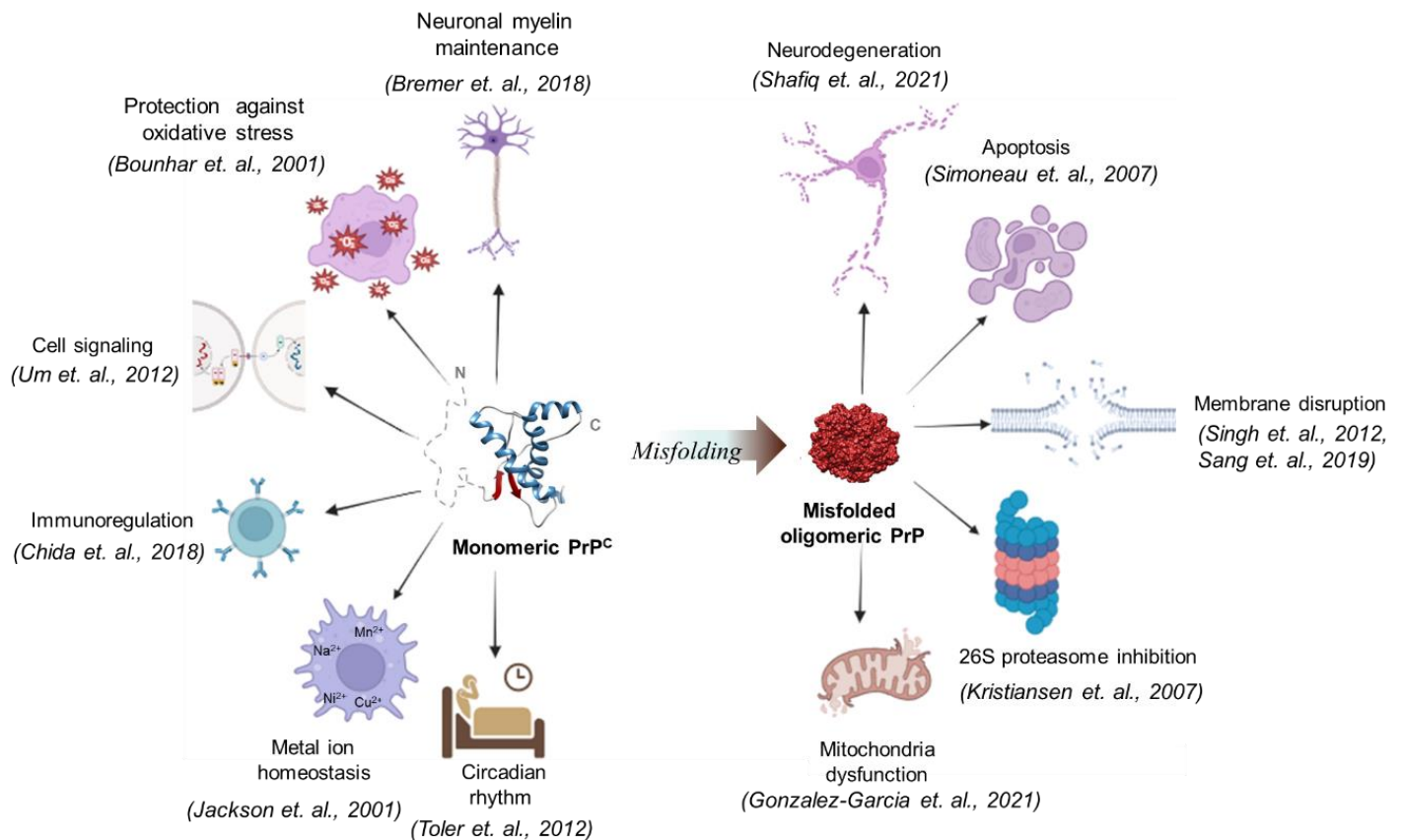


Figure 1.2. The monomeric cellular isoform of the prion protein consists of an unstructured N-terminal domain (depicted as a dashed line) and a structured α -helical C-terminal domain. PrP^C is reported to play roles in various physiological processes. Misfolding of PrP^C can lead to the formation of β -rich oligomers, which can damage cells through different mechanisms as shown. Created with [Biorender.com](https://biorender.com).

of neurodegeneration (Chesebro et al., 2005). On the other hand in some forms of GSS syndrome, transmembrane forms of PrP were found to lead to neurodegeneration even when fibrils were undetectable in the brain (Hegde et al., 1998b). PrP also can exist in cytosolic (Ma et al., 2002) and secreted forms (Hay et al., 1987), which have been shown to induce neurotoxicity in the absence of significant accumulation of amyloid fibril, both in cultured neuronal cell lines and transgenic mouse models (Morales et al., 2012; Taylor et al., 2009). Taken together, all these studies indicate that while PrP amyloid fibrils are indeed infectious, they may not be directly responsible for neurotoxicity. Other forms of misfolded PrP may play a critical role in causing neurotoxicity.

1.4.2 PrP-oligomer mediated neurotoxicity

Recent research has suggested that the neurotoxicity associated with neurodegenerative diseases, including prion diseases, is linked to protease-sensitive forms of aggregates, such as soluble oligomers, which are distinct from the amyloid fibrils that are protease-resistant (Forloni, 2023; Gutierrez and Limon, 2022; Rinauro et al., 2024; Wang et al., 2015). While there is extensive evidence of their neurotoxic effects, there is also evidence of their seeding properties facilitating propagation in the nervous system (Zampar et al., 2024). These protease-sensitive, soluble, small aggregates may be particularly neurotoxic due to their ability to pass through the brain parenchyma and synaptic clefts, disrupting their structure and function (Bartels et al., 2010; Winner et al., 2011). Soluble oligomers of α -synuclein (Kalia et al., 2013), tau (Cárdenas-Aguayo et al., 2014), FUS (Sun et al., 2011), and A β (Watson et al., 2005) have also been found to be highly neurotoxic. In the case of PrP, a correlation between the toxicity of hamster 263K PrP^{Sc} and particle size was observed, with non-fibrillar oligomeric assemblies containing 14 to 28 PrP molecules (300–600 kDa) exhibiting the highest toxicity, while larger fibrils were less toxic (Silveira et al., 2005b). Similarly, protease-sensitive, soluble oligomeric forms comprising from 20 to 78 PrP monomeric units were isolated from the brains of individuals with sporadic CJD (sCJD) and found to be highly cytotoxic (Kim et al., 2012). The seeding activity of brain-derived oligomeric aggregates was further examined using RT-QuIC and PMCA assays, revealing that small human PrP^{Sc} oligomers are the most potent seed (Kim et al., 2012). Similar results have been observed with soluble oligomeric forms isolated from cell cultures (Anaya et al., 2011). In addition, the susceptibility to sporadic prion disease appears to strongly correlate with the propensity of recombinant PrP to form misfolded β -rich oligomers (Khan et al., 2010b). PrP oligomers have been also shown to induce cellular

apoptosis (Simoneau et al., 2007), to inhibit the β subunits of the 26S proteasome (Kristiansen et al., 2007), to induce mitochondrial dysfunction, and to form pores in lipid membranes (Chich et al., 2010; Sang et al., 2019; Singh et al., 2012a), causing toxicity (Figure 1.2). Another possible mechanism involves PrP-mediated modulation of the N-methyl-D-aspartate (NMDA) receptor, which plays a key role in the proper functioning of the nervous system (Sassoon et al., 2004). Excessive NMDA receptor activity can result in neurotoxicity (Khosravani et al., 2008; Sassoon et al., 2004). These findings indicate that it is the protease-sensitive soluble misfolded oligomeric forms of PrP, rather than the protease-resistant fibrils, that are responsible for neurodegeneration. However, the heterogeneity and transient nature of these species have made it challenging to isolate and characterize them.

1.5 Molecular mechanism of PrP oligomer formation

1.5.1 Formation of misfolded PrP oligomers *in vitro*

The observation that protease-resistant, insoluble amyloid PrP fibrils do not strongly correlate with neurodegeneration, while soluble PrP oligomers, whether extracted from diseased brain (Kim et al., 2012; Silveira et al., 2005b) or generated *in vitro* from recombinant PrP (Sang et al., 2019; Simoneau et al., 2007), show a better correlation, implies that it is the soluble oligomers that are primarily responsible for the toxicity. Even though soluble oligomers have been found in the brain lysates of disease patients (Esparza et al., 2016; Kim et al., 2012; Paleologou et al., 2009), these soluble aggregates occur at very low concentrations and their characterization has been challenging, due to a lack of sensitive methods. Furthermore, brain samples are generally homogenized, and hence include large amounts of insoluble aggregates, which are largely inert, potentially complicating the interpretation of *in vivo* results. These difficulties have led to attempts to produce synthetic soluble oligomers *in vitro* by altering the conformation of recombinant mammalian PrP produced in bacteria. The oligomers formed *in vitro* are highly toxic, often disrupting lipid membranes (Sang et al., 2019; Singh et al., 2014a; Singh et al., 2012a). Moreover, the oligomers form more readily in species with high susceptibility to prion diseases (Khan et al., 2010b), supporting their relevance as models for studying prion misfolding mechanisms. Understanding the molecular mechanism leading to oligomerization is crucial not only for comprehending the molecular basis of prion diseases, but also for developing effective anti-prion therapies, including those that could prevent misfolding by stabilizing key structural regions of PrP.

Misfolded PrP oligomers can be generated under various *in vitro* conditions, including low pH (Baskakov et al., 2002b), chemical denaturants (Khan et al., 2010b), high temperatures

(Eghiaian et al., 2007), metal-induced oxidation (Redecke et al., 2007), and high pressures (Torrent and Lange, 2012). The oligomers exhibit structural and size heterogeneity but shared a common feature—a β -rich core region and a lack of substantial helical content. Computational studies have shown that the conversion of the $\alpha 2$ - $\alpha 3$ region to β -sheet is crucial for oligomer formation (Baillod et al., 2012; Chen and Thirumalai, 2013). Experimental studies using NMR (Hosszu et al., 2009), HDX-MS (Singh et al., 2012a), and electron spin resonance (Yang et al., 2015) further confirmed that the $\alpha 2$ and $\alpha 3$ regions convert to β -sheet, while the $\alpha 1$ and $\beta 2$ regions become unfolded. Notably, PK-sensitive soluble oligomers share fundamental structural characteristics with PK-resistant PrP^{Sc}, as revealed by limited proteolysis, despite variations in their PK digestion sensitivity and sizes (Sajnani et al., 2012).

1.5.2 Sequence and structure peculiarities of PrP

Earlier studies showed that the misfolding propensity of proteins strongly depends on the structural instability of the monomeric protein, arising due to specific structural features including amphiphilic helices (Takahashi et al., 2000), alternating polar and non-polar sequence pattern (Broome and Hecht, 2000), discordant α -helices (Kallberg et al., 2001) as well as edgy β -strands (Richardson and Richardson, 2002b). This leads to the question, of whether there any structural features of the monomeric prion protein that make it prone to misfolding. Surprisingly, all three helices of the prion protein are highly unusual, if not unique. For example, $\alpha 1$ of PrP is composed mostly of hydrophilic residues and is stabilized by two internal salt-bridges, making no external (tertiary) salt bridges with the rest of the protein (Morrissey and Shakhnovich, 1999b). High hydrophilicity, unusual charge distribution, and lack of tertiary contacts with the hydrophobic core of the protein make it prone to conformational change. The C-terminal end of $\alpha 2$, which includes the highly amyloidogenic ¹⁸⁶TVTTT¹⁹² motif, as well as a few N-terminal residues of $\alpha 3$, are energetically frustrated in their helical conformation, and have a high β -structure propensity (Dima and Thirumalai, 2002). $\alpha 1$ and $\alpha 3$ of the monomeric prion protein contain a high concentration of underwrapped hydrogen bonds (insufficiently shielded from water attack), making these regions prone to structural rearrangement (Fernández et al., 2003). Because of these unusual properties, $\alpha 1$ appears to have a strong propensity to detach from the ($\alpha 2$ - $\alpha 3$). Not surprisingly, the protein is shown to readily form domain-swapped dimers during crystallization, by rearranging the $\alpha 2$ - $\alpha 3$ sub-domain (Hafner-Bratkovič et al., 2011; Knaus et al., 2001).

1.5.3 Effect of pH on native monomer: PrP is prone to misfolding at low pH

PrP is expressed at the cell surface, and is endocytosed into lysosomes (Harris, 2003), while some PrP can also leak into the cytosol (Mironov et al., 2003). PrP experiences a change in pH from 7.4 (in the cytosol) (Roos and Boron, 1982) to 4.5 (in the endosome) (Aubry et al., 1993) during its cellular lifetime. Interestingly, the formation of *in vitro* PrP oligomers, characterized by β -rich structures, is also favored at low pH (Gerber et al., 2008; Singh and Udgaonkar, 2016e), suggesting that PrP likely misfolds when it encounters the low pH in the endosomes (Arnold et al., 1995; Borchelt et al., 1992a). The monomeric prion protein is more stable at neutral pH than at low pH (Calzolari and Zahn, 2003; Singh and Udgaonkar, 2016e). Differential scanning calorimetry studies also indicate that the heat capacity of native protein at acidic pH, is twice that of globular proteins of similar size, suggesting that it undergoes significant structural fluctuations at low pH (Moulick and Udgaonkar, 2014). MD simulations have also suggested that low pH increases the flexibility of $\alpha 1$, promotes the unfolding of the C-terminal end of $\alpha 2$ and destabilizes the hydrophobic core of the protein (Alonso et al., 2001; DeMarco and Daggett, 2007; Van der Kamp and Daggett, 2010a). HDX and NMR studies have provided experimental confirmation of the pH-induced structural changes observed in the simulations (Calzolari and Zahn, 2003). At neutral pH, the human prion protein retains a well-defined globular structure, with stable $\alpha 1$ and $\alpha 2$. However, at low pH, both helices become destabilized (Calzolari and Zahn, 2003). In $\alpha 1$, this destabilization is attributed to the protonation of His154 at acidic pH, which destabilizes $\alpha 1$ by introducing charge repulsion near Arg156 (Langella et al., 2004). Similarly, the C-terminal end of $\alpha 2$ displays increased flexibility and a tendency to unfold at low pH, where protonation of His186 likely destabilizes $\alpha 2$ because of unfavorable interactions with the helix dipole (Garrec et al., 2013). Similar results have been obtained in the case of mouse prion protein, where the protonation of either H186 or D201 at low pH leads to the subtle structural changes: increased structural dynamics in $\alpha 1$ and in the loop between $\alpha 1$ and $\beta 2$, which makes the protein more prone to misfolding (Singh and Udgaonkar, 2016e). Earlier studies had also highlighted changes in the electrostatic potentials of PrP upon a change in pH (Calzolari and Zahn, 2003), while a remarkably uneven distribution of positively and negatively charged residues was observed at low pH. Recent temperature dependent NMR studies have indicated that both ends of $\alpha 2$ are prone to unfolding at pH 4, and that residues in $\beta 1$, the $\beta 1$ - $\alpha 1$ loop, and Gln211 in $\alpha 3$ sample alternative conformations (Bhate et al., 2021a). PET-FCS studies conducted at pH 4 have also identified structural fluctuations at both ends of $\alpha 2$, suggesting increased susceptibility to conformational changes in these regions (Goluguri et al., 2019b). These fluctuations have been shown to further

influence dynamics of adjacent regions, with the C-terminal end of $\alpha 2$ positioned near $\alpha 1$, while the N-terminal end of $\alpha 2$ interacts with the C-terminal end of $\alpha 3$ (Goluguri et al., 2019b).

Identification of PUFs and folding intermediates at low pH

Diffusion across the misfolding landscape for the prion protein has been shown to be 1000-fold slower in comparison to that across the folding landscape, indicating a rugged misfolding landscape (Yu et al., 2015). There has therefore been extensive research carried out into identifying misfolding-prone partially unfolded forms (PUFs) of PrP, which are sampled transiently during its folding or unfolding, or due to local fluctuations of the N state (Figure 1.3A). The identification and characterization of these partially unfolded intermediates are challenging due to their low abundance as well as their transient nature. The folding and unfolding of PrP occurs in the microsecond time domain (Wildegger et al., 1999), making it difficult to implicate intermediates as precursors of misfolding. Folding intermediates have been detected for the human prion protein, especially at acidic pH, and for pathogenic mutations like F197S. Notably, misfolding-resistant variants, such as Q167R, do not form such intermediates (Robinson and Pinheiro, 2009). Pressure-induced unfolding studies (Kachel et al., 2006) and single-molecule force-spectroscopy (Raspadori et al., 2022) studies have also identified partially unfolded intermediates of PrP (Gupta et al., 2016). In the ovine prion protein, susceptible variants have been shown to populate similar β -rich intermediates, while resistant variants do not (Rezaei et al., 2002). Equilibrium unfolding studies done at low pH have also identified off-pathway β -rich intermediates (Hornemann and Glockshuber, 1998; Swietnicki et al., 1997).

Earlier HDX-MS and HDX-NMR studies have identified two distinct PUFs (PUF1 and PUF2) in the case of the mouse prion protein based on the local stability (ΔG_{op}) and surface exposure (m value) of different

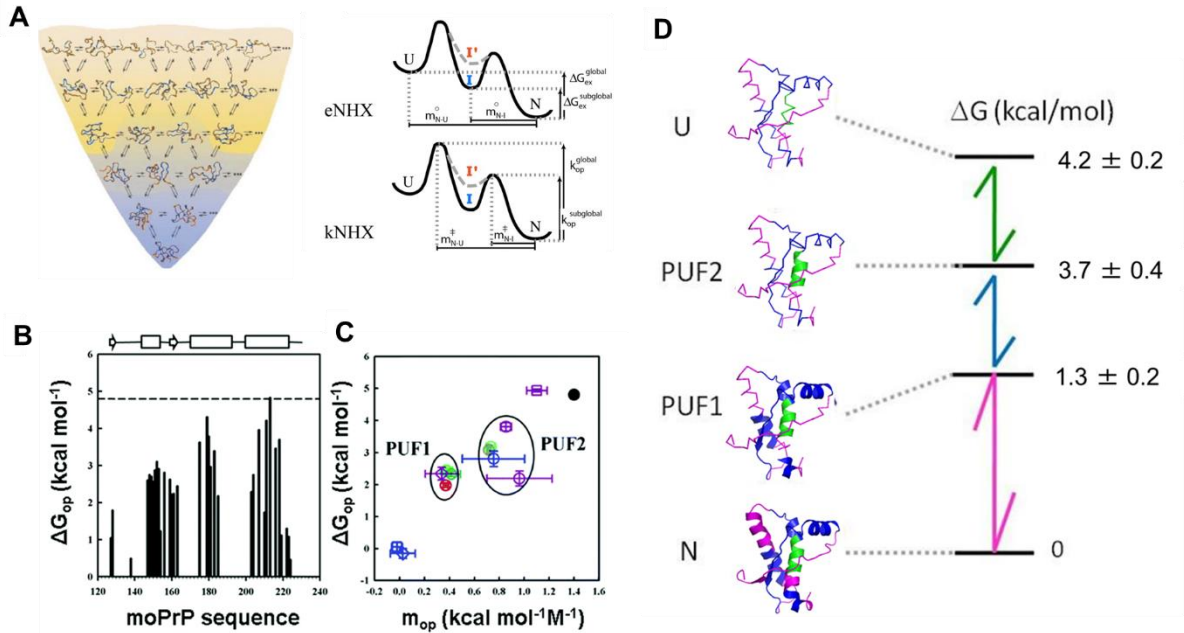


Figure 1.3. Detection of aggregation-prone PUFs of PrP populated at equilibrium using native state HX experiments. (A) Under native conditions, the N state cycles through various high-energy intermediates, which exist in minuscule amounts. These intermediates can be identified through native state HDX experiments. An amide hydrogen only exchange when their protective H-bond is broken in high-energy intermediate states. While equilibrium HDX (eNHX) can identify PUFs based on their local stability (ΔG_{op}) and surface exposure (m_{op} value) in denaturant, temperature, and pressure-dependent HX experiments, kinetic HDX identify them by their unfolding rate constants. (Reproduced from Oliveberg, & Wolynes, 2005 and Hoang, Linh, et al, 2002) (B) 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) (Reproduced from Moulick, et al, 2015) (C) Partially unfolded forms PUF1 and PUF2, populated in equilibrium with the N state, which differ not only in the extent to which they are destabilized but also in the extent they have exposed surface area, due to partial unfolding. (Reproduced from Moulick, et al, 2015) (D) Schematic representations of PUF1 and PUF2. In PUF1, sequence segments 127-143, 168-173, 182-196, 210-226 and 225-231 (segments shown in pink) have unfolded. In PUF2, sequence segments 144-167, 177-183, 197-204 and 217-223 have unfolded (segments shown in blue). In U sequence segment 205-212 has also unfolded (segment shown in green).

segments of the protein. These PUFs exist in dynamic equilibrium with the N state at pH 4 (Moulick et al., 2015c; Moulick and Udgaonkar, 2017) (Figure 1.3B, C). In the case of PUF1, $\beta 1$, the loop between $\beta 1$ and $\alpha 1$, and the C-terminal end of $\alpha 2$ were found to have become unstructured and solvent-exposed. PUF2 includes $\alpha 1$, the loop between $\alpha 1$ and $\beta 2$, and $\beta 2$, as having become unfolded and solvent-accessible. PUF1 is characterized by a ΔG_{op} of $1.3 \pm 0.2 \text{ kcal mol}^{-1}$ and a m_{op} value of $0.4 \pm 0.03 \text{ kcal mol}^{-1} \text{ M}^{-1}$, whereas PUF2 is characterized by a higher ΔG_{op} of $3.7 \pm 0.4 \text{ kcal mol}^{-1}$ and a m_{op} value of $0.8 \pm 0.1 \text{ kcal mol}^{-1} \text{ M}^{-1}$ (Moulick et al., 2015). Since the ΔG_{op} value of PUF2 was found to be higher than that of PUF1 (Moulick et al., 2015), it is possible that PUF1 is on the pathway of unfolding of the N state to PUF2. Consequently, the sequence segments that are unfolded in PUF1 would also be unfolded in PUF2 (Figure 1.3D). Little was, however, known whether misfolding originates directly from the identified PUFs.

Identification of MG-like intermediates at low pH

Several mutant variants of PrP, such as those with cysteine-to-alanine substitutions (Maiti and Surewicz, 2001), or with mutations of the conserved aromatic residues (Pal and Udgaonkar, 2023) as well as of the conserved methionine residues (Younan et al., 2012), tend to adopt molten globule (MG) like conformations, which are known to be highly prone to misfolding into β -rich aggregates (Fink, 1995; Iram et al., 2013). MG-like intermediates have also been observed in equilibrium studies of single tryptophan mutants of Syrian hamster PrP (Jenkins et al., 2008). At pH 2, the isolated CTD of PrP adopts a MG-like A-state (Honda et al., 2014b). The population of this A-state correlates directly with the rate of oligomerization. The T182A mutant variant, which is linked with familial prion disease, also adopts a MG-like conformation at low pH (Singh and Udgaonkar, 2016a). Interestingly, all these MG-like states are characterized by destabilization of the ($\alpha 2$ – $\alpha 3$) subdomain, suggesting that this is a critical step in the misfolding and oligomerization pathway.

Change in monomer structure and dynamics at the initiation of misfolding

While the prion protein undergoes significant structural perturbation at low pH, that alone is insufficient to trigger misfolding and oligomerisation. The presence of salt (e.g., NaCl) is essential for the process to occur (Sengupta et al., 2017a). This is partly because, at pH 4, monomeric PrP is positively charged, which leads to strong electrostatic repulsion between monomers. Salt at 150 mM concentration is necessary to screen these repulsive forces, allowing the monomers to associate (Sengupta et al., 2017a). Furthermore, real-time NMR

measurements have demonstrated that salt also perturbs the K193–E195 salt bridge, a crucial component of the electrostatic network connecting the C-terminal end of $\alpha 2$ with $\alpha 1$ (Sengupta et al., 2017a) (Figure 1.4). PET-FCS studies revealed enhanced fluctuations across the N-terminal end of $\alpha 2$ and the C-terminal end of $\alpha 3$ upon the addition of 150 mM salt, indicating the loss of critical interactions between these regions (Goluguri et al., 2019b). Upon salt addition, alternative conformations identified by NMR become more prevalent, as a consequence of increased structural perturbation (Bhate et al., 2021a).

1.5.4 Role of PUFs in misfolding

Evolutionary selection of ‘gatekeeper’ residues to impede PrP misfolding

It is known that protein sequences have evolved to prevent misfolding and aggregation (Reumers et al., 2009b; Rousseau et al., 2006). Evolutionarily conserved residues at specific sequence positions serve as ‘gatekeeper’ residues, helping to impede the misfolding process (Richardson and Richardson, 2002b). One approach to understand the mechanism of prion misfolding is to study the role of evolutionarily conserved amino acid residues in regulating this process. Mammalian prion proteins display overall highly conserved structures across species, despite some sequence variability, particularly at the interface of $\beta 2$ - $\alpha 2$ loop and the C-terminal end of $\alpha 3$ (Bernardi and Bruni, 2019; Biljan et al., 2011; Slapšak et al., 2019). Interestingly three aromatic residues (Tyr168, Phe174, and Tyr217) present at this packing interface are found to be highly conserved (Bartz et al., 1994; Huang and Caflisch, 2015a; Kurt et al., 2014b; van Rheede et al., 2003). Tyr168 and Tyr217 are strictly conserved, while Phe174 is conserved in all but two mammalian species (Huang and Caflisch, 2015b; Zhang, 2015). Molecular dynamics simulations suggest that favorable interactions between these aromatic residues stabilize the $\beta 2$ - $\alpha 2$ loop and the C-terminal end of $\alpha 3$ (Cheng and Dagget, 2014; Huang and Caflisch, 2015b), suggesting that these residues have been evolutionarily selected to stabilize the N state. Proline residues are also often found to be strongly conserved and known to play an important role in stabilizing native structures by providing backbone rigidity (Richardson and Richardson, 2002b). More importantly, they can prevent aggregate formation by blocking intermolecular β - β interactions (Richardson and Richardson, 2002b).

The role of the three conserved aromatic residues (168, 174 and 217) as well as the three conserved proline residues (136, 157 and 164) in the CTD of mammalian PrP were studied through mutagenesis. The secondary structures of the mutant variants remained largely

unaffected, resembling that of wt moPrP (Pal and Udgaonkar, 2022b, 2023), but the mutations cause a significant destabilization of the N state (Pal and Udgaonkar, 2022b, 2023). More importantly, these mutant variants show significantly faster misfolding rate constants compared to the wt protein, suggesting they have been evolutionarily selected for reducing the probability of misfolding (Pal and Udgaonkar, 2022, 2023) (Figure 1.4). While the mutations significantly accelerate misfolding, the oligomers formed by the mutant variants exhibit a similar size and structural characteristics similar to those of the wt protein, indicating that the mutations primarily influence misfolding and oligomerization, rather than directly influencing the final oligomer structure.

Gatekeeper residues modulate the formation of PUFs

HDX-MS studies of the proline mutant variants showed that these mutations primarily affect four sequence segments: 144–148, 149–153, 154–167 (which encompass the α 1- β 2 sub-domain), and 197–204 (spanning the α 2- α 3 loop and the N-terminal end of α 3) (Pal and Udgaonkar, 2022b). These segments exhibit faster kinetics of deuterium incorporation (Figure 1.5A) and a significant reduction in the ΔG_{op} values (Figure 1.5B) suggesting that the Pro-to-Ala mutations destabilize these regions and promote their unfolding (Pal and Udgaonkar, 2022b). While all four sequence segments belong to PUF2, these mutations caused marginal changes in the ΔG_{op} values of sequence segments 177–183 and 217–223, (Pal and Udgaonkar, 2022b), which are also found to be unfolded in PUF2.

In contrast, mutations of the conserved aromatic residues predominantly affect the deuterium incorporation kinetics (Figure 1.5C) and ΔG_{op} values of another distinct set of segments (Figure 1.5D) (Pal and Udgaonkar, 2023). These include sequence segments including β 1 (96–144), the loop between α 1 and β 2, β 2 (154–167), the loop between β 2 and α 2 (168–173), the C-terminal end of α 2 (182–196), α 3 (205–212, 210–216, and 217–223) and the C-terminal end of the protein (225–231). Among the sequence segments that have become unfolded, sequence segments 96-144, 168-173, 182-196, and 225-231 belong to PUF1, whereas sequence segments 154-167 and 217-223 belong to PUF2, suggesting that the aromatic mutations have impacted the stability of both the PUFs (Pal and Udgaonkar, 2023). Interestingly, the aromatic mutations do not significantly impact the sequence segments of PUF2 that are affected by the proline mutations (Pal and Udgaonkar, 2022b).

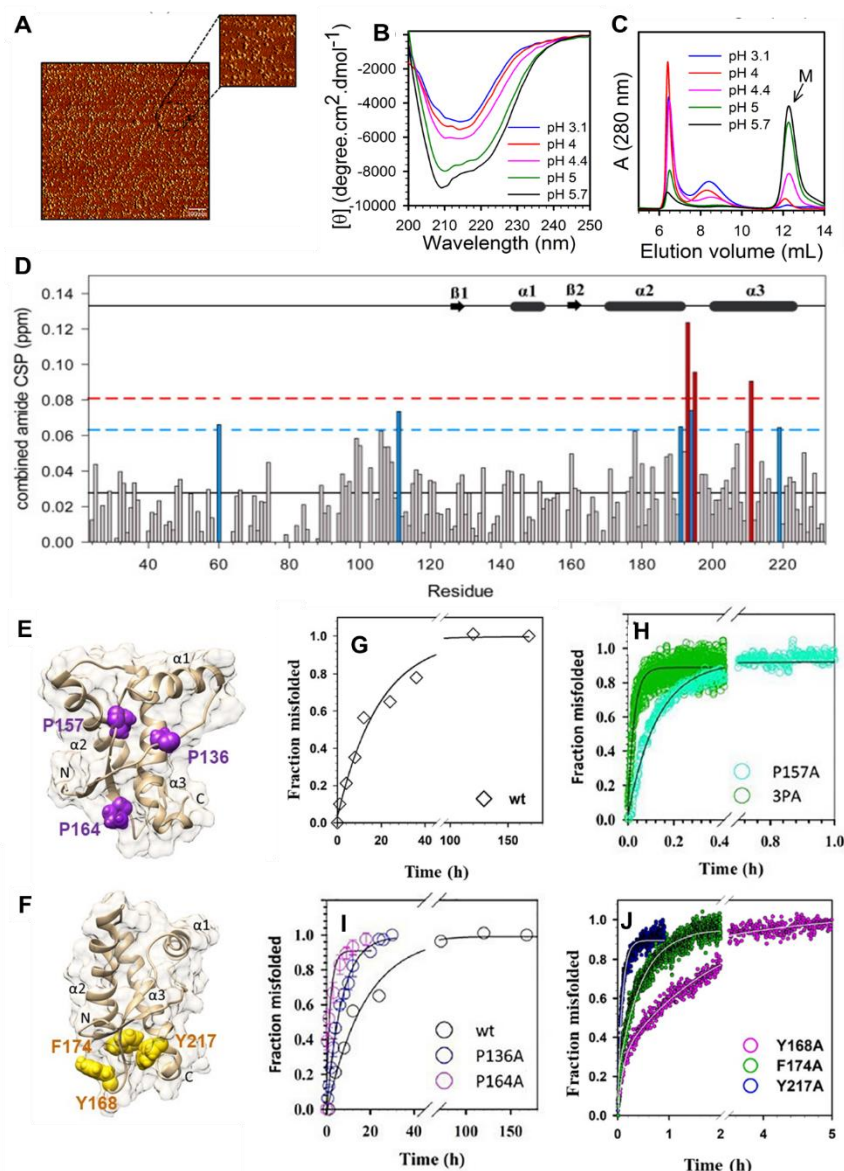


Figure 1.4. Conserved Gatekeeper residues impede salt-mediated misfolding and oligomerization of PrP at low pH (A) AFM images of PrP oligomers. (B) Far-UV CD spectra. (C) SEC profiles after 24 h of oligomerization at different pH values, showing the pH dependence of misfolding and oligomerization (Reproduced from Singh & Udgaonkar, 2016). (D) Salt binds specifically to positively charged moPrP at low pH, causing chemical shift perturbations (CSPs) throughout the protein, with the largest CSPs located in the loop between $\alpha 2$ and $\alpha 3$ (Reproduced from Sengupta & Udgaonkar, 2017). (E) Positions of the evolutionarily conserved proline residues and (F) aromatic residues, which serve as gatekeepers against misfolding. (G, H, I, J) Mutations of these conserved gatekeeper residues accelerate PrP misfolding leading to oligomer formation (Reproduced from Pal & Udgaonkar, 2022, 2023).

Identification of heterogeneity in PUFs

When PUF1 and PUF2 were first identified, it was assumed that each PUF consists of a structurally homogenous population of molecules (Moulick et al., 2015c). However, the observation that in both sets of mutant variants, with the proline and with the aromatic mutations, only specific sequence segments of PUF2 get affected, suggesting that PUF2 is not a single conformational state but an ensemble of multiple iso-energetic sub-populations, each with distinct structural features (Pal and Udgaonkar, 2022b, 2023). The sub-population, which becomes destabilized upon the mutation of the conserved proline residues, is referred to as PUF2*, and characterized by the disordered segments 144–148, 149–153, 154–167, and 197–204 (Pal and Udgaonkar, 2022b). On the other hand, the sub-population of PUF2, which becomes destabilized upon the mutation of the conserved aromatic residues, is referred to as PUF2** and features disordered segments 154–167 and 217–223 (Pal and Udgaonkar, 2023) (Figure 1.6A). Despite their structural differences, both sub-populations share similar local stabilities, with ΔG_{op} values of 3.7 ± 0.4 kcal/mol for PUF2* and 3.7 ± 0.1 kcal/mol for PUF2**. The discovery of these iso-energetic sub-populations in PUF2 suggests that the N state of PrP is in equilibrium with multiple PUFs, which have different structural features but similar overall stabilities. These findings align well with the concept of an energy landscape for protein folding, where distinct conformations with similar energies can coexist in equilibrium.

Identification of the PUFs as the direct precursors to misfolding

The logarithm of the observed misfolding rate constant for the proline mutant variants increases linearly with a decrease in the average ΔG_{op} values (ΔG_{op}^{av}) for the sequence segments that exchange in PUF2* (144–148, 149–153, 154–167, and 197–204), reflecting the stability of PUF2* (Pal and Udgaonkar, 2022b). This indicates that the misfolding rate constant is directly proportional to the equilibrium population of PUF2* (Figure 1.6B). In contrast, the logarithm of the observed misfolding rate constants for the aromatic mutant variants increases linearly with a decrease in the average ΔG_{op} values (ΔG_{op}^{av}) for the sequence segments that exchange in PUF1 (96–144, 168–173, 182–196, 210–216, and 225–231) as well as those in PUF2** (154–167 and 217–223) (Pal and Udgaonkar, 2023) (Figure 1.6C). This suggests that the misfolding rate constant for the aromatic mutants is directly proportional to the equilibrium populations of both PUF1 and PUF2**. The linear correlation between the misfolding rate

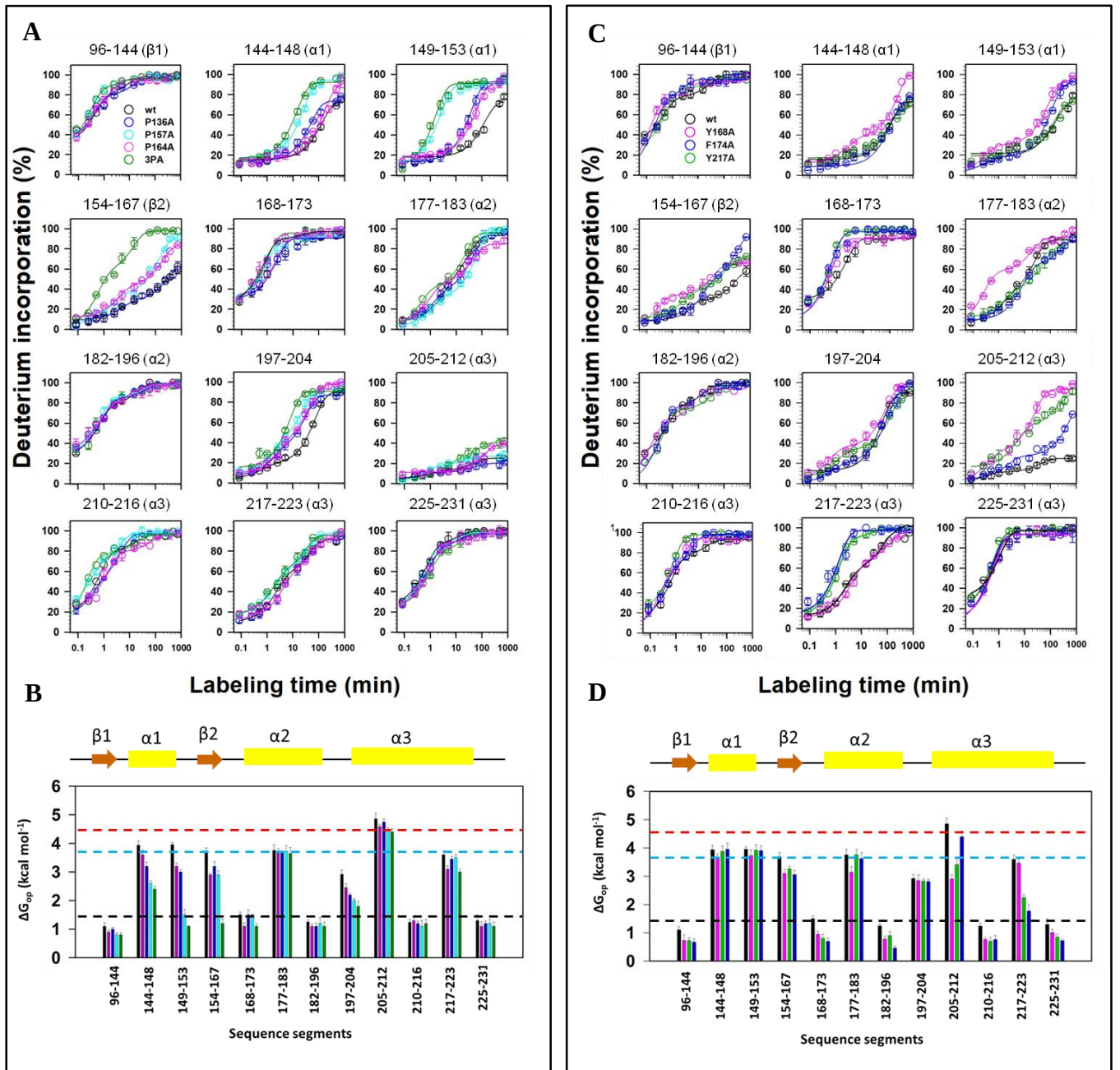


Figure 1.5. Gatekeeper residues modulate the formation of PUFs. The kinetics of HDX and ΔG_{op} values for different sequence segments of the conserved proline (A, B) and aromatic mutant variants (C, D), compared to wt moPrP, are shown. In both panels B and D, the black, cyan, and red dashed lines represent the energy required to form PUF1, PUF2, and U, respectively, from the N state in the case of wt moPrP. In the mutant variants, segments that have undergone unfolding in the PUFs exhibit significantly reduced ΔG_{op} values, suggesting that the conserved gatekeeper residues maintain the energy barrier between N and the PUFs in the wt protein (Reproduced from Pal & Udgaonkar, 2022, 2023)

constant and the free energy difference (ΔG_{op}) between the N state and the PUFs (PUF1, PUF2*, and PUF2**) strongly implies that the PUFs are the direct precursor conformations from which misfolding begins. Mutations of the conserved proline residues increase the sampling of PUF2*, while mutations of the conserved aromatic residues increase the sampling of PUF1 and PUF2** from the N state (Pal and Udgaonkar, 2022b, 2023), by decreasing the values of ΔG_{op} separating the PUFs from the N state. Hence, misfolding can commence in multiple ways from structurally distinct precursor conformations, PUF1, PUF2* and PUF2** (Pal and Udgaonkar, 2023). In the case of the wt protein, misfolding would initiate primarily from PUF1, as it is much more stable than PUF2* and PUF2**, being over 30 times more populated at equilibrium (Pal and Udgaonkar, 2023).

Pathways to initiate misfolding

It has been proposed that the separation of the $\alpha 2$ - $\alpha 3$ and $\beta 1$ - $\alpha 1$ - $\beta 2$ subdomains must occur before the conversion of $\alpha 2$ and $\alpha 3$ to a β -conformation in the PrP oligomers at low pH (Goluguri et al., 2019b; Hadži et al., 2015b; Sengupta and Udgaonkar, 2019b). Anti-prion drugs that lock the ($\alpha 2$ - $\alpha 3$) subdomain to the ($\beta 1$ - $\alpha 1$ - $\beta 2$) subdomain prevent misfolding and oligomerization completely (Kuwata et al., 2007b; Mashima et al., 2013). Similarly, the introduction of a disulfide bond between the $\beta 2$ - $\alpha 2$ loop and the C-terminal end of $\alpha 3$ reduces misfolded oligomer formation significantly, suggesting that interactions between these subdomains help guard against misfolding (Prigent and Rezaei, 2011a). Formation of the PUFs can trigger the separation of the subdomains in two different ways. In pathway 1, the formation of PUF2* promotes subdomain separation by the disruption of conserved non-local salt bridges between the ($\beta 1$ - $\alpha 1$ - $\beta 2$) and ($\alpha 2$ - $\alpha 3$) regions, which have been observed in several pathogenic mutant variants (Singh and Udgaonkar, 2015b). Mutations in the conserved proline residues within the CTD can contribute to subdomain separation using a similar mechanism (Pal and Udgaonkar, 2022b). In pathway 2, the formation of PUF1 triggers subdomain separation by disrupting aromatic and hydrophobic interactions between the $\beta 2$ - $\alpha 2$ loop and the C-terminal end of $\alpha 3$, as seen in mutant variants in which aromatic residues are substituted with alanine (Pal and Udgaonkar, 2023).

Spontaneous misfolding can be slowed down by rigidifying the $\beta 2$ - $\alpha 2$ loop

HDX-MS studies have shown that the conformation of the $\beta 2$ - $\alpha 2$ loop, which connects the $\beta 1$ - $\alpha 1$ - $\beta 2$ sub-domain with the $\alpha 2$ - $\alpha 3$ sub-domain, also plays a critical role in the dynamics and misfolding pathways of prion protein (Pal and Udgaonkar, 2024a). In a mutant variant of

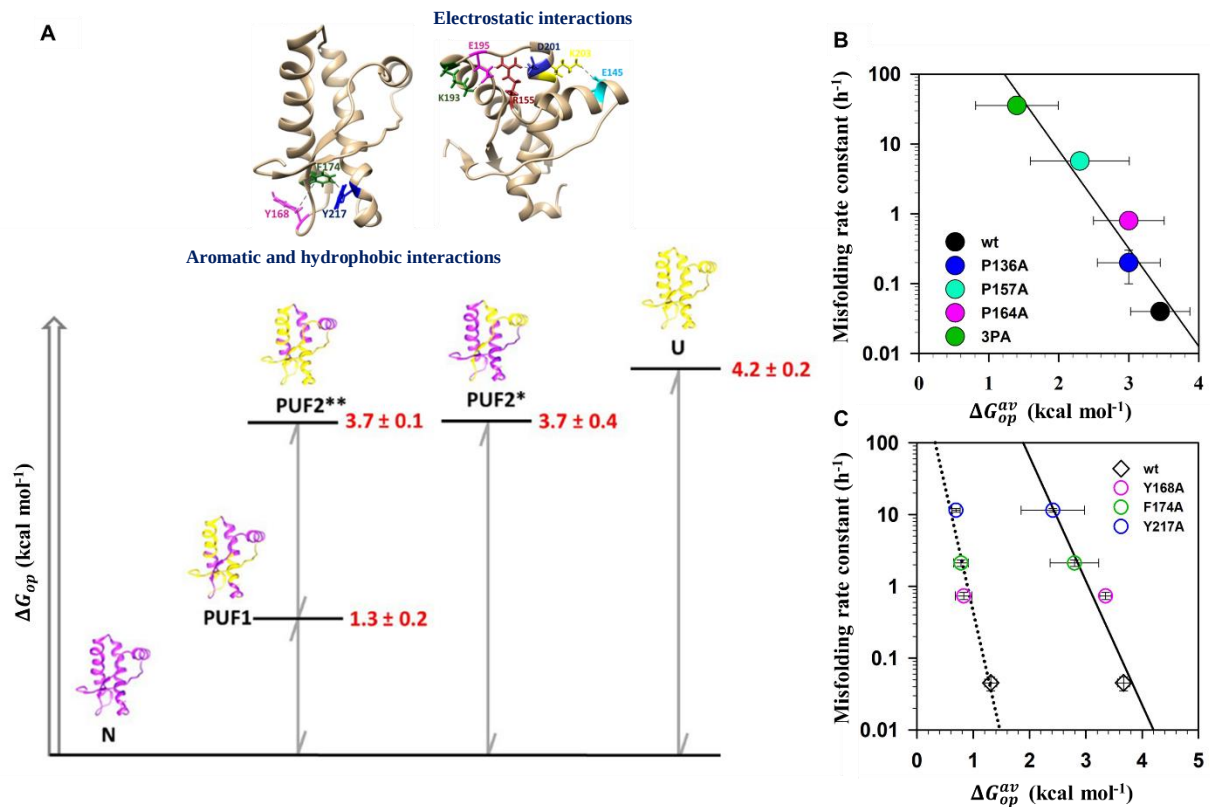


Figure 1.6. PUFs act as the direct precursors to misfolding resulting in oligomer formation. (A) PUF1 appears to be a homogenous population of molecules, while PUF2 comprises of two isoenergetic sub-populations of molecules, PUF2* and PUF2**, which differ in structure. For each PUF, segments that are intact in structure are shown in purple, while those that have unfolded are shown in gold. The stabilities of the individual PUFs are shown in red. (B) Plot of the dependence of the observed rate constant of misfolding of the proline mutant variants on the stability (ΔG_{op}^{av}) of PUF2* and (C) Plot of the dependence of the observed rate constant of misfolding of the aromatic mutant variants on the stability (ΔG_{op}^{av}) of PUF1 (dotted line) and PUF2** (solid line). The different forms (PUF1, PUF2*, PUF2** and U) in dynamic equilibrium with the N state will be populated in accordance with Boltzmann's Law. The linear relation of the logarithm of the observed misfolding rate constant and the stability of the PUFs suggest that misfolding can potentially commence from any of the three PUFs shown (Reproduced from Pal & Udgaonkar, 2022, 2023).

the mouse prion protein, [S169N, N173T] moPrP, which contains elk/deer-specific residues that result in a rigid $\beta 2-\alpha 2$ loop, the stability of the N state is similar to that of wt moPrP, which has a flexible loop (Pal and Udgaonkar, 2024a). However, the [S169N, N173T] variant misfolds into β -rich oligomers at a rate that is two times slower than that of wt moPrP. The rigid $\beta 2-\alpha 2$ loop stabilizes long-range intramolecular interactions between it and the C-terminal end of $\alpha 3$ (Pal and Udgaonkar, 2024a). This stabilization reduces the structural dynamics at the interface, which in turn increases the free energy gap between the N state and the PUFs, which act as precursors for spontaneous misfolding. As a result, these PUFs are less accessible from the N state, reducing their equilibrium populations, and slowing spontaneous misfolding (Pal and Udgaonkar, 2024a). Additionally, the rigidity of the $\beta 2-\alpha 2$ loop in [S169N, N173T] moPrP, compared to the more flexible loop of wt moPrP, makes the monomer less amenable to adding on to preformed fibrils (Pal and Udgaonkar, 2024a).

The role of diseases-linked mutations in misfolding and oligomerization

Most pathogenic mutations linked to inherited prion diseases are primarily found in the structured domain, particularly within the $\alpha 2-\alpha 3$ region, although some are also present in the intrinsically disordered NTD (Bernardi and Bruni, 2019a). Given that PrP aggregation involves both misfolding and multimerization, assessing the combined effects of these mutations is important. HDX-MS studies have shown that several mutations, including F197S, D177N, D201N, R207H, and Q216R result in unfolding of $\alpha 1$, leading to destabilization of the $\alpha 2-\alpha 3$ subdomain (Singh and Udgaonkar, 2015b). Interestingly, in all cases, the pathogenic mutations were found to affect regions of the protein that were found to be unfolded in the PUFs, suggesting that these mutations promote misfolding by stabilizing the PUFs with respect to the N state (Pal and Udgaonkar, 2022b, 2023). These results suggest that prion variants with pathogenic mutations follow the same mechanism of misfolding as the wt protein, and the mutations merely increase the probability of misfolding by increasing the sampling of the PUFs from the N state. It should be noted that the $\alpha 2-\alpha 3$ subdomain can be destabilized independently while the $\alpha 1$ helix remains stable (Chebaro and Derreumaux, 2009; Pal and Udgaonkar, 2023). NMR and simulation studies have shown that the pathogenic mutations Q225E, Q211P, E210Q, Y217N, and V209I, destabilize the $\alpha 2-\alpha 3$ subdomain, by breaking key connections between the $\beta 2-\alpha 2$ loop and the C-terminal end of $\alpha 3$ (Biljan et al., 2011; Cheng and Dagget, 2014; Ilc et al., 2010; Meli et al., 2011a; Slapšak et al., 2019b).

Unlike mutations in the CTD, which primarily impact the stability, dynamics, and in some cases structural integrity of monomeric PrP, NTD mutations mainly affect protein association and interaction with other co-factors. The conserved palindromic sequence ¹¹¹AGAAAAGA¹²⁰ in the NTD is particularly sensitive (Norstrom and Mastrianni, 2005), with disease-linked mutations A116V and G113V accelerating misfolding and oligomerization by facilitating dimer formation (Sabareesan and Udgaonkar, 2016a). Furthermore, the mutations P101L and P104L increase the glycosaminoglycan (GAG)-binding affinity and interactions with N-terminal-specific antibodies, thereby promoting aggregation (Yin et al., 2007).

1.5.5 Role of the dimer in misfolding and oligomerization

Initial rate-limiting step is dimer formation

The apparent rate constant of monomer loss during oligomer formation by moPrP has a reaction order of 2 indicating that the rate-limiting step of oligomerization is dimerization (Sabareesan and Udgaonkar, 2016a; Sengupta et al., 2017a). A study on the misfolding of human PrP misfolding in the presence of lipids also found a reaction order of 2.0 ± 0.1 (Lührs et al., 2006), emphasizing that dimerization is the rate-limiting step in the oligomerization process. An earlier study of oligomer growth in Syrian hamster PrP reported a reaction order of 3 (Sokolowski et al., 2003), while an AFM-based study on the misfolding of human PrP misfolding identified β -rich oligomers made up of 3–4 monomers as the first stable species (Cho et al., 2011). Prion dimers have been detected in brain samples from humans with inherited prion diseases (Gao et al., 2019) as well as in a murine neuroblastoma cell line expressing hamster PrP (Priola et al., 1995). However, the role played by the dimer in prion disease has so far appeared uncertain, as PrP dimers have also been observed in the brains of non-infected hamsters and mice (Bendheim and Bolton, 1986).

Role of the palindromic sequence in dimer formation

In the case of moPrP, dimer formation appears to be driven by association of the palindromic regions of two monomers (Sabareesan and Udgaonkar, 2016a). The conserved palindromic sequence, ¹¹¹AGAAAAGA¹²⁰, is the most conserved sequence element across mammalian and non-mammalian prion proteins and plays a crucial role in the interaction between PrP^C and PrP^{Sc} (Norstrom and Mastrianni, 2005). Replica exchange molecular dynamics simulations indicate that this segment has a strong propensity to adopt β -hairpin structures (Ning et al., 2015). The pathogenic mutations such as G113V and A116V are known

to accelerate misfolding by enhancing monomer-monomer interactions at the dimer interface (Sabareesan and Udgaonkar, 2016a).

Structure formed during dimer formation

In the case of a mutant variant of the moPrP, which adopts a molten-globule-like conformation, and which spontaneously misfolds into a dimer even in the absence of salt, certain sequence segments gain protective structure in two distinct kinetic phases (Pal and Udgaonkar, 2024b). The 96–144 segment, which includes the conserved palindromic sequence ¹¹¹AGAAAAGA¹²⁰ gains structure in the faster phase, confirming its role in establishing the initial contact between two monomers during dimer formation. Additionally, the sequence segments 159–166 (β 2), 168–173 (β 2- α 2 loop), and 182–196 (C-terminal of α 2) gain structure in both the faster and the slower phases, which appears to contribute to stabilization of the monomer–monomer interface, marking the transition toward stable dimer formation (Pal and Udgaonkar, 2024b). As dimerization progresses, several regions undergo further structural rearrangements. Notably, the sequence segments 197–204 (α 2- α 3 loop) and 210–216 (C-terminal of α 3) undergo conformational conversion only in the slower kinetic phase, adopting β -rich structure (Pal and Udgaonkar, 2024b). Interestingly, the segments undergoing this conformational change are the same regions that were previously identified as unfolded in the PUFs. These findings suggest that the formation of the PUFs is critical for the formation of stable dimers, as the loss of native structure enables monomer–monomer interactions and subsequent dimerization.

Oligomer appears to form by assembly of dimers

The fact that the dimers transform very rapidly into oligomers upon the addition of salt suggests that the oligomers are formed through the assembly of dimers (Pal and Udgaonkar, 2024b). The structure of the dimer closely resembles that of the oligomers except for sequence segment 205-212, in the middle of α 3 which appears to be unfolded in the dimer, but which becomes structured in the oligomer, suggesting that this region may be involved in dimer-dimer interactions within oligomers (Pal and Udgaonkar, 2024b). It has been reported that prion assemblies extracted from transgenic mouse brains expressing prion proteins from various mammalian species, naturally dissociate into dimers, aligning with the suggestion that misfolded oligomers form through dimer assembly (Bohl et al., 2023).

1.5.6 Sequence of conformational conversion events during oligomer formation

The molecular mechanism underlying prion protein misfolding at low pH in the presence of salt involves a series of structural changes that destabilize the native fold and promote oligomer formation (Figure 1.7). At acidic pH, structural fluctuations render both ends of $\alpha 2$ prone to unfolding, as evidenced by computational (Dima and Thirumalai, 2004), temperature-dependent NMR (Bhate et al., 2021a), and PET-FCS studies (Goluguri et al., 2019b). While low pH induces instability, the addition of salt is essential for triggering misfolding and oligomerization by perturbing the K193–E195 salt bridge (Sengupta et al., 2017a), destabilizing $\alpha 1$, and enhancing fluctuations at the N-terminal end of $\alpha 2$ and the C-terminal end of $\alpha 3$ (Goluguri et al., 2019b). This loss of stabilizing interactions leads to the dissociation of the $\alpha 2$ – $\alpha 3$ subdomain from the $\beta 1$ – $\alpha 1$ – $\beta 2$ subdomain (Sengupta and Udgaonkar, 2019a), exposing hydrophobic surfaces that lead to further destabilization and the formation of β -rich dimers. The conserved palindromic sequence $^{111}\text{AGAAAAGA}^{120}$ in the NTD plays a critical role in promoting dimer formation. The α -to- β structural conversion primarily involves segments from $\alpha 2$ and $\alpha 3$. Only segment 205–212, remains unfolded in the dimer and adopts a structured conformation only when oligomers form when dimers interact through this segment (Pal and Udgaonkar, 2024b). Accretion of the dimers leads to the formation of stable, β -rich oligomers. Pathogenic mutations in CTD can accelerate misfolding by destabilizing the monomeric protein, either by disrupting interactions between $\alpha 1$ and the $\alpha 2$ – $\alpha 3$ loop (Singh and Udgaonkar, 2015b) or between the $\beta 2$ – $\alpha 2$ loop and the C-terminal end of $\alpha 3$, (Meli et al., 2011b) enhancing destabilization and aggregation.

1.5.7 Role of PUFs in fibril formation

The stabilization of the C-terminal end of $\alpha 2$ (182-196) in moPrP enhances overall protein stability and prevents misfolding and oligomer formation (Singh et al., 2014a). This increased stability raises the energy barrier between the N state and PUF1, thereby decreasing the equilibrium population of PUF1. Mutant proteins with a stabilized $\alpha 2$ formed long, straight amyloid fibrils at pH 7 similar to wt moPrP, but with extended lag phases (Singh et al., 2014a), implying that PUF1 may be involved in fibril formation. Additionally, in the case of [S169N, N173T] moPrP variant, it was found that the monomer is less likely to add on to preformed fibrils in comparison to wt moPrP. The mutations lead to the transformation of the flexible $\beta 2$ – $\alpha 2$ loop into a rigid structure, which increases the free energy barriers and reduces the populations of aggregation-prone PUF1 and PUF2* (Pal and Udgaonkar, 2024a). These results suggest that the PUFs are not only precursors to oligomerization but also to amyloid fibril

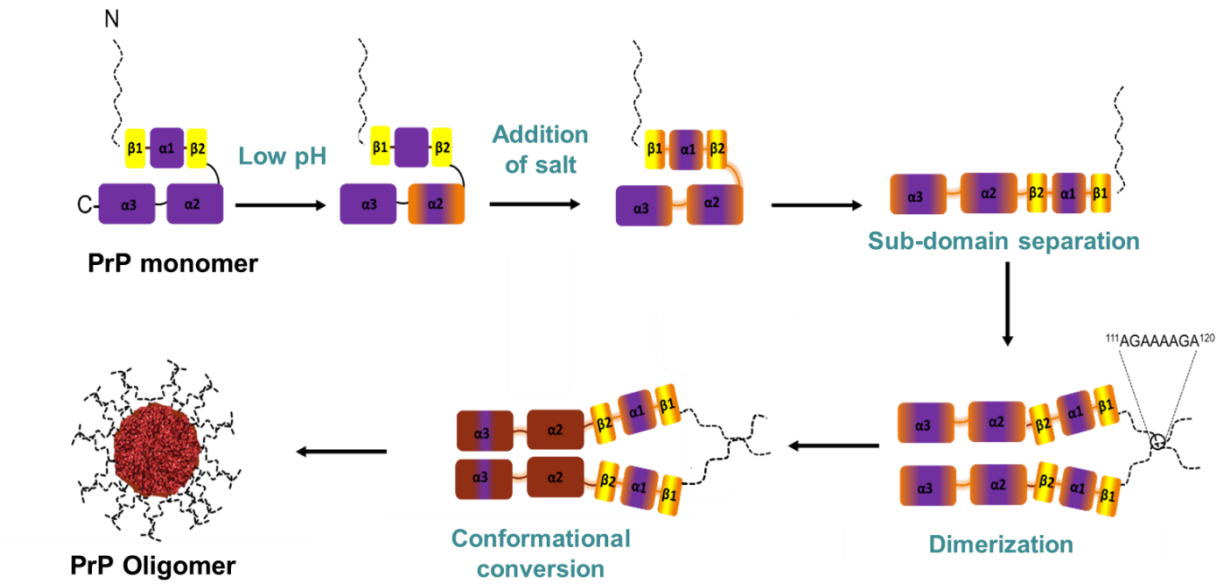


Figure 1.7. Molecular mechanism of spontaneous misfolding of PrP leading to oligomer formation at low pH in the presence of salt. Monomeric PrP consists of an unstructured NTD [represented by a dashed line] and a structured CTD, which includes three α -helices ($\alpha1$, $\alpha2$, and $\alpha3$) [shown in purple] and two short antiparallel β -strands ($\beta1$ and $\beta2$) [shown in yellow]. At low pH, structural fluctuations, particularly at both ends of $\alpha2$ [indicated by a color change to orange], increase the propensity for PrP misfolding. The addition of salt further destabilizes $\alpha1$ and induces fluctuations in the $\beta2$ - $\alpha2$ loop, disrupting interactions between the $\beta1$ - $\alpha1$ - $\beta2$ and $\alpha2$ - $\alpha3$ subdomains. This disruption exposes hydrophobic surfaces of $\alpha2$ - $\alpha3$, further destabilizing the protein and promoting the formation of β -rich dimers. The α to β conformational conversion majorly involves the $\alpha2$ and $\alpha3$ regions [shown in brown]. Additionally, the conserved palindromic sequence $^{111}\text{AGAAAAGA}^{120}$ in the NTD plays a critical role in dimer formation, facilitating the formation of stable β -rich oligomers through dimer accretion.

formation. In 3A moPrP, which adopts a MG-like conformation, the energy gap between the MG state and PUFs is significantly reduced, leading to increased equilibrium populations of PUFs (Pal and Udgaonkar, 2024b). The 3A moPrP mutant variants exhibited a lag phase for fibril formation nearly three times shorter than that of wt moPrP (Pal and Udgaonkar, 2024b), further supporting the role of PUFs as key precursors in fibril formation.

1.5.8 Structural landscape of PrP aggregates: insights from HDX and Cryo-EM

Misfolded PrP forms various types of aggregates, including oligomers (Sang et al., 2019; Singh et al., 2012a), worm-like fibrils (Jain and Udgaonkar, 2011a), and straight fibrils (Baskakov and Bocharova, 2005; Singh and Udgaonkar, 2013a; Yamaguchi and Kuwata, 2018). All three aggregate forms exhibit β -sheet structures, some of which confer protection against HDX. Previous HDX studies revealed that the β -rich core region of the aggregates is most protected against HDX. In worm-like (Singh et al., 2012a) and straight fibrils (Singh and Udgaonkar, 2013a) the β -rich core extends from residues 160 to 225. In contrast, the oligomeric core, spanning residues 168 to 212 in wt moPrP (Pal and Udgaonkar, 2022b; Singh and Udgaonkar, 2015b), shows weaker protection against HDX compared to fibrillar forms. Recent advancements in cryogenic electron microscopy (cryo-EM) have enabled near-atomic resolution imaging of highly pathogenic PrP fibrils, revealing the structural diversity of disease linked prion protein aggregates (Hallinan et al., 2022a; Kraus et al., 2021a; Manka et al., 2022a; Wang et al., 2020a). For instance, straight fibrils formed *in vitro* from huPrP exhibit a β -rich core extending from residues 169 to 224 (Wang et al., 2020a). On the other hand, in the case of PrP fibrils isolated from diseased brains, the β -rich core spans residues 94–225 in mice (Manka et al., 2022a), 94–226 in hamsters (Kraus et al., 2021a), and 79–140 in humans (Hallinan et al., 2022a). These cores consist of 15, 11, and 8 β -strands, respectively. Notably, HDX studies on prion fibrils isolated from diseased brains have shown that not all β -strands identified in cryo-EM structures are equally protected against HDX (Smirnovas et al., 2011a), suggesting structural heterogeneity. Unfortunately, detailed structural characterization of PrP oligomers remains elusive, leaving open questions about the similarity between the β -rich structures in oligomers and brain-derived aggregates. Additionally, over 30 distinct types of PrP^{Sc} has been reported so far in mammals, including various strains and sequences (Rossi et al., 2019). Thus, further research is essential to fully understand their structural diversity and how those structures underpin strain-specific disease phenotypes.

1.5.9 Therapeutic implications of the molecular mechanism of PrP oligomer formation

The lack of effective treatments for prion diseases, coupled with the difficulty of early diagnosis and the rapid progression of these diseases (with an average time from onset to death of just six months to a year), underscores the urgent need for further research into potential therapeutics. This includes identifying new therapeutic targets and strategies, as well as improving screening methods for prion diseases. A major challenge in developing drugs against prion diseases is the complex misfolding process of PrP. This misfolding process involves the formation of multiple metastable misfolding-prone intermediates, which facilitate misfolding into toxic β -rich oligomers. Characterization of these intermediates is challenging due to their transient and heterogeneous nature. High-resolution structures of misfolded oligomers is still lacking, and an understanding of the molecular mechanisms behind oligomer formation is key to suggesting potential targets on PrP for therapeutic intervention.

The naturally occurring G126V and E218K mutations in huPrP are considered protective against kuru and sporadic Creutzfeldt-Jakob disease (sCJD), respectively (Asante et al., 2015; Hizume et al., 2009). The protective effect of the E219K mutation is attributed to subtle structural rearrangements, particularly in the β 2- α 2 loop, which enhances its interactions with residues at the C-terminal end of the α 3 through long-range contacts (Biljan et al., 2012a). MD simulation studies suggest that the G126V mutation weakens main-chain hydrogen bond interactions, hindering dimer formation and the development of a stable aggregate (Zhou et al., 2016). Quantitative analyses of fibril formation kinetics revealed that the G126V moPrP mutant variant exhibits a higher critical concentration, a longer lag phase, and a slower fibril growth rate compared to wt moPrP (Sabareesan and Udgaonkar, 2017). The protective effect of the G126V mutation has been attributed to the increase in the critical concentration below which fibril formation does not occur. Additionally, since toxic oligomers appear to form by the assembly of dimers, with certain regions playing a critical role in the dimer-dimer interface, blocking dimer formation could be an effective strategy to prevent oligomer formation.

The native monomeric structure of PrP has been well-characterized, but the details of the structures of the high-energy PUFs, which serve as precursors to oligomer formation were lacking initially until now. Recent HDX-MS/NMR studies have provided important structural insights into these PUFs, revealing regions of PrP that become unfolded and participate in initial misfolding events (Moulick et al., 2015c; Pal and Udgaonkar, 2024b). Targeting these regions could prevent the spontaneous misfolding of PrP. In general, any compound, whether a chemical chaperone or an antibody, that binds to the misfolding-prone regions of the native conformation, would reduce the propensity of misfolding.

In the past years, significant efforts have been made on identifying small molecules that reduce PrP misfolding, but with limited success. These include porphyrins (Caughey et al., 1998), Congo red derivatives (Sellarajah et al., 2004), acridine and phenothiazine derivatives (Korth et al., 2001), and polyamines (Korth et al., 2001). While the precise mechanisms by which these compounds inhibit PrP misfolding are still unclear, the majority of them bind to misfolding-prone regions of native PrP that become unfolded in the PUFs. For example, two of the most effective anti-prion drugs GN8 and GJP49 intercalate between the $\alpha 1$ and $\alpha 2$ - $\alpha 3$ loop, stabilizing regions of the N state that become unfolded in PUF2 (Kuwata et al., 2007b). Similarly, stabilization of the $\beta 2$ - $\alpha 2$ loop (Kamatari et al., 2013) as well as the C-terminal end of $\alpha 2$ (Baral et al., 2014) in the N state, both of which become unfolded in PUF1, using small chaperone molecules is shown to prevent PrP^{Sc} production. While PrP^C structures are nearly identical across species, PrP^{Sc} forms multiple different ‘strains’ (Bartz, 2016). A major advantage of using a chemical chaperone is its strain-independent action on PrP^C, which has a common structure across mammals.

Simulation studies have shown that PrP^C is a challenging target for small-molecule drugs, largely due to the absence of clear binding sites. Non-small-molecule technologies, such as oligonucleotide-based therapeutics, could be a promising strategy for inhibiting PrP misfolding (Reidenbach et al., 2020). Indeed, an RNA aptamer with a G-quadruplex structure has been shown to bind tightly to PrP^C and reduce PrP^{Sc} levels in mouse neuronal cells infected with the human TSE agent (Mashima et al., 2013). Interestingly, this RNA aptamer binds to segment 108-119, which is unfolded in PUF1. Such binding would prevent its unfolding.

Another approach being explored to prevent prion misfolding is to modulate the trafficking of PrP^C since misfolding of PrP^C has been shown to occur in the endocytic pathway, where it enters lysosomes having a low internal pH (Borchelt et al., 1992a; Filesi et al., 2007). Compounds such as chlorpromazine are able to redirect PrP^C to cytoplasm by affecting clathrin-mediated endocytosis (Stincardini et al., 2017). However, the concentrations required to induce this relocalization are close to those that cause cytotoxicity, suggesting that these compounds might have an intrinsic tendency to reroute multiple surface proteins, potentially compromising cell viability.

Eliminating or reducing PrP^C expression *in vivo* has emerged as one of the most effective strategies for combating PrP misfolding so far. This approach could block the replication of all prion strains and reduce brain pathology associated with prion infection, as PrP^C is necessary for the formation of PrP^{Sc}. Indeed, removing neuronal PrP^C has been shown

to reverse prion-related brain damage and cognitive decline in PrP^{Sc}-infected mice (Mallucci et al., 2007). Additionally, mice lacking PrP^C in brain cells are able to clear PrP^{Sc} (Safar et al., 2005). Postnatal knockout of the Prnp gene in mice, either by RNA interference (White and Mallucci, 2009) or by using the cre/loxP system (Federoff and Mhyre, 2007) prevents the formation of PrP^{Sc} and reverses existing pathology. Mice without endogenous PrP do not develop prion disease even after prolonged exposure to PrP^{Sc} (Autenried et al., 2004). Furthermore, mice expressing a thermodynamically stabilized mutant form of PrP^C, with reduced misfolding propensity, are more resistant to prion infection (Kong et al., 2013).

1.6 PrP misfolding through the condensate pathway

1.6.1 The impact of LLPS in protein misfolding

Recent studies have provided significant insights into how protein molecules undergo LLPS and form liquid-like condensates, influencing both their functional and pathological roles, including misfolding and aggregation (Forman-Kay et al., 2018). Protein misfolding within/at condensates is influenced by multiple factors such as increased local concentration, altered viscosity, interfacial effects, and changes in protein conformation. Enhanced local concentration within condensates accelerates aggregation by increasing protein interactions (Weber et al., 2019; Visser et al., 2024). On the other hand, the high viscosity of condensates reduces protein diffusivity, sometimes inhibiting aggregation, although intermediate viscosities can stabilize partially folded conformations and enhance aggregation (Wang et al., 2021; Munishkina et al., 2004). Protein concentration may be high at condensate, and which might promote heterogeneous nucleation by inducing conformational changes (Grigolato and Arosio, 2021; Lipiński et al., 2022). Additionally, the crowded environment inside heterotypic condensates can alter protein stability, either stabilizing or promoting aggregation-prone β -structures (Menon and Mondal, 2023; Vagenende et al., 2009). Structural changes, influenced by intermolecular interactions, are key to understanding the role of condensates in protein misfolding and their implications for PMDs (Wen et al., 2021; Linsenmeier et al., 2023).

1.6.2 Formation of PrP condensates under physiological conditions.

PrP forms liquid-like condensates, driven primarily by its sequence features, but also by interactions with metal ions, and nucleic acids (Agarwal and Mukhopadhyay, 2022; Matos et al., 2020). Cu²⁺ ions play a key role in modulating PrP condensate formation through ionic interactions, both *in vitro* and *in vivo* (do Amaral et al., 2023). Prolonged Cu²⁺ exposure generates reactive oxygen species (ROS) through Fenton reactions, promoting PrP β -cleavage

and a liquid-to-solid phase transition, leading to amyloid-like aggregation. Non-oxidizing conditions prevent such aggregate formation (do Amaral et al., 2023).

Macromolecular crowding and pH also influence PrP phase separation. PEG8000 reduces the protein concentration required for LLPS, emphasizing cellular crowding effects, while acidic pH (5.2–6.5) inhibits the propensity to form homotypic condensates (Kostylev et al., 2018). The presence of A β oligomers promotes structural changes in PrP condensates, inducing a disorder-to-order transition and potentially contributing to neurotoxicity by trapping the metabotropic glutamate receptor-5 (Kostylev et al., 2018). G126V/I mutations modulate LLPS and amyloid fibril formation of human PrP. Condensates formed by the mutant proteins are incapable of fusion and revert back to solution over time, highlighting the regulatory role of these mutations in phase behavior (Huang et al., 2020).

Nucleic acids also significantly influence the phase behavior of PrP. DNA aptamers induce partial unfolding and reentrant phase separation in rPrP90–231, with hairpin aptamers preserving the liquid-like state and single-stranded aptamers promoting fibril formation (Matos et al., 2020). RNA interactions, particularly with the Y145Stop fragment, trigger gel-like condensate formation, tuning PrP phase behavior by lowering the saturation concentration of LLPS (Agarwal et al., 2021). The presence of RNA regulates the phase separation of PrP in a concentration-dependent manner, with low levels enhancing and high levels inhibiting it (Agarwal et al., 2021). Additionally, heterotypic phase separation between tau and PrP forms complex condensates that mature into intermixed aggregates, with RNA-inducing multiphasic, immiscible condensates that dissolve at high RNA concentrations (Rai et al., 2023).

1.6.3 Intrinsically disordered NTD drives PrP phase separation

Intrinsically disordered proteins (IDPs) or regions (IDRs) are highly dynamic and lack stable secondary and tertiary structures, enabling them to access a broad conformational space and facilitate numerous interactions (Wright and Dyson, 1999). This flexibility makes IDPs/IDRs highly prone to undergo LLPS (Abyzov et al., 2022; Wright and Dyson, 2015). IDRs often contain multiple post-translational modification (PTM) sites, which regulate phase separation behavior (Owen and Shewmaker, 2019). The NTD of PrP (23–123), an intrinsically disordered region, plays a crucial role in LLPS (Kamps et al., 2021; Polido et al., 2021). The fragments N1 (residues 23–114) and N2 (residues 23–89) undergo phase separation under physiological conditions, while structured C-terminal fragments (C1 and C2) do not (Polido et al., 2021). These results are consistent with other studies that show that the Csat (threshold concentration required to undergo LLPS) of the full-length PrP is much higher than the

truncated NTD, suggesting that the presence of the structured CTD reduces the overall LLPS propensity of full-length PrP (Agarwal et al., 2021).

Aromatic residues play a critical role in LLPS by facilitating interactions such as cation- π and π - π bonds (Das et al., 2020; Saar et al., 2021). Mutations in these residues impair LLPS in proteins like FUS and hnRNPA2 (Kato et al., 2012; Xiang et al., 2015a). The "stickers-and-spacers" model explains how the distribution of aromatic ("stickers") and polar ("spacers") residues in IDRs controls phase behavior (Ruff et al., 2021). The NTD has regularly spaced tryptophan and tyrosine residues acting as stickers and glycine/proline residues as spacers, enabling efficient intermolecular interactions (Saar et al., 2021; Wang et al., 2018). Glycine, comprising one-third of the sequence of the NTD of PrP, enhances condensate fluidity, and its mutation reduces LLPS propensity by increasing Csat (Xu et al., 2024; Wang et al., 2018).

1.6.4 Structured CTD plays an important role in driving PrP phase separation

The structured CTD plays a critical role in regulating LLPS, alongside the unstructured NTD. Mutations in the CTD can significantly impact the LLPS propensity of PrP. For example, substitutions of His177 and Asn159 increase the Csat, thereby inhibiting LLPS in the case of human PrP (Li et al., 2023). A recent study also suggests that the pathogenic mutation E200K, not only regulates the LLPS propensity of moPrP, but it also modulates the maturation of condensates (Liu et al., 2024). Mutations of conserved gatekeeper residues of the CTD, including Pro residues at positions 136, 157, and 164, as well as aromatic residues at positions 168, 174, and 217, drastically decrease the Csat of moPrP (Pal & Udgaonkar, unpublished data).

Additionally, CTD mutations that promote LLPS, might alter the conformation of the CTD in a way that favors phase separation. Interestingly, CD spectroscopy has revealed a loss of helical content in PrP upon LLPS in the presence of nucleic acids, indicating the unfolding of certain structural segments (Matos et al., 2020). In the NMR spectrum of the monomeric protein, Thr residues in $\alpha 2$ and $\alpha 3$ exhibit chemical shifts expected for α -helices (Kostylev et al., 2018). In condensates, however, the C α and C β chemical shifts of these Thr residues have shifted towards those expected in a random coil, suggesting that the unfolding of the C-terminal helices is a key feature of LLPS in the case of PrP (Kostylev et al., 2018). This unfolding would lead to the destabilization of the structured CTD, inducing disorder and a greater propensity for intermolecular interactions that drives phase separation. Hence, it appears that while the unstructured NTD of PrP is essential for phase separation, the structured CTD plays an

important regulatory role by influencing the saturation concentration and undergoing conformational changes in the condensates.

1.6.5 Maturation of condensates: liquid-like to solid-like transitions

Biomolecular condensates are inherently metastable states and exhibit liquid-like properties, such as the ability to coalesce, to deform under shear stress, to adopt spherical shapes, and to show rapid recovery times in fluorescence recovery after photobleaching (FRAP) experiments (Alberti and Dormann, 2019). These properties are attributed to the weak, multivalent attractive interactions between the biomolecules within the condensate. Such weak interactions allow dynamic binding and unbinding, leading to free molecular diffusion within the condensate and enabling the exchange of molecules with the surrounding environment (Garaizar et al., 2022). However, liquid-like condensates undergo many changes after formation. While the protein concentration in the dense phase remains constant for a given condition, the condensates begin to grow. This growth results from Ostwald ripening, as well as from fusion events between droplets.

While condensates were initially associated with liquid-like properties, studies using FRAP recovery (Taylor et al., 2019), coalescence (Garaizar et al., 2022), and microrheology techniques (Luo et al., 2022) have shown that condensates can mature into gel-like or solid-like states over time. Mature condensates exhibit reduced fusion tendencies, longer recovery times (or no recovery) after photobleaching, and slower diffusion, indicating that molecular movement within the condensate becomes significantly restricted. Liquid-like condensates can transition into solid-like states through different mechanisms, such as gelation (Winter and Mours, 1999), glass formation (Berthier and Biroli, 2011) as well as aggregation (Patel et al., 2015). Gelation occurs when physical cross-links between condensate components reach a critical density, forming a connected polymer network that spans the entire system, resulting in a shift from a viscoelastic fluid to a solid (Winter and Mours, 1999). Another mechanism by which a liquid becomes more solid-like is through glass formation, remaining in a non-equilibrium state (Berthier and Biroli, 2011; Roth, 2016). Unlike gels, glassy-systems do not undergo a sudden change in material properties and never reach a true solid-like state. Liquid-like condensates can also transition to a more solid-like state through aggregate formation, as observed in the case of multiple proteins linked to neurodegenerative disorders (Kato et al., 2012; Kostylev et al., 2018; Xiang et al., 2015). Using FRAP, it has been shown that homotypic

full-length moPrP condensates lose their liquid-like nature over the course of 12 h (Pal & Udgaonkar, unpublished data).

While the maturation of condensates is a common phenomenon observed for all phase-separating proteins (Lu and Weitz, 2013), it has been shown that aggregation-prone proteins mature at a much faster rate. When comparing LLPS of aggregation-prone IDRs, it was found that the majority of the condensates mature over the course of 24 h (Lin et al., 2015). Intuitively, hyper-concentrated condensates should facilitate the formation of fibrillar aggregates, which follows the nucleated polymerization mechanism (Babinchak and Surewicz, 2020). Indeed, growing evidence suggests that amyloid aggregates can develop from matured condensates, in the case of aggregation-prone proteins associated with neurodegenerative disorders including TDP-43 (Babinchak et al., 2019), FUS (Patel et al., 2015), tau (Wegmann et al., 2018), α -synuclein (Ray et al., 2020). However recent findings suggest that condensates may also suppress fibril formation (Lipiński et al., 2022) and can act as sinks for soluble protein (Das et al., 2024). This suggests that condensate-mediated fibrillation is a highly complex process, depending on various factors. In the case of full-length PrP, Th-T positive β -rich aggregates appear to emerge from matured condensates formed in the presence of crowding agents (Kostylev et al., 2018) and kosmotropic salt (Tange et al., 2021). However, these aggregates lack the autocatalytic activity observed in the amyloid aggregates formed through the canonical non-LLPS conditions. Interestingly, the Y145Stop mutant variant of PrP, which lacks the structured CTD, can form amyloid fibrils from matured condensates, which show autocatalytic activity as well (Agarwal et al., 2021). Similarly, LLPS of PrP in the presence of nucleic acids (Matos et al., 2020) or metal ions such as Cu^{2+} (do Amaral et al., 2023) or other aggregation-prone proteins, such as tau (Rai et al., 2023) and α -synuclein (Agarwal et al., 2022), has been shown to promote the aggregation of PrP, in the majority of cases, the aggregates were shown to be Th-T positive. However, whether full-length PrP can form amyloid aggregates from homotypic condensates without any crowding agents, nucleic acids, or other proteins is still an open question.

1.7 Multiple misfolding pathways of PrP

The structural diversity of PrP aggregates has important implications for understanding the pathogenesis of prion diseases. Additionally, PrP can form different PrP^{Sc} strains in the same host expressing the same PrP^{C} , with each strain exhibiting distinct structural motifs linked to various pathological outcomes. Similarly, PrP has been shown to utilize different misfolding

pathways that lead to the formation of different kinds of misfolded conformations. The misfolding process is highly context-dependent, with different environmental conditions influencing the type of aggregates that form. For instance, at pH 2 (Jain and Udgaonkar, 2011a) PrP forms small, curly amyloid fibrils, whereas at pH 4 (Rezaei et al., 2005; Singh et al., 2014a), in the presence of physiological salt concentrations, spherical oligomers are the dominant species. At pH 7 and under partially denaturing conditions, straight amyloid fibrils are formed (Baskakov and Bocharova, 2005; Singh and Udgaonkar, 2013a)

While the mechanisms of aggregation, size, and morphology of these misfolded PrP species vary, they all share a common structural feature: the conformational conversion of the structured CTD of the protein. Specifically, $\alpha 2$ and $\alpha 3$ undergo conformational changes and form the β -rich core of the aggregates, which require the prior separation of the $\beta 1$ - $\alpha 1$ - $\beta 2$ sub-domain from the $\alpha 2$ - $\alpha 3$ sub-domain (Figure 1.8). Interestingly, the unstructured NTD of PrP remains unstructured in all of these aggregates.

Earlier studies have focused on the canonical aggregation pathways leading to the formation of different kinds of misfolded aggregates, more recent research has proposed that aggregates can also form through LLPS, where the liquid-like condensates can mature and transition into gel-like or solid-like states. For instance, in the case of the TDP-43, amyloid fibrillar aggregates have been shown to develop from the maturation of LLPS condensates (Babinchak et al., 2019). On the other hand, the formation of non-fibrillar aggregates with amyloid-like characteristics under LLPS conditions has been observed in the case of FUS (Monahan et al., 2017). In the case of full-length PrP, maturation of condensates has been shown to induce structural changes in the unstructured NTD, while the structured CTD appears to remain largely unchanged (Figure 1.8) (Pal & Udgaonkar, unpublished data). This is in sharp contrast to the canonical aggregation pathway, where the CTD undergoes significant conformational changes, particularly at $\alpha 2$ and $\alpha 3$. It is crucial to investigate whether different LLPS conditions, could lead to the formation of structurally distinct PrP aggregates. While recent studies suggest that LLPS may enable alternative misfolding pathways for PrP, much remains to be explored regarding how LLPS influences PrP aggregation and the resulting structural properties of the aggregates.

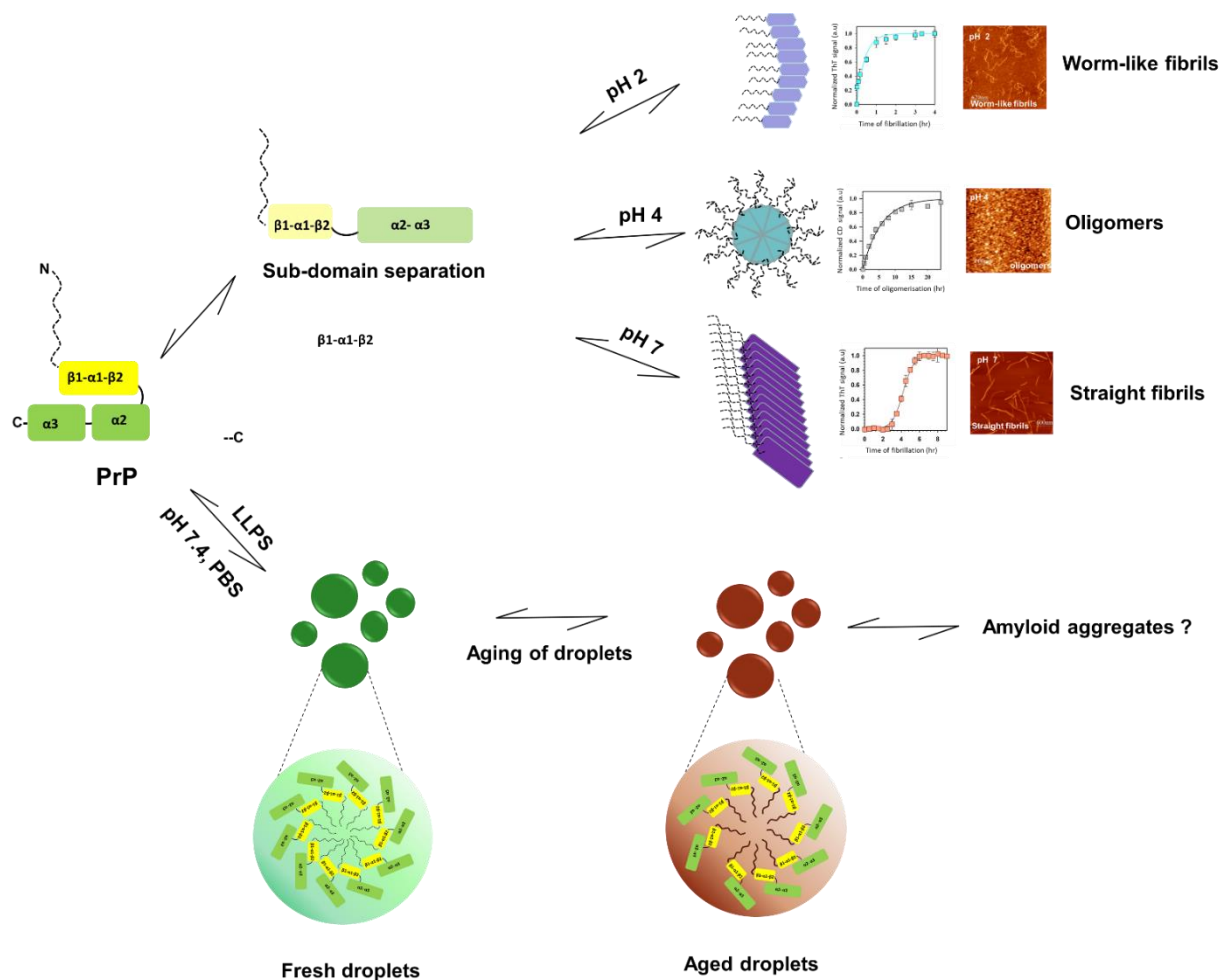


Figure 1.8. Phase transitions of mammalian PrP. The mammalian prion protein can undergo different types of phase transitions. In one type of phase transition, which is highly pH-dependent, the protein forms different kinds of misfolded aggregates: curly amyloid fibrils at pH 2, spherical oligomers at pH 4, and straight fibrils at pH 7. The NTD remains unstructured in all of these aggregates (shown as a dashed black line). In another type of phase transition, the prion protein can undergo liquid-liquid phase separation under physiological conditions, forming condensates that solidify over time. The NTD gains significant structure during maturation (shown as a solid brown line). The mature condensates might lead to the formation of amyloid aggregates.

Chapter 2

Evolutionarily conserved proline residues impede the misfolding of the mouse prion protein by destabilizing an aggregation-competent partially unfolded form

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Introduction

The mammalian prion protein (PrP) is a highly conserved glycoprotein expressed mainly on the neuronal cell surface (Kretzschmar et al., 1986a). The mouse prion protein (moPrP) is 208 residues long, GPI (glycophosphatidylinositol)-anchored, and α helix rich (Riek et al., 2021; Stahl et al., 1987a). It consists of two distinct domains, an unstructured N-terminal domain (NTD) and a structured C-terminal domain (CTD). The CTD consists of three α helices ($\alpha 1$, $\alpha 2$, $\alpha 3$) and two anti-parallel β -strands ($\beta 1$ and $\beta 2$) (Riek et al., 2021). A disulfide bond between C178 and C213 (mouse numbering is used throughout) links $\alpha 2$ and $\alpha 3$ (Ning et al., 2014b; Riek et al., 2021). The physiological function of the prion protein is yet to be determined. Studies done so far suggest that the prion protein may not be an essential protein and might have a redundant function (Castle and Gill, 2017; Richt et al., 2007a; Steele et al., 2007c; Yu et al., 2009). Misfolding of the prion protein has been linked to several fatal neurodegenerative diseases, collectively known as Transmissible Spongiform Encephalopathies (TSE) (Moore et al., 2009b).

The misfolded prion protein can exist in at least two β -sheet-rich forms: an amyloid fibrillar form and a soluble oligomeric form (Thody et al., 2018). *In vitro* studies have shown that the prion protein readily undergoes misfolding and oligomerization at acidic pH, upon either H186 or D201 becoming protonated, with a characteristic transition midpoint at pH 4.7 (Singh and Udgaonkar, 2016e). Hence, while oligomerization is observable at one pH unit above the transition midpoint, it is not observable at physiological pH at which only a miniscule fraction of prion protein molecules would have either of these two residue side-chains in their protonated forms. It is important to note that the propensity of different mammalian prion proteins to form β -sheet-rich oligomers at acidic pH correlates well with susceptibility to prion disease [Khan et al, 2010]. Oligomers are also known to be more infectious than fibrils (Tixador et al., 2010). Hence, understanding the molecular mechanism of prion misfolding leading to oligomer formation is important for the development of therapeutics against prion diseases, where little success has been achieved so far despite extensive efforts.

One approach that can be taken to understand the mechanism of misfolding of the prion protein is to study the role of evolutionarily conserved amino acid residues in controlling the misfolding process. It is known that the sequences of proteins have evolved to safeguard against misfolding and aggregation (Beerten et al., 2012; Reumers et al., 2009a; Richardson and Richardson, 2002a; Rousseau et al., 2006). Evolutionarily conserved residues (Reumers et al., 2009a) at specific sequence positions, which act as “gatekeeper” residues (Beerten et al., 2012)

that impede misfolding, include the charged amino residues Arg, Lys, Asp and Glu (Beerten et al., 2012; Reumers et al., 2009a; Rousseau et al., 2006), as well as Pro (Beerten et al., 2012; Richardson and Richardson, 2002a; Rousseau et al., 2006).

It is well known that a Pro residue may play an important role in protein folding reactions (Levitt, 1981; Osváth and Gruebele, 2003), but Pro also appears to be the most effective gatekeeper residue against misfolding (Richardson and Richardson, 2002a; Rousseau et al., 2006). Since its backbone nitrogen is linked covalently to its side chain, generating a cyclic pyrrolidine ring (Theillet et al., 2013), its presence disrupts secondary structural units, both α -helices and β -strands, by introducing a strong local twist to the peptide bond (Richardson and Richardson, 2002a). Consequently, Pro residues are often found at the ends of α -helices and β -strands, and they disfavor intermolecular β - β interactions when present at the end of the β -strands (Richardson and Richardson, 2002a). A Pro residue also introduces rigidity to the peptide backbone (Richardson and Richardson, 2002a; Theillet et al., 2013), by restricting the main chain dihedral angle, ϕ , of the peptide bond; thus, it can play an important role in the misfolding process, as observed in the case of fibronectin type-III (Steward et al., 2002) and α -synuclein (Meuvis et al., 2010). Notably, the cyclic pyrrolidine ring of Pro often causes the *trans* conformer of the peptide bond to be not as strongly favored as it is by other amino acid residues (Wedemeyer et al., 2002); consequently, a Pro residue can adopt a *cis* conformation more readily than any other amino acid residue (Wedemeyer et al., 2002). *Cis/trans* peptidyl proline isomerization can modulate protein misfolding and aggregation in multiple ways. For example, an increase in the *trans* population of Pro32 in the case of β 2-microglobulin induces the formation of higher-order structures (Maschio et al., 2021). By altering the binding of phosphatase or kinase enzymes, proline isomerization can also indirectly contribute to the propensity to form aggregates (Zhou et al., 2000). Elucidation of the role played by conserved Pro residues in modulating the misfolding of the prion protein, is expected to provide a better molecular understanding of the misfolding process, in which α -helical structure is converted into β -sheet structure.

The sequence of the mammalian prion protein has several conserved Pro residues: residues 50, 59, 67, 75, 83, 101 and 104 in the NTD, as well as 136, 157, and 164 in the CTD. Of the seven Pro residues in the NTD, five are present in the highly conserved five-octapeptide repeat region that spans residues 50-90. This repeat region is thought to be important in prion function (Burns et al., 2002b) and in modulating aggregation (Gielnik et al., 2019; Moore et al., 2006a). Proline residues 101 and 104 are present in the highly conserved central lysine

cluster spanning residues 100-109. Mutation of Pro101 and Pro104 is associated with the most common prion disease, Gerstmann-Straussler-Scheinker disease (Kraus et al., 2015). All three Pro residues in the CTD are highly conserved across different mammalian species; moreover, sequence segment XPNXVY that contains Pro157 has a higher than average sequence conservation (Shamsir and Dalby, 2007). Pro136 is present in the loop between β 1 and α 1, Pro157 is present in the loop between α 1 and β 2 (Figure 2.1a and b), and Pro164 is located on the edge of β 2. β 2 is flanked by Pro157 and Pro164 (Figure 2.1a). It seems that Pro164 may terminate the expansion of the β 2 strand due to conformational constraints imposed on the peptide backbone. Unlike in the case of the conserved Pro residues in the NTD, which have been implicated in misfolding (Bernardi et al., 2017; Gielnik et al., 2019; Kraus et al., 2015; Moore et al., 2006a; Popova et al., 2012), little is understood of the role of highly conserved Pro residues in the CTD of the prion protein.

In the current study, it is shown that the conserved proline residues in the structured CTD of moPrP play an important role in maintaining the integrity of the protein structure. Replacement of the proline residues by alanine destabilizes the native protein, and accelerates the misfolding process by up to 900-fold. Hydrogen deuterium exchange (HDX) studies coupled with mass spectrometry (MS) show that the native (N) state of wt moPrP is in dynamic equilibrium with a partially unfolded form (PUF2*) in which α 1, the loop between α 1 and β 2, β 2 and the N-terminal end of α 3 are disordered. PUF2* is stabilized and therefore becomes more accessible from the N state upon replacement of the Pro residues with Ala. It is also shown that the Pro residues play an important role in determining the boundary of the structural core of the misfolded oligomers, and that the Pro to Ala mutations result in an expanded and more ordered core. The results of the current study suggest that the Pro residues in the CTD have been evolutionarily selected for reducing the probability of misfolding of the prion protein.

Materials and methods

Buffers and Reagents

All the reagents used in this study were of high purity grade, and were purchased from Sigma. Ultra-pure GdnHCl was obtained from United States Biochemicals, and was of the highest purity grade.

Site-directed mutagenesis

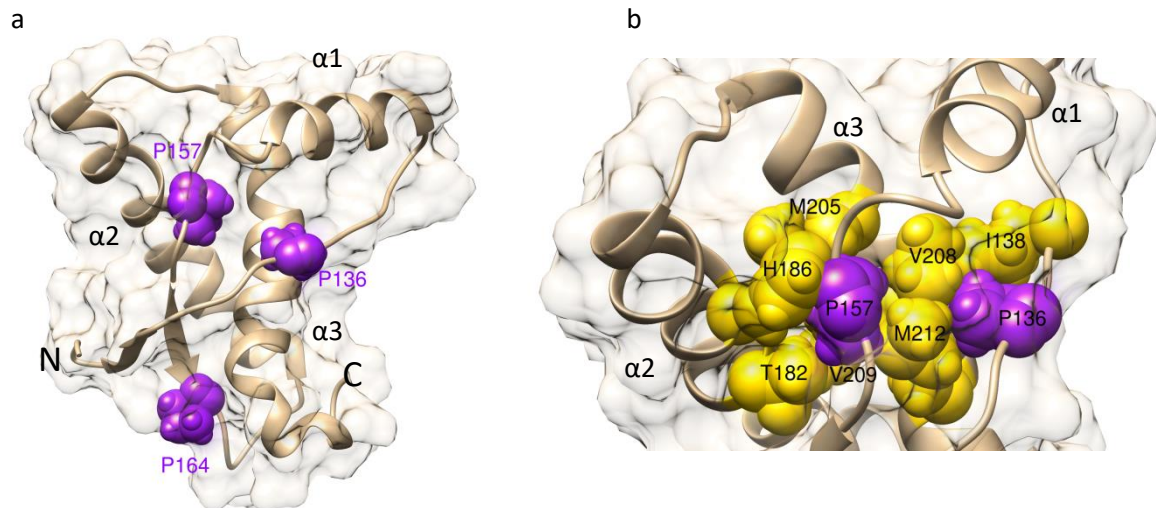


Figure 2.1. Structure of the C-terminal domain of the mouse prion protein showing the locations of the three proline residues (Pro136, Pro157 and Pro164). The N-terminal domain comprises residues 23-120, and is unstructured in the full-length protein. (a) The three proline residues that were mutated in this study are colored purple. All three are in the *trans* conformation. N and C represent the termini of the CTD. (b) The side-chains of Pro136 and Pro157 are buried to the extent of 92% and 97%, respectively, and appear to interact with Ile138, His186, Thr182, Met205, Val208, Val209, and Met212 (yellow). The side-chain of Pro164 is buried to the extent of 60%. The figure was drawn using Chimera and Protein Data Bank entry 1AG2.

The mutant variants of full-length moPrP were generated using the Quickgene site-directed mutagenesis kit (Stratagene). Primers containing 1 or 2 nucleotide changes were obtained from Sigma. Four mutant variants were prepared: P136A, P157A, P164A and 3PA (P136A P157A P164A). The mutations were confirmed by DNA sequencing.

Protein Expression and Purification

Wt moPrP and the mutant variants were expressed in *Escherichia coli* BL21 (DE3) codon plus cells (Stratagene) transformed with a pET17b plasmid containing the full-length sequence (23-231) of the moPrP gene. All the moPrP variants were purified as described previously (Jain and Udgaonkar, 2008; Singh et al., 2014b). The purity of each moPrP variant preparation was confirmed by mass spectrometry using a Synapt G2 HD mass spectrometer (Waters Corporation). Each moPrP variant had its expected mass.

Far-UV CD measurements

Far-UV CD spectra were collected using a Jasco J-815 spectropolarimeter. They were recorded using a protein concentration of 10 μ M, in a 1 mm cuvette, using a scan speed of 50nm/min, a digital integration time of 2 s, and a bandwidth of 1 nm. The wavelength was scanned from 200 to 250 nm, and a total of 15 spectra were averaged. Far-UV CD spectra under native conditions were acquired in 10 mM sodium acetate, pH 4, at 25 $^{\circ}$ C.

Urea induced equilibrium unfolding studies

Urea-induced equilibrium unfolding was carried out at pH 4, at 25 $^{\circ}$ C in 10 mM sodium acetate buffer. For these studies, 10 μ M protein was incubated in different denaturant concentrations for 1 h at 25 $^{\circ}$ C before the far-UV CD signal at 222 nm was monitored. The data were fit to a two-state (N $\longleftrightarrow$ U) equilibrium unfolding model, and the thermodynamic parameters were obtained (Agashe and Udgaonkar, 1995).

Misfolding studies at pH 4

Misfolding was monitored by the measurement of ellipticity at 222 nm (θ_{222}). The protein in 10 mM sodium acetate buffer (pH 4) was diluted two-fold with 2x misfolding buffer (10 mM sodium acetate buffer containing 300 mM NaCl, pH 4) so that it was finally in 1x misfolding buffer (10 mM sodium acetate buffer containing 150 mM NaCl, pH 4). The protein concentration used for most of the experiments was 10 μ M, except for the temperature-dependent kinetic studies of the misfolding of wt moPrP where it was 100 μ M. The samples

were incubated at 37 °C. At different times of misfolding, aliquots of the protein sample were withdrawn for analysis by measurement of θ_{222} .

To study the misfolding kinetics of P157A and 3PA moPrP, a 1 mm quartz cuvette maintained at 37 °C was used. The kinetics were monitored by measurement of θ_{222} . Both the protein sample and 2x misfolding buffer were incubated at 37 °C before starting the reaction. The reaction was started by mixing 20 μ M moPrP solution with an equal volume of 2x misfolding buffer. The time from the mixing of the protein solution with 2x misfolding buffer to the first reading was 20 s. The fraction misfolded form (f_m) was calculated using the equation:

$$f_m = \frac{\theta_t - \theta_0}{\theta_\infty - \theta_0}$$

θ_t and θ_0 are the ellipticities at 222 nm at time t and at time $t = 0$ of the misfolding process, respectively and θ_∞ is the ellipticity at 222 nm of the completely misfolded form.

Dynamic Light Scattering (DLS) measurements

DLS measurements were carried out on a DynaPro-99 instrument (Wyatt Technology Corp.). All the buffers were filtered through 0.02 μ m filters (Whatman). The scattering intensity at 90 °, and its autocorrelation, were acquired simultaneously using a laser at 829.4 nm to illuminate the sample. 50 acquisitions were collected in each experiment. The acquisition time was set at 5 s, the signal-to-noise ratio threshold at 2.5, the temperature at 25 °C, and the sensitivity at 80%. Data with uneven autocorrelation functions were excluded. The data were resolved into a Gaussian distribution using DynaLS (Protein Solutions Ltd.)

Peptide mapping

To generate a peptide map of moPrP, the protein, in 10 mM sodium acetate buffer at pH 4, was subjected to online pepsin digestion in 0.05% formic acid using an immobilized pepsin cartridge (Applied Biosystems) at a flow rate of 40 μ l/min on a nanoAcquity UPLC (Waters). Fragments were collected in a peptide trap column (C18 reversed-phase chromatography column), washed to remove salt, and eluted on an analytical C18 reversed-phase chromatography column using a gradient of 3 to 40% acetonitrile (0.1% formic acid) at a flow rate of 40 μ l/min. The peptides were analyzed using the coupled Synapt G2 HD mass spectrometer (Waters Corporation). Peptide sequencing was done using the MS/tandem MS method, followed by

analysis with the Protein Lynx Global Server software (Waters Corporation), and manual inspection.

HDX-MS measurements

(a) for native monomers

To initiate deuterium labeling, 100 μ M protein was diluted into a labeling buffer [10 mM sodium acetate buffer in D₂O (pH 4), corrected for the isotope effect], so that HDX occurred in 95% D₂O and at 25 °C. At different times, a 50 μ l aliquot was mixed with 50 μ l ice-cold 20 mM glycine-HCl buffer (pH 2.5) to quench the labeling. These samples were then immediately injected into the HDX module (Waters Corporation) coupled to a nanoAcquity UPLC for online pepsin digestion using an immobilized pepsin cartridge (AppliedBiosystems) at a flow rate of 40 μ l/ min of water (0.05% formic acid). The peptides eluting from the pepsin column were collected using the trap column, washed to remove salt, and eluted on an analytical C18 reversed-phase chromatography column with a gradient of 3 to 40% acetonitrile (0.1% formic acid) at a flow rate of 40 μ l/ min (total elution time of 12 min). All columns were kept at 4 °C in the cold chamber of the HDX module to minimize back exchange. The mass spectrometer parameters were as follows: source temperature, 35 °C; desolvation temperature, 100 °C; capillary voltage, 3.0 kV.

Peptide masses were calculated from the centroid of the isotopic envelope using the MassLynx software, and the shift in mass of labeled peptides relative to unlabeled peptides was used to determine the extent of deuterium incorporation at each time point of HDX. Since the sample was in 95% D₂O during labeling, and exposed to H₂O during quenching, control experiments were carried out to correct for back exchange. To this end, the protein was incubated in 10 mM sodium acetate, pH 4 (in 95% D₂O), and deuterated by unfolding at 65 °C for 15 min, followed by refolding on ice. Refolded moPrP was shown to behave like native moPrP, using CD spectroscopy and thermal equilibrium unfolding studies. The fully (95%) deuterated moPrP samples were processed in the same way as the labeling reaction samples. The extent of deuterium incorporation in each peptide fragment, % D was calculated using the equation:

$$\%D = \frac{m_t - m_0}{m_{95} - m_0} \times 100$$

where m_t is the measured centroid mass at time t , m_0 is the measured mass of the undeuterated reference peptide, and m_{95} is the measured mass of a fully deuterated reference

peptide (in 95% D₂O). For calculation of the free energy of opening (ΔG_{op}), the observed HDX rate constant (k_{obs}) for a peptide fragment, which was obtained by fitting the kinetic curve of HDX to either a single exponential or a two-exponential equation depending upon the goodness of fit, was compared to the intrinsic rate constant (k_{int}) expected for the same peptide fragment in a random coil state (Bai et al., 1993a; Nguyen et al., 2018). The HDX protection factor ($P_f = k_{int}/k_{obs}$) for the peptide was used to calculate ΔG_{op} for the sequence segment corresponding to the peptide ($\Delta G_{op} = RT \ln P_f$).

(b) For oligomers

Oligomers prepared by incubation of 10 μ M protein in 150 mM NaCl, 10 mM sodium acetate, pH 4, at 37 °C, (for a time corresponding to 5 time constants of the misfolding reaction) were concentrated to 500 μ M by centrifugation (10000g) using a 10 kDa molecular mass cut-off filter. Deuterium labeling was initiated by diluting 5 μ l of oligomers with 45 μ l of 1x misfolding buffer prepared in D₂O (pH 4, corrected for isotope effect). Labeling was allowed to occur at 25 °C for 300 s or 3000 s. After that, 450 μ l of ice-cold quenching buffer (8 M guanidine hydrochloride (GdnHCl), 100 mM glycine, pH 2.5) were added to stop the labeling reaction and dissolve the oligomers. After 2 min of incubation on ice, the samples were desalted using a Sephadex G-25 HiTrap desalting column pre-equilibrated with water at pH 2.5, in conjunction with an AKTA Basic HPLC. The desalted samples were injected into the HDX module (Waters) coupled with the nanoAcquity UPLC, for online pepsin digestion (see above). Exchange experiments of native moPrP were also carried out in a similar way after 10-fold dilution of the protein in 10 mM sodium acetate into D₂O containing buffer (pH 4, corrected for the isotope effect). To mimic the digestion conditions used for oligomers, 50 μ l of deuterium labeled sample of native moPrP were mixed with 450 μ l of ice-cold quenching buffer containing 8 M guanidine hydrochloride (GdnHCl), 100 mM glycine, pH 2.5, and processed in the same way as were the oligomers.

Peptide masses were calculated from the centroids of the isotopic envelopes, using the MassLynx software, and the shift in the mass of labeled peptides relative to that of the unlabeled peptides was used to determine the extent of deuterium labeling after 300 s and 3000 s of HDX. As the sample was in 90% D₂O during labeling and exposed to H₂O after dissolution in GdnHCl, control experiments were carried out to correct for back exchange. To this end, the protein was incubated in 10 mM sodium acetate, pH 4 (in 90% D₂O), and deuterated by unfolding at 65 °C for 15 min, followed by refolding on ice. The fully deuterated (90%) moPrP

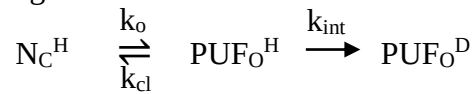
samples were processed in the same way as the labeling reaction samples. The extent of deuterium incorporation in each peptide, % D was calculated using the equation:

$$\%D = \frac{m_t - m_0}{m_{90} - m_0} \times 100$$

where m_t is the measured centroid mass at time t , m_0 is the measured mass of the undeuterated reference peptide, and m_{90} is the measured mass of the fully deuterated reference peptide (in 90% D_2O).

Mechanism of hydrogen exchange (HDX)

The following model is used to describe the HDX data:



The native state N is assumed to be in rapid equilibrium with a partially unfolded form, PUF . PUF is locally unfolded in four sequence segments (144-148, 149-153, 154-167 and 197-204). Hence, the amide sites of these four sequence segments that are protected (closed) in N_C^H , are fully exposed (open) to exchange in PUF_O^H . The equilibrium between N_C^H and PUF_O^H is defined by the equilibrium constant, $K_{op} = k_{op}/k_{cl}$.

Exchange occurs into PUF_O^H with an intrinsic rate constant, k_{int} , which is sequence-dependent (Bai et al., 1993a) and hence different for each of the four sequence segments. Previous mass spectrometry studies had shown that HX into moPrP at pH 4 occurs in the EX2 limit [12]. This was not surprising since the rate constant of refolding of moPrP at pH 4, 25 °C, which would be a lower bound for k_{cl} , is higher than $10,000 \text{ s}^{-1}$ (Moulick et al., 2019), while the average intrinsic rate constant of exchange, k_{int} , is more than 10^5 -fold slower at pH 4, 25 °C. For HX occurring in the EX2 limit, the observed rate constant of exchange, $k_{obs} = K_{op} \cdot k_{int}$. The protection factor, P_f , for each sequence segment is defined as $P_f = k_{int}/k_{obs} = 1/K_{op}$. The free energy of opening (unfolding) of a sequence segment to exchange, ΔG_{op} is given by :

$$\Delta G_{op} = -RT \ln K_{op} = RT \ln P_f$$

ΔG_{op} is therefore the free energy of unfolding of the N state to the partially unfolded form in which the four sequence segments are locally unfolded.

Results

The Pro to Ala mutations do not affect secondary structure but destabilize the native state

All four proline mutant variants (P136A, P157A, P164A and 3PA) showed far-UV CD spectra that are very similar to those of wt moPrP (Figure 2.2a). This suggests that the Pro to Ala mutations did not affect the secondary structure of the protein in any significant manner.

However, urea-induced equilibrium studies indicated that the Pro to Ala mutations led to significant destabilization of the N state of the protein (Figure 2.2b). P157A moPrP showed the highest degree of destabilization among the single proline mutant variants ($\Delta\Delta G_u = 0.6 \text{ kcal mol}^{-1}$), suggesting that it plays an important role in maintaining the stability of the N state of the protein.

The Pro to Ala mutations result in rapid misfolding of moPrP

The prion protein remains in its native α helical monomeric form at pH 4, but becomes prone to misfolding at low pH because of the protonation of a critical residue, H186 (Singh and Udgaonkar, 2016d; Van der Kamp and Daggett, 2010b). Misfolding is triggered by the addition of a physiological concentration (150 mM) of salt. The addition of salt is known to disrupt a stabilizing interaction between K193 and E195 in the monomeric protein (Sengupta et al., 2017b; Van der Kamp and Daggett, 2010b). At pH 4, monomeric wt moPrP has an overall charge of +27, and the addition of salt would also screen the electrostatic repulsion between monomers, facilitating monomer association (Sengupta et al., 2017b). In the absence of salt, misfolding is very slow (10^{-4} h^{-1}) (Sengupta et al., 2017b). Hence, the effects of the Pro to Ala mutations on misfolding were studied at pH 4, 37 °C in 150 mM NaCl. The kinetics of misfolding was monitored by measuring the change in the CD signal at 222 nm. The observed misfolding kinetics of all moPrP variants studied here, appeared monophasic and fit well to a single exponential equation (Figures 3a and b). It should be noted that the misfolding of the prion protein at pH 4 is accompanied by oligomer formation. In the case of wt moPrP, for which the observed misfolding rate constant is relatively slow, the observed rate constant of misfolding matched the observed rate constant of oligomer formation, as probed by size exclusion chromatography (SEC) (data not shown). All four Pro to Ala mutant variants showed much faster misfolding rate constants compared to wt moPrP (Figure 2.3 and Table 2.1), suggesting that Pro residues play important role in impeding the misfolding process. P157A and 3PA moPrP showed the most drastic effects.

Far-UV CD spectra showed that the oligomers formed by the different Pro to Ala mutant variants have a β -sheet content similar to that in the oligomers formed by wt moPrP (Figure 2.4a). The hydrodynamic radii (R_H) of the oligomers formed by the different moPrP variants were also very similar, in the range of 10 to 15 nm, as measured by DLS (Figure 2.4b).

Dependence of the rate constants of misfolding on native state stability

To quantify the relationship between the misfolding propensity and thermodynamic destabilization, the observed misfolding rate constants are plotted against the decrease in free energy of unfolding ($\Delta\Delta G_u$) of the mutant variants with respect to wtmoPrP (Figure 2.3c). The logarithm of the observed misfolding rate constants showed a linear dependence on $\Delta\Delta G_u$ (Figure 2.3c).

Effect of the Pro to Ala mutations on protein dynamics

To understand why the Pro to Ala mutations accelerate the misfolding of moPrP and destabilize the native state of the protein, it was important to determine the effect of mutations not only on the structure but also on the dynamics of the protein. HDX-MS studies were carried out to obtain sequence specific information about changes in the structure and dynamics that occur upon mutation.

In the HDX-MS experiment, the unstructured, solvent-exposed amide sites on the main chain will become labeled by deuterium, whereas the amide sites in the structured buried regions of the protein will become deuterated to a lower extent and more slowly. Since the sequence segments which become deuterated will have a higher mass, they are easily identifiable by MS of the corresponding peptide fragments obtained by carrying out peptic fragmentation after HDX. For this purpose, a peptide map was generated using controlled proteolysis by pepsin as described in Materials and Methods (Figure 2.5). Since the HDX experiments were carried out at pH 4, where the intrinsic rate constant of HDX is slow, structural changes in all the secondary structural elements could be monitored.

Figure 2.5 shows the kinetics of deuterium incorporation into different sequence segments of the CTD of moPrP, for the different mutant variants. In some sequence segments, the kinetics of deuterium incorporation were very similar for the different variants while some sequence segments showed small variations in the kinetics. However, the sequence segments 144-148, 149-153 ($\alpha 1$), 154-167 (the loop between $\alpha 1$ and $\beta 2$ and the $\beta 2$) and 197-204 (the N terminal end of $\alpha 3$) showed pronounced increases in the observed rate constants of deuterium incorporation for the slowed down HDX into the sequence segment, and $\Delta G_{op} = RT \ln P_f$, is the free energy of opening (unfolding) of the local structure of the sequence segment to HDX. ΔG_{op} is therefore the free energy of stability of the local structure. It is the difference in the free

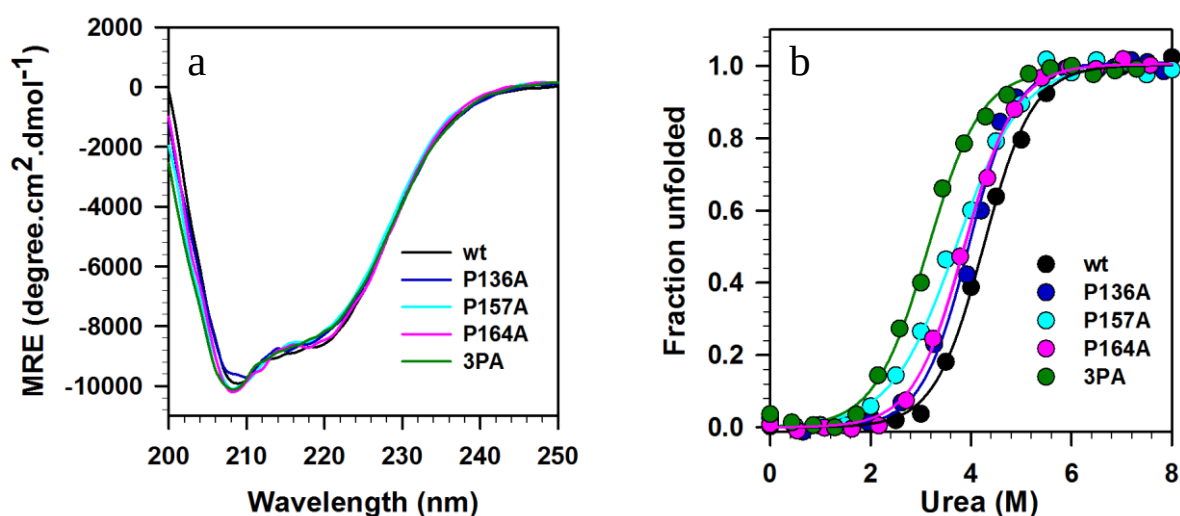


Figure 2.2 Effect of Pro to Ala mutations on secondary structure and stability (a) Far-UV CD spectra of the native monomeric forms of the moPrP variants acquired at pH 4, at 25 °C . (b) Urea-induced equilibrium unfolding transitions of the moPrP variants at pH 4, at 25 °C, as monitored by measurement of the far-UV CD signal at 222 nm. The signal change was normalized to obtain the fraction unfolded. The colors of the lines represent the different moPrP variants as indicated. The solid lines through the data points are fits to an equation describing a two state unfolding transition (Agashe and Udgaonkar, 1995), and the values obtained for the thermodynamic parameters are listed in Table S1. The urea-induced equilibrium unfolding transitions were measured in three independent experiments, and representative data from one experiment is shown for each moPrP variant. The standard deviation in measurement determined from the three independent experiments for each moPrP variant is given in Table 2.1

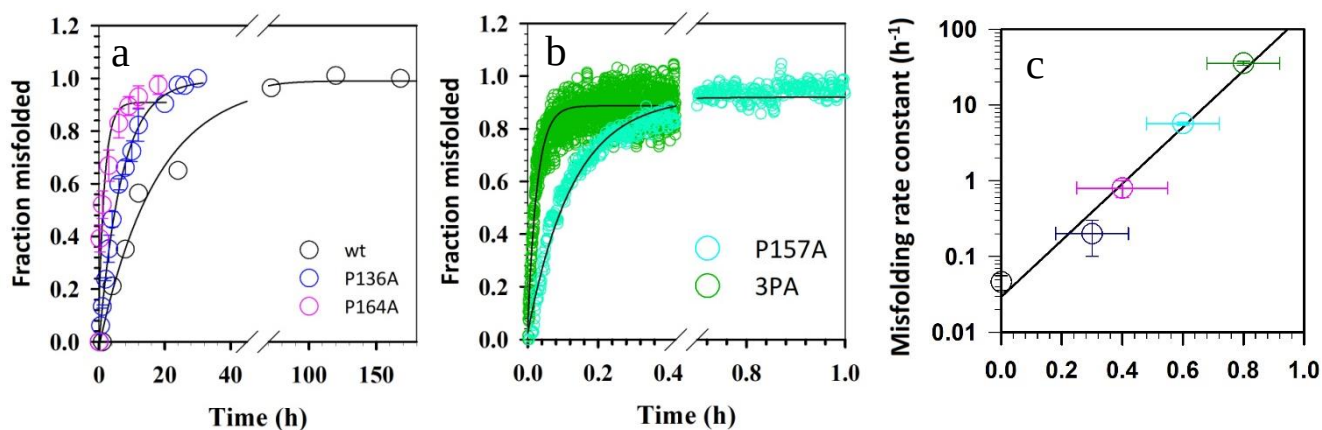


Figure 2.3. Dependence of the observed rate constant of misfolding on native state stability (a) The fraction misfolded form at different times of misfolding of 10 μM wt, P136A and P164A moPrP in 150 mM NaCl at pH 4, at 37 $^{\circ}\text{C}$ is shown. (b) The fraction misfolded form at different times of misfolding of 10 μM P157A and 3PA moPrP in 150 mM NaCl at pH 4, at 37 $^{\circ}\text{C}$ is shown. The fraction misfolded form was calculated from the fractional change in the CD signal at 222 nm. The continuous lines through the data points in both panels a and b are fits to a single exponential equation. (c) Plot of the observed rate constant of misfolding of the different moPrP variants at 10 μM concentration in 150 mM NaCl at pH 4, at 37 $^{\circ}\text{C}$ versus the decrease in free energy of unfolding of the variants ($\Delta\Delta G_u$) with respect to that of wt moPrP. The colors for the different moPrP variants are as indicated. The error bars represent the standard deviations from at least two independent experiments.

Table 2.1. Stabilities and misfolding rate constants of the different moPrP variants

moPrP variant	ΔG_u (kcal mol ⁻¹)	Misfolding rate constant (h ⁻¹)
wt	4.2 ± 0.2	0.04 ± 0.01
P136A	3.9 ± 0.1	0.2 ± 0.1
P157A	3.6 ± 0.1	5.7 ± 0.3
P164A	3.8 ± 0.2	0.8 ± 0.2
3PA	3.4 ± 0.1	35.6 ± 2.0

The free energy of unfolding (ΔG_u), was obtained from urea induced equilibrium studies at pH 4, at 25 °C (Figure 2.2b). The m value was constrained to the same value (1.02 kcal mol⁻¹M⁻¹) for all the moPrP variants. The observed rate constant of misfolding was determined at 10 µM concentration in 150 mM NaCl at pH 4, at 37 °C (Figure 2.3a and b). The standard deviation from the mean of the ΔG_u values was determined from three independent experiments. The standard error of measurement of the misfolding rate constants was determined from two independent experiments.

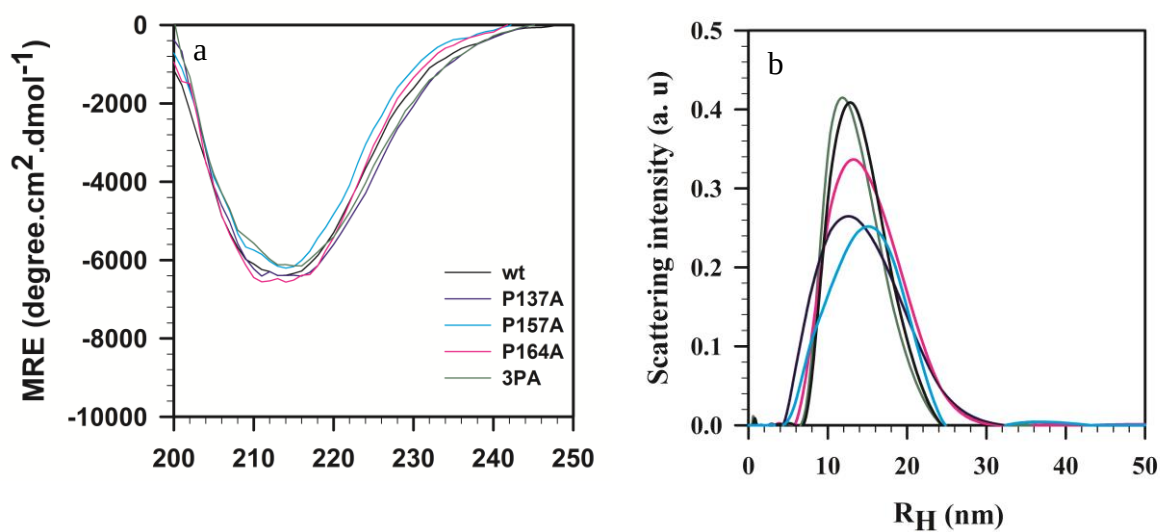


Figure 2.4. Secondary structure and size of moPrP oligomers (a) Far-UV CD spectra. (b) Distribution of hydrodynamic radii of the misfolded oligomers of the different moPrP variants. Misfolding and oligomerization were carried out at 10 μ M concentration in 150 mM NaCl at pH 4, at 37 $^{\circ}$ C. The differently colored lines represent the different moPrP variants as indicated.

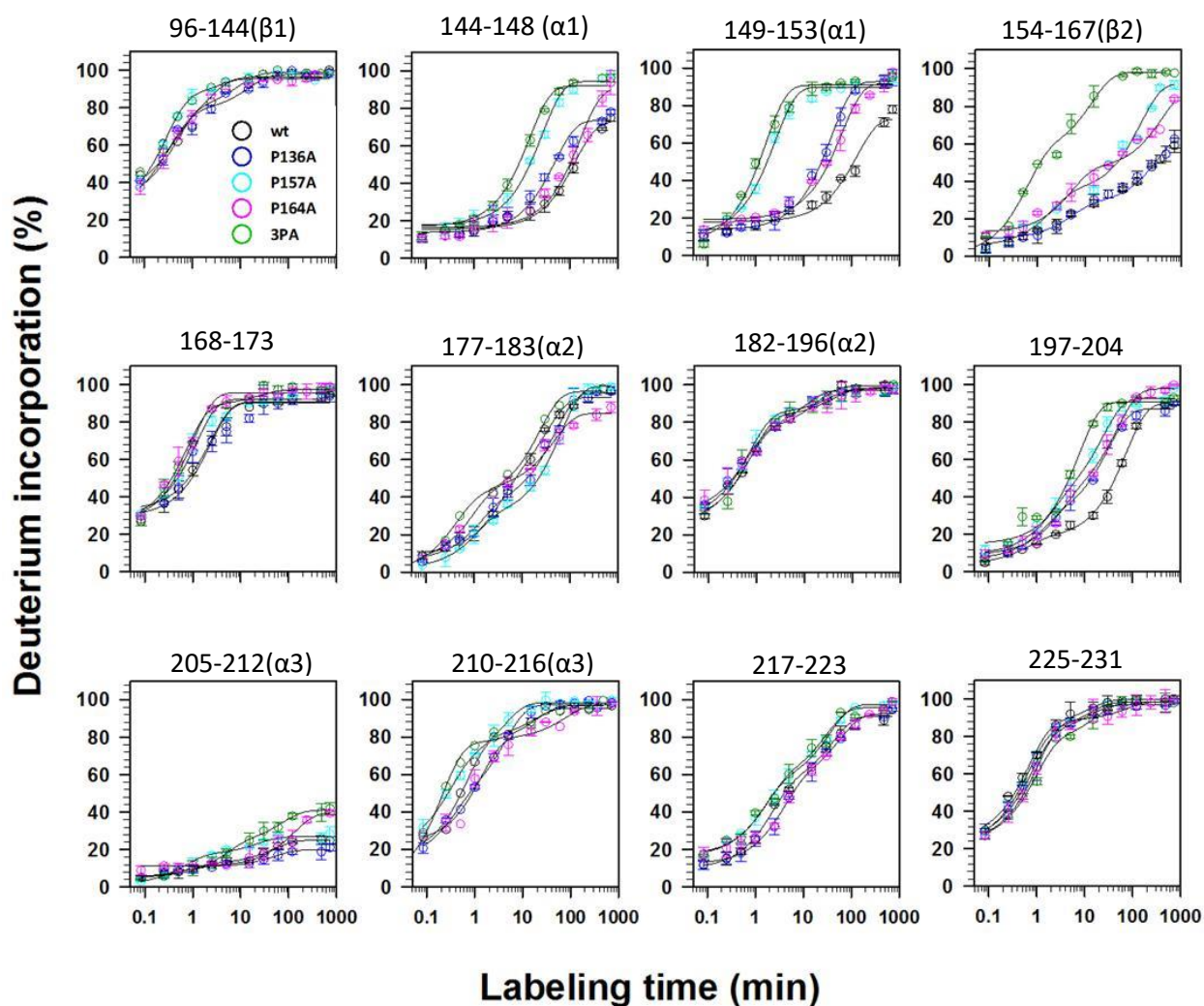


Figure 2.5. Effect of Pro to Ala mutations on the native state dynamics. The time courses of HDX into different sequence segments of the native monomeric moPrP variants at pH 4, at 25 °C. The differently colored circles represent the different moPrP variants, as indicated. The error bars represent the standard deviations determined from two independent experiments. The solid lines through the data represent fits to either monoexponential or biexponential equations.

Table 2.2. Local stabilities (ΔG_{op}) of sequence segments encompassing PUF2* for the moPrP variants:

Sequence segment	ΔG_{op} (kcal mol ⁻¹)				
	wt	P136A	P157A	P164A	3PA
144-148	3.6	3.1	2.6	3.6	2.4
149-153	3.6	2.9	1.3	3.2	1.1
154-167	3.3	3.2	2.9	2.9	1.2
197-204	2.7	2.2	2.0	2.4	1.6
ΔG_{op}^{av}	3.4 ± 0.4	3.0 ± 0.4	2.3 ± 0.7	3.0 ± 0.5	1.4 ± 0.6

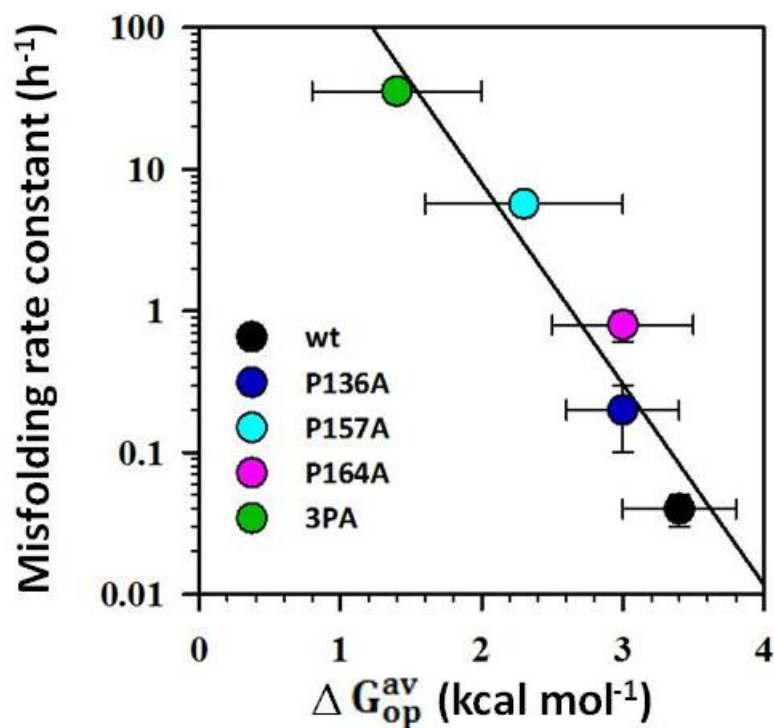


Figure 2.6. Dependence of the observed rate constant of misfolding of the different moPrP variants on ΔG_{op}^{av} . Misfolding of the different moPrP variants at 10 μ M concentration was carried out in 150 mM NaCl at pH 4, at 37 °C. ΔG_{op}^{av} is the average of the ΔG_{op} values obtained for the four sequence segments 144-148, 149-153, 154-167 and 197-204 for each of the moPrP variants (Table S4). The colors of the symbols for the different moPrP variants are as indicated. The error bars are the standard deviations determined from two independent experiments.

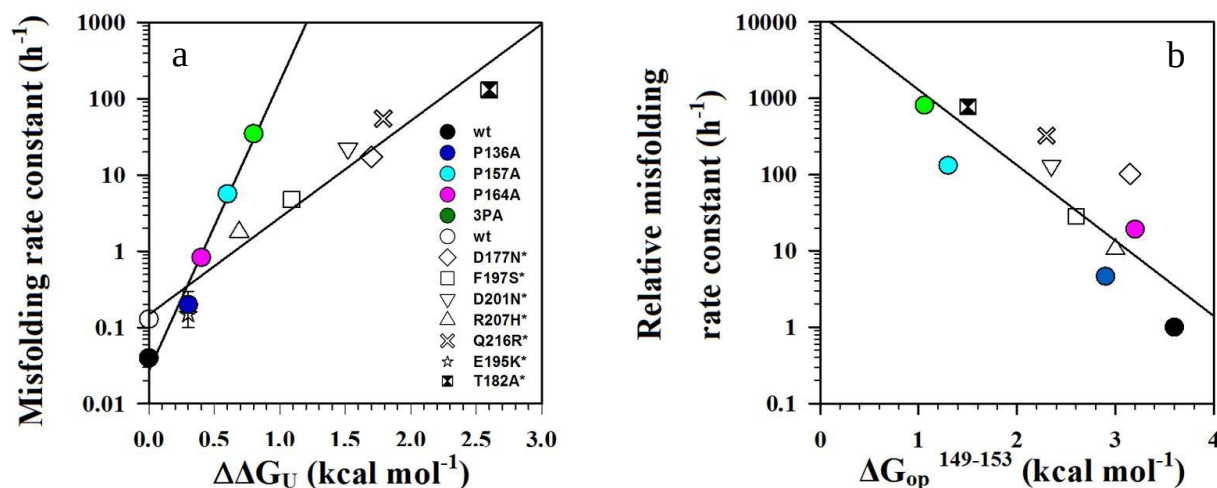


Figure 2.7. Proline mutations versus disease-linked mutations. (a) Plot of the observed rate constant of misfolding of different moPrP variants in 150 mM NaCl at pH 4, at 37 °C *versus* the decrease in global stability of the mutant variants ($\Delta\Delta G_U$) with respect to wt moPrP. The misfolding of the Pro to Ala mutant variants was studied at 10 μM concentration (filled symbols), and that of the disease-linked mutant variants was studied at 100 μM concentration (empty symbols). The continuous lines through the data points are linear fits to the data points. (b) Plot of the relative observed rate constant of misfolding of the different moPrP variants with respect to that of wt moPrP in 150 mM NaCl at pH 4, at 37 °C *versus* ΔG_{op} values for the 149-153 segment calculated from HDX labeling rates. The error bars represent the standard deviations from two independent experiments. Asterisks denote disease-linked mutant variants, for which the data was taken from references (Sengupta et al., 2017b; Singh and Udgaonkar, 2015c; Singh and Udgaonkar, 2016c).

energy of the form with the local structure unfolded and the free energy of the native state with the local structure folded. Table 2.2 show the effects of the Pro to Ala mutations on the ΔG_{op} values of the sequence segments 144-148, 149-153, 154-167 and 197-204. It shows that the mutations primarily decrease the values of ΔG_{op} , thereby increasing the sampling of the partially unfolded form (PUF) in which the four segments are unfolded. It should be noted that ΔG_{op} is the difference in free energies of the N state and the partially unfolded form (PUF) into which HDX occurs.

Dependence of the rate constants of misfolding on local segment stability

The Pro to Ala mutations maximally perturb the rate constants of HDX into four sequence segments: 144-148 and 149-153 ($\alpha 1$), 154-167 (the loop between $\alpha 1$ and $\beta 2$, and $\beta 2$) and 197-204 (the N terminal end of $\alpha 3$) (Figure 2.5). Hence the local segmental stabilities of these four sequence segments are decreased (Figure 2.6). For each of the moPrP variants, the ΔG_{op} values for the four sequence segments were averaged to yield ΔG_{op}^{av} . Figure 2.6 shows that when the logarithm of the observed rate constants of misfolding is plotted against the ΔG_{op}^{av} values, the linear dependence was still observed. This indicated that each mutation similarly perturbs the stability of the four sequence segments in the PUF into which HDX occurs.

Local and not the global stability dictates the misfolding rate constant

Several disease-linked mutant variants are known to misfold rapidly under similar conditions, and for them too, a linear dependence of the logarithm of the observed misfolding rate constants on the decrease in the free energy of unfolding ($\Delta \Delta G_u$) had been observed (Figure 2.7a).

Interestingly, the logarithm of the observed rate constant of misfolding of the disease-linked mutant variants also correlates linearly and well with the local stability of sequence segment 149-153 (Figure 2.7b). The change in the local stability of sequence segment 149-153 is the more appropriate probe of the misfolding propensity, as the observed rate constant of misfolding has the same dependence on ΔG_{op} for both the Pro to Ala mutant variants and the disease-linked mutant variants.

The core of the oligomers formed by 3PA moPrP is more extended than that formed by wt moPrP

Figure 2.8 compares the extent of deuterium incorporation into the different sequence segments of oligomers formed by the moPrP variants, when the oligomers were subjected to

300 s and 3000 s labeling pulses. Also shown in Figure 2.8, for comparison, is the extent of deuterium incorporation into the different sequence segments of the monomers of the all different moPrP variants. The core of oligomers was defined as comprising those sequence segments that had a protection factor, P_f , of at least 10. Hence, all sequence segments that had less than 40% deuterium incorporation, when subjected to the 300 s labelling pulse (at pH 4, 25 °C), were assumed to be in the core of the oligomers. In oligomers formed by wt moPrP, it was seen that the sequence stretch 182-212 had less than 40% deuterium incorporation ($P_f > 10$), while in oligomers formed by 3PA moPrP, the sequence stretch 149-212 had less than 40% deuterium incorporation ($P_f > 10$), when they were subjected to the 300 s labeling pulse. By this criterion, it appeared that the core of the oligomers had expanded from residues 182-212 in wt moPrP, to residues 149-212 in 3PA moPrP.

Effect of the Pro to Ala mutations on the energy barrier of misfolding

Figure 2.9a shows the CD-monitored kinetics of misfolding of wt moPrP at different temperatures. At each temperature the kinetic curves fit well to a single-exponential equation. The observed misfolding rate constants increased with an increase in temperature (Figure 2.9a). Similar behavior was also observed for the 3PA mutant variant (Figure 2.9b). The logarithm of the observed rate constant of misfolding was plotted against $1/T$; the Arrhenius plot (Figure 2.9c) indicated that the energy barrier misfolding slowing down misfolding was 19 kcal mol⁻¹ for wt moPrP. Similarly large energy barriers have been observed to slow down the misfolding of other proteins, including α -synuclein (Uversky et al., 2001b), amyloid- β (Kusumoto et al., 1998), human insulin (Manno et al., 2007) and barstar (Kumar et al., 2007). The energy barrier observed for the misfolding of wt moPrP was also very similar to that observed for the proline isomerization reaction (Theillet et al., 2013). All three Pro residues in the structured CTD are known to be in the *trans* conformation in the moPrP monomer (Riek et al., 2021), and it should be noted that the NMR spectra at pH 4 of the monomer were found to be indistinguishable from those reported in the earlier study. It is possible therefore that the misfolding of moPrP is accompanied by *trans* to *cis* isomerization of one or more of these Pro residues. When the three Pro residues were however mutated to Ala, the energy barrier of misfolding reduced only to 14 kcal mol⁻¹ in 3PA moPrP. This observation suggests

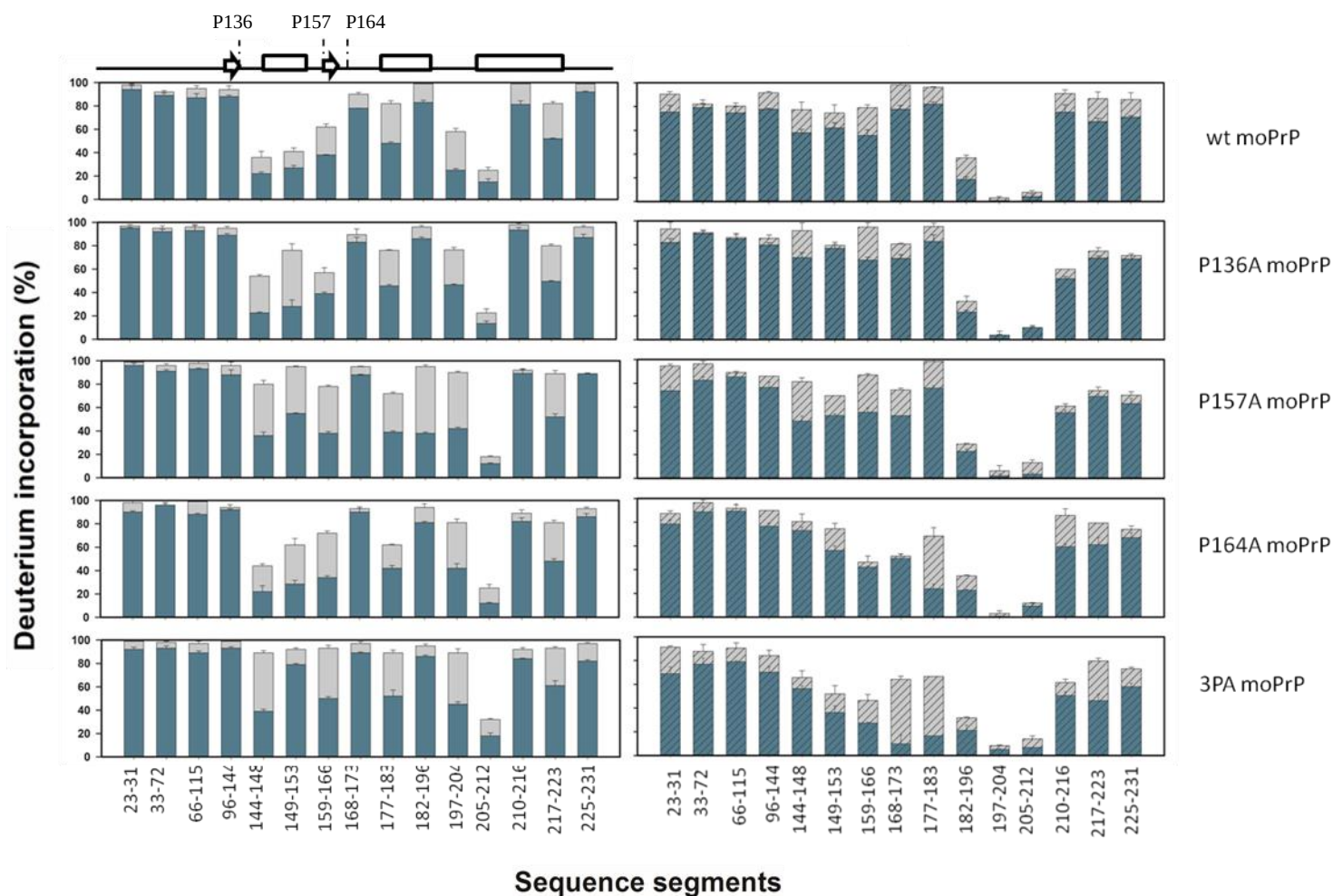


Figure 2.8. HDX-MS characterization of the monomers (left) and oligomers (right) of the different moPrP variants formed by 10 μ M protein in 150 mM NaCl at pH 4, at 37 °C. The histogram bars show the extents of HDX labeling at 300 s (dark cyan) and 3000 s (grey), at pH 4, at 25 °C. The error bars represent the standard deviations from two independent experiments.

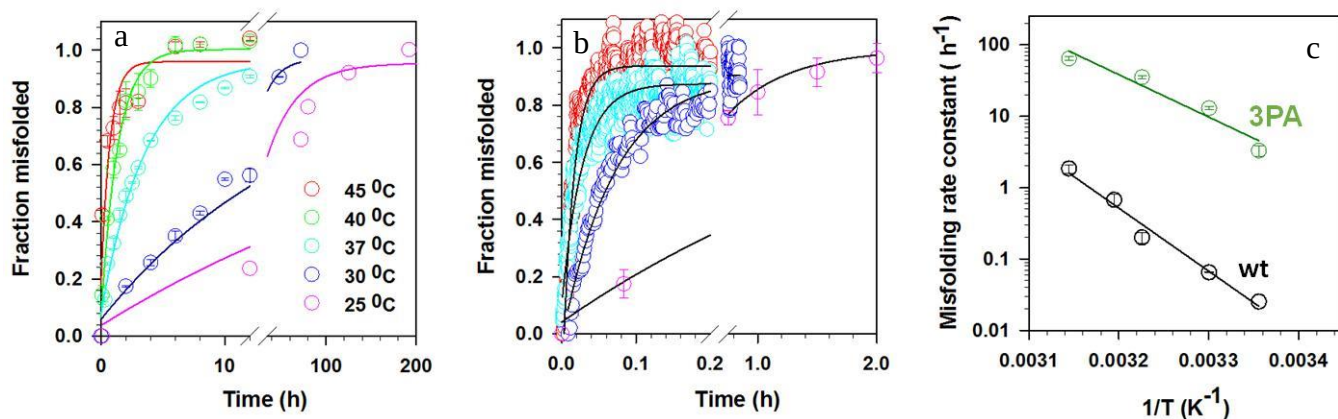


Figure 2.9. Temperature dependence of the kinetics of misfolding of 100 μM wt (a) and 10 μM 3PA (b) moPrP in 150 mM NaCl, at pH 4. Representative plots are shown for each temperature. The continuous lines through the data points are fits to a single-exponential equation. (c) Observed rate constants of misfolding are plotted against 1/T. Linear fits through the data points yield activation energies of 19 kcal/mol and 14 kcal/mol for wt and 3PA moPrP, respectively. The error bars in the panels represent the standard deviations from two independent experiments.

that it is unlikely that any of the Pro residues switch from a *trans* to *cis* conformation during misfolding.

DISCUSSION

The effect of mutating a Pro residue on the stability of a protein is difficult to predict. The effect on the N state may be stabilizing or destabilizing, or neither, depending on the location of the Pro residue in the protein structure, on the nature of the neighboring residues, and on the nature of the residue replacing it (Choi and Mayo, 2006). A Pro residue cannot participate in hydrogen bonding, whereas any residue that replaces it would be able to. If the substituting residue does engage in hydrogen bonding, the mutation would be expected to have a stabilizing effect; if it does not, the unsatisfied hydrogen bonding potential would be expected to have a strongly destabilizing effect on the N state (Rose, 2021). The effect on the U state is expected to be stabilizing, as the conformational entropy of U would be reduced because the pyrrolidine ring of a Pro residue restricts its main chain dihedral angle, ϕ , to $-63 \pm 15^\circ$ (MacArthur and Thornton, 1991) and also restricts the conformation of the preceding residue (MacArthur and Thornton, 1991). Hence, replacing a Pro residue with any other residue would entropically destabilize the protein, that is reduce ΔG_U , the free energy difference between the U and N states.

The Pro to Ala mutations result in the destabilization of moPrP

The observation that the Pro to Ala mutations reduce ΔG_U can be rationalized to be the result of entropic stabilization of the U state, as discussed above. It is instructive, however, to examine whether ΔG_U could have also been reduced because the N state is enthalpically destabilized. The side-chains of Pro136 and Pro157 are buried in the hydrophobic core, and form van der Waal contacts with multiple residues present in $\alpha 2$ and $\alpha 3$ (Figure 2.1b). Substitution of these Pro residues with the smaller Ala residues could perturb the packing in the core and thereby destabilize the protein (Lee et al., 2019a). It would be unlikely that the substituting Ala residues remain buried, as hydrogen bonding partners for their main chain amides would not be available. Since burial of a main chain amide without a hydrogen bonding partner would significantly destabilize the protein (Rose, 2021), it is likely that the substituting Ala residues in the N state have their main chain amides not buried, but solvent-exposed, thereby resulting in a perturbation of core packing and consequent destabilization.

The buried side-chains of Pro157 and Pro164 also appear to confer rigidity to the sequence stretch 144-167, anchoring it in the hydrophobic core. This anchoring may be

important for the positioning of Glu145 and Arg155 in the structure such that they can participate in the salt-bridges that comprise the highly conserved electrostatic network that stabilizes the interaction of the α 1- β 2 sub-domain with α 2- α 3 sub-domain (Figure 2.10). Substitution by Ala might perturb the positioning of Glu145 and Arg155, and thereby the critical electrostatic network, resulting in the destabilization of the N state. It should be noted that the α 1- β 2 sub-domain has very few hydrophobic contacts with the α 2- α 3 sub-domain, as a result of the unusually high hydrophilicity of α 1 (Morrissey and Shakhnovich, 1999a), which has been reported to be one of the most soluble helices found in any protein (Morrissey and Shakhnovich, 1999a).

The Pro to Ala mutations stabilize a HX-competent partially unfolded form

Previous HDX-MS and HDX-NMR measurements had identified two sparsely populated, partially unfolded forms (PUFs) into which HDX occurs in regions that have become unstructured and fully solvent-exposed (Moulick et al., 2015b). β 1, the loop between β 1 and α 1, as well as the N-terminal end of α 3, were found to be such regions in PUF1. α 1, the loop between α 1 and β 2, as well as β 2 were found to be additional such regions in PUF2. It appeared that the α 1- β 2 sub-domain is separated from the α 2- α 3 sub-domain in PUF2. It was shown previously that the free energy of opening, ΔG_{op} , of structure in N to form PUF2, was 3.1 ± 0.5 kcal mol⁻¹ in the case of the wt moPrP (Moulick et al., 2015b). The previous studies could not, however, determine, whether PUF1 and PUF2 were unique conformations, or were ensembles of sub-populations that formed in parallel, and which differed in their conformations.

The current study shows that the Pro to Ala mutations predominantly affect four sequence segments encompassing the α 1- β 2 sub-domain and the N-terminal end of α 3. These regions show increased dynamics and significant changes in ΔG_{op} (Figures 2.6 and, Table 2.4). The same regions were shown to be unstructured and solvent accessible in PUF2 (Moulick et al., 2015c). The Pro to Ala mutations do not, however, have any significant effect on other regions that were also shown to be unstructured in PUF2. In particular, the kinetics of deuterium incorporation into these other regions (Figure 2.5) were affected minimally; consequently, values of ΔG_{op} for these regions were also affected only marginally (Figure 2.6). This result suggests that PUF2 is an ensemble consisting of sub-populations of distinct conformations, each of which has become unstructured in different regions, but which are nevertheless very similar in energy. The sub-population of PUF2 which has become

unstructured at the $\alpha 1$ - $\beta 2$ sub-domain and the N-terminal end of $\alpha 3$, and which is affected by the Pro to Ala mutations, is referred to as PUF2*. The value of ΔG_{op}^{av} for PUF2* in the case of wt protein, determined by averaging over the ΔG_{op} values of the four sequence segments encompassing the $\alpha 1$ - $\beta 2$ sub-domain and the N-terminal end of $\alpha 3$, is found to be 3.4 ± 0.4 kcal mol⁻¹, qualifying PUF2* to be a sub-population of the PUF2 ensemble.

The Pro to Ala mutations result in a significant reduction in the value ΔG_{op}^{av} for PUF2* (Figure 2.6). This reduction cannot be accounted for by the relatively small destabilization of the N state (see above). It can be accounted for only if the mutations significantly increase the stability of PUF2* with respect to the U state. At the present time, it is possible only to speculate how such stabilization might occur in PUF2*. It would appear that the substituting Ala residues have their main chain amides hydrogen bonded in PUF2*, either intra-molecularly with another part of the protein, or inter-molecularly with water (Bajaj et al., 2007; Jain et al., 2002). This non-native hydrogen bonding would not be present in PUF2* formed by the wt protein, because the Pro residues do not have amides that can hydrogen bond. If the hydrogen bonding, and the stabilization afforded by it is absent in the U state, then PUF2* would be stabilized with respect to the U state.

PUF2* appears to be the precursor state from which misfolding commences

In an earlier study, an intermediate, which was populated at equilibrium during the urea-induced unfolding of a mutant variant of the isolated CTD of moPrP, was identified as the precursor conformation from which misfolding initiates (Moulick and Udgaonkar, 2017). This identification was on the basis of the observed rate constant of misfolding being linearly proportional to the population (concentration) of the intermediate. On the basis of similarities in stability and in gross structure, the equilibrium intermediate was inferred to be the same as PUF2 (Moulick et al., 2015b). However, in the case of full length moPrP, such an intermediate has not been identified, and it was not possible to show that PUF2 is the direct precursor to misfolding. In this study, it has been shown that the logarithm of the apparent misfolding rate constant is linearly proportional to ΔG_{op}^{av} for PUF2* (Figure 2.6), the free energy difference between the N state and PUF2*. In other words, the observed misfolding rate constant is directly proportional to the equilibrium population (concentration) of PUF2*. This observation strongly indicates that PUF2*, a sub-population of the PUF2 ensemble, is the direct precursor conformation from which misfolding commences.

The defining structural difference between PUF2* and N appears to be that in the former, the α 1- β 2 sub-domain is separated from the α 2- α 3 sub-domain (Moulick et al., 2015b). It is known that separation of the α 1- β 2 sub-domain from the α 2- α 3 sub-domain is a critical event in the initiation of misfolding (Hadži et al., 2015a; Singh and Udgaonkar, 2015c). Several disease-linked mutations have been shown to facilitate sub-domain separation, increasing the population of PUF2*, and they thereby speed-up misfolding (Singh and Udgaonkar, 2015c). It is interesting to note that two anti-prion drugs that were found to be effective against prion conversion (Kamatari et al., 2013; Kuwata et al., 2007a) bind to the N state between α 1 and the α 2- α 3 loop, thereby presumably stabilizing the N state with respect to PUF2*. It is therefore not surprising that the effect of the Pro to Ala mutations is largest in the two non-contiguous sequence stretches, 144-167 spanning the α 1- β 2 sub-domain, and 197-204 at the N-terminal end of α 3 (Figure 2.10a). These two structural regions are both unfolded and solvent-accessible in PUF2*. In the N state, these two regions are close to each other, and are stabilized by an electrostatic interaction network comprised of salt bridges formed by residues in the two sequence stretches (Figure 2.10b).

Indeed, a distributed network of electrostatic interactions play a critical role in stabilizing the N state of the prion protein and guarding against misfolding. It is only when His186, Asp201 and Asp177 become protonated at pH 4, leading to the perturbation of the electrostatic network, that the α 1- β 2 sub-domain becomes destabilized (Lee and Chang, 2019; Singh and Udgaonkar, 2016d). Consequently, PUF2* becomes populated, albeit to a very sparse extent for wt moPrP, possibly driven by structural fluctuations between α 1 and α 2 (Goluguri et al., 2019b). The electrostatic network is destabilized by the Pro to Ala mutations, which appear to perturb the structure in such a way so as to affect the positioning of charged residues that participate in critical salt-bridges that comprise the stabilizing electrostatic network (see above). The destabilization of the N state results in the reduction in ΔG_{op}^{av} , increases the population of PUF2* relative to N. A much larger reduction in ΔG_{op}^{av} occurs, however, as a consequence of the stabilization of PUF2* relative to the U state (see above).

Misfolding does not commence from the fully unfolded U state

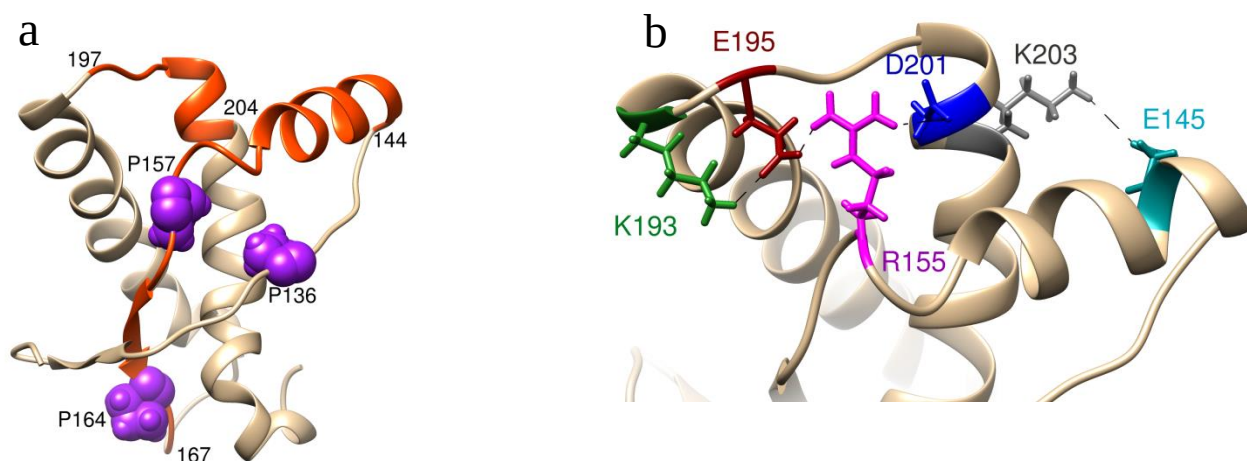


Figure 2.10. Effect of the Pro to Ala mutations on protein dynamics (a) Sequence segments 144-148, 149-153, 154-167 and 197-204 showing increased structural dynamics in native monomeric moPrP, upon Pro to Ala mutations are shown in red. The structural dynamics were determined from the kinetics of HDX into the different sequence segments at pH 4, at 25 °C (Figure 4). The proline residues which were mutated in this study are colored purple. (b) An expanded view of the well-conserved non-local salt bridges formed between Arg155 (purple) and Glu195 (brown), Arg155 and Asp201 (blue), Lys193 (green) and Glu195, Lys203 (grey) and Glu145 (cyan). The distances between the side-chains are shown in Å. The figure was drawn using Chimera and the Protein Data Bank entry 1AG2.

The observation that the logarithm of the observed misfolding rate constant is also linearly proportional to $\Delta\Delta G_U$, for the Pro to Ala mutant variants (Figure 2.7a), could be interpreted to mean that the U state can also act as the precursor conformation from which misfolding initiates. However, if such an interpretation were true, then an identical dependence would be observed for all mutant variants that have been studied previously. The observation that while the logarithm of the apparent misfolding rate constant determined for disease-linked mutant variants is also linearly proportional to $\Delta\Delta G_U$, its dependence is nevertheless very different (Figure 2.7a), suggests that misfolding does not commence from the U state. It should also be noted that some of the disease-linked mutations destabilize the protein significantly more than do the Pro to Ala mutations, but yet cause less acceleration of the misfolding reaction. On the other hand, the observation that the logarithm of the observed misfolding rate constant for the Pro to Ala mutant variants and the disease-linked mutant variants (Figure 2.7b) studied earlier, has the same linear dependence on ΔG_{op} , (determined as the free energy of opening of structure to HDX in the C-terminal part of $\alpha 1$) (Singh and Udgaonkar, 2015c), is expected when PUF2* is the precursor conformation from which misfolding commences. It is clear that the probability of misfolding depends on ΔG_{op} , which dictates the extent to which PUF2* is populated, at equilibrium, and not on $\Delta\Delta G_U$, which dictates the extent to which U is populated in equilibrium with N.

The Pro to Ala mutations lower the energy barrier for oligomerization

While one mechanism by which the Pro to Ala mutations lead to faster misfolding is through their stabilization of the aggregation-competent PUF (PUF2*) from which misfolding commences, another mechanism could be that they lower the free energy barrier that slows down misfolding. This free energy barrier would have an entropic component arising primarily from molecules having to assemble together during the oligomerization reaction that occurs concomitantly with misfolding. There would also be an enthalpic component arising from having to break the interactions that stabilize the structure, so that conformational conversion can occur. In particular, $\alpha 2$ and $\alpha 3$, which convert into β -sheet, are stabilized by electrostatic interactions between them (Lee and Chang, 2019). The protonation of His186 perturbs these interactions but not sufficiently for the two helices to start converting to β -strand, even though the gatekeeper helix $\alpha 1$ has become disordered in PUF2*. For misfolding to commence, the electrostatic interactions have first to be further destabilized. This happens upon the addition of 150 mM NaCl, which screens electrostatic charges and destabilizes N (Goluguri et al.,

2019b). The immediate effect is the disruption of the electrostatic network that includes the salt bridge between Lys193 and Glu195 and electrostatic interactions involving Glu210-Gln211 and Lys219 in $\alpha 3$ (Sengupta et al., 2017b), as well as many other polar residues (Bhate et al., 2021b). Consequently, the stabilizing tertiary electrostatic contacts between $\alpha 2$ and $\alpha 3$ break, and the helices are destabilized. Once helical structure is lost, conformational conversion to β -sheet is initiated (Sengupta and Udgaonkar, 2019b). Conformational conversion is favored, as the sequence segments spanning $\alpha 2$ and $\alpha 3$ have low helical and high β -sheet propensities in the first place (Fooks et al., 2006). It is possible that the Pro to Ala mutations lower the activation energy for misfolding (Figure 8c) and accelerate misfolding by perturbing the structure of PUF2* in such a manner that the stabilization afforded by electrostatic interactions is decreased.

It should be noted that the activation energy for misfolding remains high even after all three Pro residues have been substituted with Ala (Figure 2.9c). Since the activation energy is similar to that seen for peptidyl proline isomerization reactions accompanying protein folding and unfolding reactions (Schmid, 1993), it is possible that the misfolding of moPrP is accompanied by peptidyl proline isomerization. The three Pro residues in the CTD are in the *trans* conformation in native moPrP (Riek et al., 2021). Since it is not known whether they are in the *cis* or *trans* conformation in the misfolded oligomers, it cannot be discerned whether misfolding is accompanied by *trans* to *cis* prolyl isomerization. The observation that the activation energy remains high even after all three Pro residues have been substituted with Ala (Figure 8c), makes it unlikely that *trans* to *cis* peptidyl proline isomerization plays a role in the misfolding of moPrP, as it does for other proteins (Kumar et al., 2007; Kusumoto et al., 1998; Manno et al., 2007; Uversky et al., 2001b). Certainly, *trans* to *cis* peptidyl proline isomerization is too slow ($< 0.01 \text{ s}^{-1}$) for it to occur during the fast unfolding reaction leading to the formation of the PUF from the N state.

PUF2* appears not to be on the direct unfolding pathway from the N to U state

Earlier studies had suggested that intermediates that are obligatory for folding (Honda et al., 2015; Honda et al., 2014b) or unfolding (Moulick et al., 2019) may initiate misfolding of the prion protein. Thus, an important question is whether PUF2* is on the direct pathway of unfolding from N to U, or whether it is a dead end, off-pathway intermediate accessible from N. Earlier studies had been unable to determine whether PUF1 and PUF2 were on- or off-pathway (Moulick et al., 2015b). In the case of cytochrome *c* (Hu et al., 2016a) and apomyoglobin (Hughson et al., 1990b), the PUFs identified in HX-NMR studies were

designated as being on-pathway, as mutations that stabilized the N state also stabilized the PUFs (Bai et al., 1995). In the case of apoflavodoxin, the identified PUFs appeared not to be present on the direct pathway of unfolding (Bollen et al., 2006). In the case of moPrP, the observation that the Pro to Ala mutations destabilize the N state but stabilize PUF2* relative to U, suggests that PUF2* is an off-pathway dead-end intermediate sampled from the N state. The conjecture that PUF2* is stabilized by non-native hydrogen-bonding enabled by the Pro to Ala mutations is consistent with it being off-pathway. In this context, it should be noted that a molecular dynamics simulation study of the prion protein identified multiple partially unfolded states stabilized by non-native hydrogen bonds (Chamachi and Chakrabarty, 2017). Single molecule force microscopy studies have also shown that the unfolded state of the prion protein can misfold along multiple pathways (Yu et al., 2012b).

The Pro residues appear to restrict the boundary of the structural core of the oligomers

Misfolded moPrP is found in different types of aggregates: oligomers (Singh and Udgaonkar, 2015c) whose formation has been studied here, worm-like fibrils (Jain and Udgaonkar, 2008) and straight fibrils (Wang et al., 2020b). All three aggregates possess β -sheet structure (Sengupta and Udgaonkar, 2018), some of which would confer protection against hydrogen exchange (Sengupta and Udgaonkar, 2018; Singh and Udgaonkar, 2013b; Singh and Udgaonkar, 2015c). Previous hydrogen exchange studies had shown that the core of the aggregate, defined as the region most protected against HX, extended from residues 168 to 223 in worm-like fibrils (Singh et al., 2012b), as well as in straight fibrils formed *in vitro* (Singh and Udgaonkar, 2013b). The previous studies had shown that the core in oligomers is weakly protected against HX, compared to in worm-like fibrils and fibrils. Hence, in the current study, the core was defined as the sequence stretch comprising sequence segments that have a protection factor against HX of greater than 10 (see Results). By this criterion, the current study suggests that the protective core in the oligomers extends from residue 182 to residue 212 in wt moPrP, as also seen in previous HX studies (Singh and Udgaonkar, 2015c), but gets expanded to extend from residues 149 to 212 in 3PA moPrP (Figure 2.8).

Pro residues are rarely found in β -sheets in proteins, but they are often found flanking β -strands because of the local twist they impart to the peptide backbone (Richardson and Richardson, 2002a). Hence, it would appear that in 3PA moPrP, the absence of Pro residues in the sequence stretch 149-181, has allowed expansion of the protective β -sheet core. But it is more likely that β sheet structure spans the same length of sequence stretch in both the wt

moPrP and 3PA moPrP oligomers, as both oligomers show the same amount of β -sheet structure in the far-UV CD spectra (Figure S1a). The presence of Pro residues in the wt moPrP oligomers might have made the structure present in sequence stretch 149-181 dynamic and hence, not protective against HX.

Recent cryo-EM studies have revealed the diversity inherent in prion aggregate structure. The β -sheet structure extends from residues 169 to 224 for straight fibrils formed *in vitro* by the human prion protein (Wang et al., 2020b), and from residues 94 to 225, residues 94 to 226 and residues 79 to 140 in the case of prion aggregates derived from diseased mouse (Manka et al., 2022b), hamster (Kraus et al., 2021b) and human (Hallinan et al., 2022b) brain, respectively. The β -sheet core comprises of 15, 11 and 8 β -strands for prion aggregates derived from diseased mouse (Manka et al., 2022b), hamster (Kraus et al., 2021b) and human (Hallinan et al., 2022b) brain. Pro136 is present at one end of β 5 of mouse (Manka et al., 2022b), β 4 of hamster (Kraus et al., 2021b), and β 8 of human (Hallinan et al., 2022b) brain-derived aggregates, whereas Pro157 and Pro164 flank the ends of β 8 of mouse (Manka et al., 2022b) and β 5 of hamster (Kraus et al., 2021b) prion aggregates. It appears that the Pro residues might be playing a role in restricting the lengths of the β -strands in these fibrillar aggregates, as they could be doing in the case of the oligomers. It is clear from a previous HX study of fibrillar prion aggregate isolated from diseased brain (Smirnovas et al., 2011b), that all the β -strands of the fibril core seen in the cryo-EM studies, are not protective against hydrogen exchange. Unfortunately, detailed structural characterization of prion oligomers is not yet possible, and it remains to be seen how similar the β -sheet structure in prion oligomers is to that in prion aggregate derived from diseased brain.

Chapter 3

Mutation of evolutionarily conserved aromatic residues suggests that misfolding of the mouse prion protein may commence in multiple ways

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Introduction

Prion diseases, also known as Transmissible Spongiform Encephalopathies, constitute a group of fatal neurodegenerative diseases, characterised by the accumulation of the misfolded isoform (PrP^{Sc}) of the mammalian prion protein, in the central nervous system (Kretzschmar et al., 1986b; Moore et al., 2009a). The mature cellular isoform (PrP^C) of the mouse prion protein (moPrP) is 208 amino acid residues long, and consists of two distinct domains: an intrinsically disordered N-terminal domain (NTD) and a structured C-terminal globular domain (CTD) (Riek et al., 1996b). The NTD has a 5-octarepeat region, which can bind metal ions and is thought to be important in prion aggregation (Burns et al., 2002a; Moore et al., 2006b). The NTD also contains a hydrophobic stretch of highly conserved residues (Coleman et al., 2014), which play an important role in determining the interaction between PrP^{Sc} and the lipid membrane (Hegde et al., 1998a). The structured CTD consists of three α -helices ($\alpha 1$, $\alpha 2$ and $\alpha 3$), as well as a β -sheet consisting of two small anti-parallel β -strands ($\beta 1$ and $\beta 2$) (Riek et al., 1996b). $\alpha 2$ and $\alpha 3$ are held together by a disulfide bond between Cys178 and Cys213 (mouse numbering is used throughout), which has been shown to be important in maintaining the stability of the protein (Ning et al., 2014a).

The exact biological role of the prion protein in mammals is still mostly unclear. The prion protein appears to play a role in several processes, including metal ion homeostasis (Rana et al., 2009; Vassallo and Herms, 2003; Watt and Hooper, 2003), cell signaling (Collinge et al., 1994a; Haigh et al., 2010; Legname, 2017), cell adhesion (Málaga-Trillo et al., 2009; Martins et al., 2002; Schmitt-Ulms et al., 2001), and protection against oxidative stress (Brown, 2006; Choi et al., 2007; Milhavet and Lehmann, 2002; Wong et al., 2001). Despite the prion protein being widely expressed in the mammalian central nervous system, transgenic mouse (*Tg*) lines in which the *Prnp* gene (*Prnp*^{0/0}) is knocked out, do not show any pathological abnormalities (Büeler et al., 1992). This suggests that the mammalian prion protein might have redundant functions and not be an essential protein.

The susceptibilities of various mammals to sporadic prion diseases correlate well with the propensity of their PrP^C to misfold into β -sheet rich oligomers at low pH (Khan et al., 2010a). *In vivo*, during its trafficking through the endocytic pathway, PrP^C enters into lysosomes where it encounters a low pH (Borchelt et al., 1992b). *In vitro* studies have shown that moPrP undergoes substantial structural fluctuations at acidic pH upon protonation of a critical residue, His186 (Singh and Udgaonkar, 2016f; Van der Kamp and Daggett, 2010b). It is therefore likely that the prion protein misfolds into its oligomeric form when it encounters the low pH in the lysosome. At neutral pH, oligomerisation cannot be studied, as only a

negligibly small fraction of moPrP molecules have His186 in the protonated form (Singh and Udgaonkar, 2016f). Importantly, oligomers formed *in vitro* at low pH are cytotoxic (Erlich et al., 2010; Huang et al., 2010; Simoneau et al., 2007) and able to disrupt lipid membranes (Caughey et al., 2009; Chich et al., 2010; Singh et al., 2014a), which suggests to a possible mechanism of their toxicity. Remarkably, a diseased human brain has recently yielded highly infectious small non-fibrillar oligomeric prion assemblies (Vanni et al., 2020). A better understanding of the disease as well as the development of therapeutic strategies against prion diseases will arise from knowledge of the misfolding of the prion protein that results in oligomerisation.

The structures of the different mammalian prion proteins have been conserved during evolution, and the prion protein sequences show a high degree of amino acid similarity (Baiardi et al., 2019; Bernardi and Bruni, 2019b). Regions that display sequence and structural variation across different species include the $\beta 2$ - $\alpha 2$ loop and the C-terminus of $\alpha 3$, which are packed against each other (Bernardi and Bruni, 2019b; Biljan et al., 2017; Slapšak et al., 2019a). Despite this variation in the local sequence and structure of the mammalian prion protein, three aromatic residues (Tyr168, Phe174, and Tyr217) present at the packing interface (Figure 3.1) are highly conserved (Bartz et al., 1994; Huang and Caflisch, 2015; Kurt et al., 2014b; van Rheede et al., 2003b). Tyr168 and Tyr217 are strictly conserved, whereas Phe174 is conserved in all but two species (Huang and Caflisch, 2015). The aromatic interactions between Tyr168-Phe174-Tyr217 are the most conserved aromatic-interactions observed in the structures of different mammalian prion proteins (Zhang, 2015). MD simulation studies have suggested that favorable interactions between Tyr168, Phe174, and Tyr217 stabilise the interface of the $\beta 2$ - $\alpha 2$ loop and the C-terminus of $\alpha 3$ (Huang and Caflisch, 2015; Jung Cheng and Daggett, 2014). It is likely that the highly conserved aromatic residues play an important role in maintaining the integrity of the PrP^C structure and in guarding against misfolding that leads to the formation of oligomers.

In the current study, it is shown by mutational studies, that the evolutionarily conserved aromatic residues Tyr168, Phe174 and Tyr217 indeed play a significant role in maintaining the structural integrity and conformation of the protein. The N state of moPrP becomes unstable when the conserved aromatic residues are mutated to alanines, which also dramatically accelerates (up to 300-fold) the misfolding process. Native state hydrogen exchange (HDX) studies coupled with mass spectrometry (MS) have identified an aggregation-competent partially unfolded form (PUF2**), which is in rapid equilibrium with the N state at

pH 4. In PUF2**, the interface between the β 2- α 2 loop and the C-terminus of α 3 is disordered, as are the α 1- β 2 loop and β 2. Mutations of the evolutionarily conserved aromatic residues reduce the energy gap between the N state and PUF2**, making the latter more accessible from the N state in the mutant variants. This study also reveals that there are multiple misfolding pathways for moPrP. The observed misfolding rate constant of the mutant variants correlates well with the extent to which PUF2** is populated at equilibrium, indicating that PUF2** directly misfolds, while a previous study had identified another aggregation-prone partially identified conformation, PUF2* (Pal and Udgaonkar, 2022a), which is very similar in energy to PUF2** but has a very different conformation.

MATERIALS AND METHODS

Buffers and Reagents

The reagents used in this study were of high purity grade, and were obtained from Sigma. Ultra-pure GdnHCl was procured from United States Biochemicals, and was of the highest purity grade.

Site-directed mutagenesis

Mutant variants of full-length moPrP were generated using the Quickgene site-directed mutagenesis kit (Stratagene). Primers containing 3 nucleotide changes were obtained from Sigma. Three mutant variants were prepared: Y168A, F174A and Y217A. The mutations were confirmed by DNA sequencing.

Protein Expression and Purification

wt moPrP and the mutant variants were expressed in *Escherichia coli* BL21(DE3) codon plus cells (Stratagene) transformed with a pET17b plasmid containing the full-length sequence (23-231) of the moPrP gene. The moPrP variants were purified as described previously (Jain and Udgaonkar, 2008; Singh et al., 2014a). The purity and mass of each moPrP variant preparation were confirmed by mass spectrometry using a Synapt G2 HD mass spectrometer (Waters Corporation).

Far-UV CD measurements

Far-UV CD spectra were recorded on a Jasco J-815 spectropolarimeter using a protein concentration of 10 μ M in a 1 mm cuvette, using a scan speed of 50 nm/min, a digital integration time of 2 s, and a bandwidth of 1 nm. The wavelength was scanned from 200 to 250

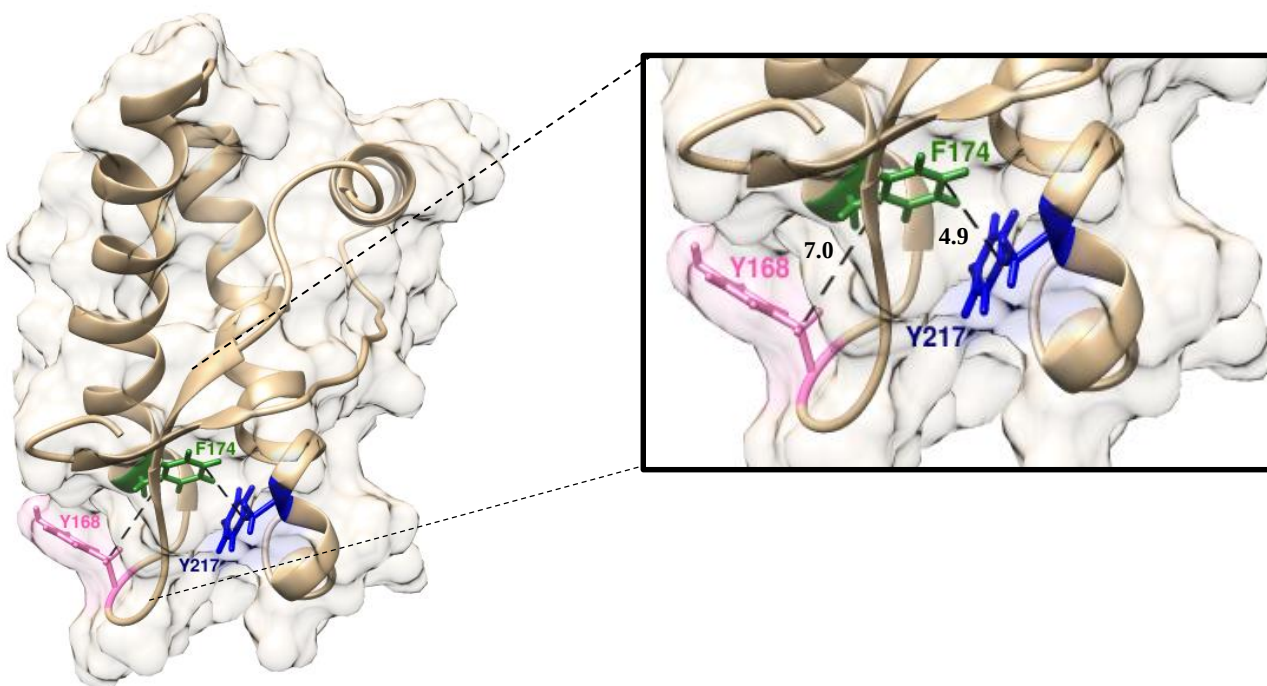


Figure 3.1. Structure of the C-terminal domain of the mouse prion protein showing the locations of the three conserved aromatic residues, Tyr168, Phe174 and Tyr217. The N-terminal domain comprises of residues 23-120, and is unstructured in the full-length protein. The three conserved aromatic residues are colored pink (Y168), green (F174) and blue (Y217). The side-chains of Y168, F174 and Y217 are buried to the extents of 40 %, 100 % and 90 %, respectively. The distances between the side-chains are shown in Å in the expanded view. The figure was drawn using Chimera and Protein Data Bank entry 1AG2.

nm, and a total of 15 spectra were averaged. Far-UV CD spectra under native conditions were acquired in 10 mM sodium acetate, pH 4, at 25 °C.

Urea-induced equilibrium unfolding studies

Urea-induced equilibrium unfolding studies were carried out in 10 mM sodium acetate buffer at pH 4, at 25 °C. 10 μ M protein was incubated in different urea concentrations for 1 h, before the far-UV CD signal at 222 nm was monitored. The data were fit to a two-state N $\rightleftharpoons$ U equilibrium unfolding model (Agashe and Udgaonkar, 1995), and the thermodynamic parameters were obtained.

Misfolding studies at pH

Misfolding was initiated by diluting 20 μ M protein in 10 mM sodium acetate buffer (pH 4) two-fold with 2x misfolding buffer (10 mM sodium acetate buffer containing 300 mM NaCl, pH 4). Both the protein sample and 2x misfolding buffer were incubated at 37 °C before starting the reaction. The time from the mixing of the protein solution with 2x misfolding buffer to the first reading was 20 s. To study the misfolding kinetics of the moPrP variants, the CD signal at 222 nm (θ_{222}) was monitored using a 1 mm quartz cuvette maintained at 37 °C.

HDX-MS measurements

The peptide map of the moPrP variants was generated as described previously (Pal and Udgaonkar, 2022b). To initiate deuterium labeling, 100 μ M protein was diluted into a labeling buffer [10 mM sodium acetate buffer in D₂O at pH 4, corrected for the isotope effect], so that HDX occurred in 95% D₂O, at 25 °C. At different times, a 50 μ l aliquot was mixed with 50 μ l ice-cold 20 mM glycine-HCl buffer (pH 2.5) to quench the labeling reaction. Online pepsin digestion was carried out by injecting the samples immediately into an immobilized pepsin cartridge (Applied Biosystems) at a flow rate of 40 μ l/ min of water (0.05% formic acid) which was placed in a HDX module (Waters Corporation) coupled to a nanoAcquity UPLC. The peptides eluting from the pepsin column were collected using a trap column, washed to remove salt, and eluted over 12 min on an analytical C18 reversed-phase chromatography column using a gradient of 3 to 40% acetonitrile (0.1% formic acid) at a flow rate of 40 μ l/ min. All columns were kept at 4 °C to minimize back exchange. The parameters of the mass spectrometer parameters were set as follows: source temperature, 35 °C; desolvation temperature, 100 °C; capillary voltage, 3.0 kV.

Peptide masses were calculated from the centroid of the isotopic envelope, using MassLynx. The shifts in the masses of labeled peptides relative to unlabeled peptides were used to determine the extent of deuterium incorporation at each time point of HDX. Since the sample was in 95% D₂O during labeling, and exposed to H₂O during quenching, control experiments were carried out to correct for back exchange. For this, the protein was incubated in 10 mM sodium acetate, pH 4 (in 95% D₂O), and deuterated by unfolding at 65 °C for 15 min, followed by refolding on ice. CD spectroscopy and thermal equilibrium unfolding studies showed that the refolded protein behaves like native moPrP. The fully (95%) deuterated moPrP samples were processed in the same way as the labeling reaction samples. The extent of deuterium incorporation in each peptide fragment, % D, was calculated using the equation:

$$\%D = \frac{(m(t) - (m(0\%)))}{(m(95\%) - m(0\%))} \times 100$$

where $m(t)$ is the measured centroid mass at time t , $m(0\%)$ is the measured mass of the undeuterated reference peptide, and $m(95\%)$ is the measured mass of a 95% deuterated reference peptide.

In the current study, native state HDX was performed in the EX2 limit (Moulick et al., 2019; Pal and Udgaonkar, 2022a) allowing the stabilities of the partially unfolded forms, in which HDX occurs to be determined. For calculation of the free energy of opening (ΔG_{op}) of a sequence segment, the observed HDX rate constant (k_{obs}) of deuterium incorporation was first obtained by fitting the kinetic curve of HDX to either a single exponential or a two-exponential equation, depending upon the goodness of fit. For each of the sequence segments, k_{obs} was compared to the rate constant (k_{int}) that would be observed in the corresponding peptide fragment in a random coil state (Bai et al., 1993b; Nguyen et al., 2018). The quantity $P_f = k_{int}/k_{obs}$ is the protection factor that slows down HDX into the sequence segment. The HDX protection factor determined for each peptide fragment was used to calculate the local stability of the corresponding sequence segment in the protein, $\Delta G_{op} = RT \ln P_f$. For each sequence segment, ΔG_{op} is therefore the free energy of opening of the N state to form a HDX-competent partially unfolded form.

Results

Mutation of the aromatic residues does not affect the secondary structure but destabilizes the protein

All three single mutant variants showed far-UV CD spectra that are very similar to that of wt moPrP (Figure 3.2a), indicating that the Y168A, F174A and Y217A mutations did

not affect the global secondary structure of the protein. However, urea-induced equilibrium studies indicated that these mutations led to significant destabilization of the N state of the protein (Figure 3.2b). Y217A moPrP showed the highest degree of destabilisation among the mutant variants ($\Delta\Delta G_{\text{NU}} = 2.4 \text{ kcal mol}^{-1}$), followed by F174A ($\Delta\Delta G_{\text{NU}} = 2.2 \text{ kcal mol}^{-1}$) and Y168A ($\Delta\Delta G_{\text{NU}} = 1.9 \text{ kcal mol}^{-1}$), suggesting that the conserved aromatic residues are important in maintaining the stability of the native protein.

Mutation of the aromatic residues results in rapid misfolding

The prion protein is known to misfold on the cell surface, as well as in the endocytic pathway when it enters lysosomes and encounters a low pH (Borchelt et al., 1992b). At pH 4, moPrP remains in its native monomeric helix-rich conformation, but becomes prone to misfolding due to protonation of the critical residue His186 (Singh and Udgaonkar, 2016f). It has been shown that misfolding begins when a crucial network of stabilising interactions between several charged residues (Arg155, His186, Asp201, Lys193, and Lys195) located in the $\beta 1$ - $\alpha 1$ - $\beta 2$ and $\alpha 2$ - $\alpha 3$ subdomains is disrupted (Sengupta et al., 2017a; Sengupta and Udgaonkar, 2018). The misfolding of wt moPrP at pH 4 in the absence of added salt is very slow ($\sim 10^{-4} \text{ h}^{-1}$) (Sengupta et al., 2017a). Hence, in the current study, the misfolding of the different moPrP variants at pH 4 was studied in the presence of a physiological concentration (150 mM) of salt at 37 °C. The kinetics of misfolding were monitored by measuring the change in the CD signal at 222 nm. For all the moPrP variants, the kinetics of misfolding appeared monophasic and fit well to a single exponential equation. It should be noted that the misfolding of moPrP at pH 4 is accompanied by oligomerisation (Sabareesan and Udgaonkar, 2016b; Sengupta and Udgaonkar, 2019a). Earlier studies had shown that the kinetics of CD-monitored misfolding and the kinetics of oligomerisation monitored by size exclusion chromatography (SEC) were identical for wt moPrP, suggesting that both processes occur concurrently (Sabareesan and Udgaonkar, 2016b). All three mutant variants studied here showed significantly faster misfolding rate constants compared to wt moPrP (Figures 3.3a and 3.3b), suggesting that the three conserved aromatic residues might have been evolutionarily selected to impede misfolding. Among the mutant variants, Y217A moPrP misfolded the fastest, followed by F174A and Y168A moPrP.

Dependence of the rate constants of misfolding on native state stability

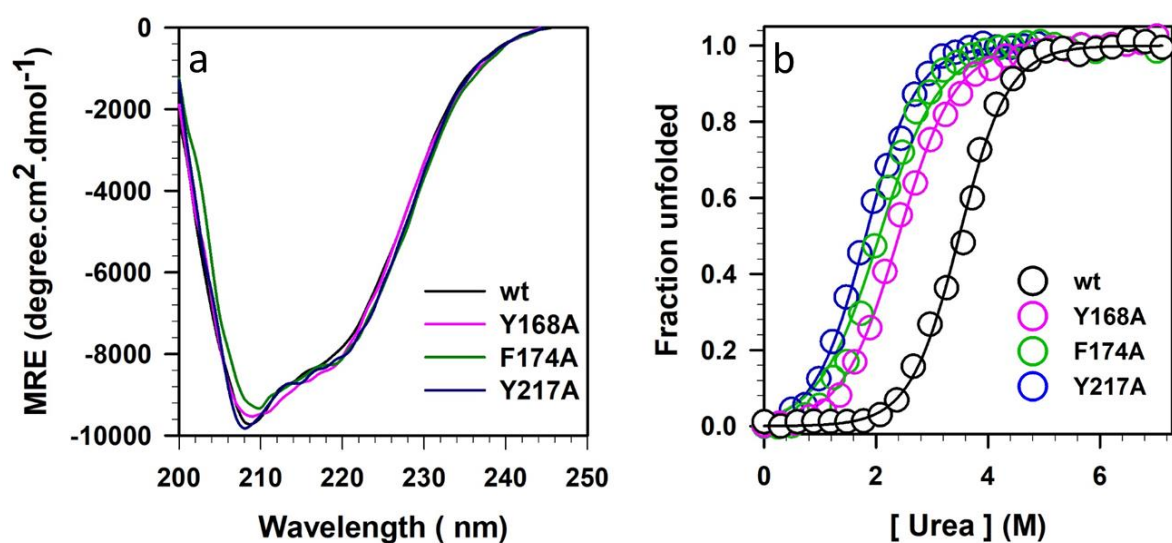


Figure 3.2. Effect of the mutations on secondary structure and stability (a) Far-UV CD spectra of the native monomeric forms of the moPrP variants acquired at pH 4, 25°C. (b) Urea-induced equilibrium unfolding transitions of the moPrP variants at pH 4, 25°C, as monitored by measurement of the far-UV CD signal at 222 nm. The signal change was normalized to obtain the fraction unfolded. The colors of the lines represent the different moPrP variants as indicated. The solid lines through the data points are fits to an equation describing a two-state unfolding transition.

In Figure 3.3c, the observed rate constants of misfolding are plotted against the decrease in free energy of unfolding ($\Delta\Delta G_{\text{NU}}$) of the mutant variants with respect to wt moPrP (Figure 3C). The logarithm of the observed misfolding rate constant increased linearly with the extent of destabilization ($\Delta\Delta G_{\text{NU}}$). Several disease-linked mutant variants are known to misfold rapidly under similar conditions, and for them too, a linear dependence of the logarithm of the observed misfolding rate constants on the decrease in the free energy of unfolding ($\Delta\Delta G_{\text{NU}}$) has been observed (Singh and Udgaonkar, 2015c, 2016b). Interestingly, the observed misfolding rate constants of the mutant variants studied here, and of the disease-linked mutant variants studied earlier, have the same dependence on $\Delta\Delta G_{\text{NU}}$ (Figure 3.3c).

Effect of the mutations on protein dynamics

To determine the effect of the mutations not only on the structure but also on the dynamics of the protein, HDX-MS studies were carried out. The HDX-MS studies revealed sequence-specific information about changes in the structure and dynamics that occur upon mutation. The HDX experiments were carried out at pH 4, where the intrinsic rate constant of HDX is slow (Bai et al., 1993b). This allowed HDX to be monitored at amide hydrogen sites spread over all the secondary structural elements of the protein.

Figure 3.4 shows the kinetics of deuterium incorporation into different sequence segments of the CTD of moPrP, for the different mutant variants. The kinetics of deuterium incorporation were found to be faster in most of the sequence segments for the mutant variants. The sequence segments covering $\beta 1$ (96-144), the loop between $\alpha 1$ and $\beta 2$, and the $\beta 2$ (154-167), the loop between $\beta 2$ and $\alpha 2$ (168-173), the C-terminus of $\alpha 2$ (182-196) and the C-terminus of $\alpha 3$ (205-212, 210-216 and 217-223) showed pronounced increases in the observed rate constants of deuterium incorporation for the all three mutant variants in comparison to wt moPrP. The rate of deuterium incorporation, however, seen to increase differently in different sequence segments. The rate of incorporation of deuterium into the sequence segments covering $\beta 1$ (96-144), the C-terminus of $\alpha 2$ (182-196), and the C-terminus of protein (225-231) of wt moPrP was, already quite fast: it was only slightly slower than the intrinsic rate of exchange (k_{int}). As a result, even though the mutation of the conserved aromatic residues did have an effect on these sequence segments, the effect appeared to be small only because the exchange was so fast in the wt protein itself. On the other hand, sequence segments covering $\alpha 1$ (144-148, 149-153), $\alpha 2$ (177-183) and the loop between $\alpha 2$ and $\alpha 3$ and the N termini of $\alpha 3$ (197-204) showed little or no variation in the kinetics of deuterium incorporation in the mutant

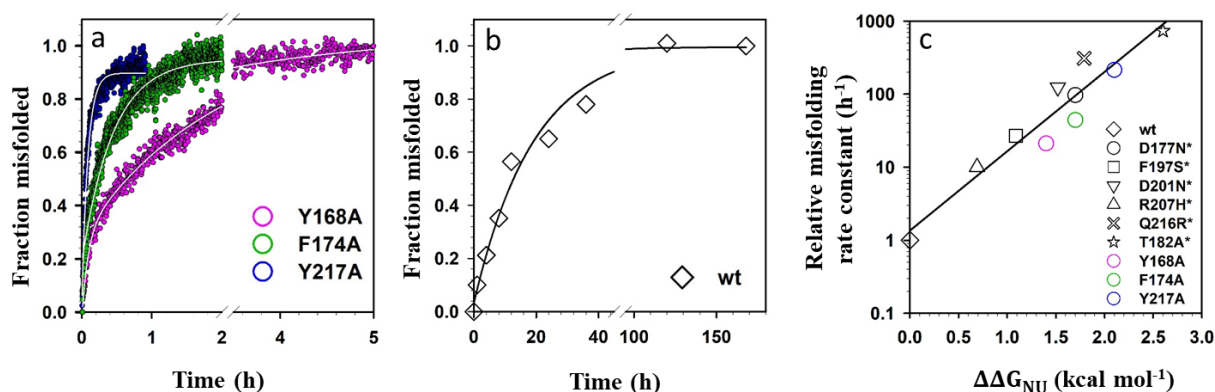


Figure 3.3. Dependence of the observed rate constant of misfolding on native state stability (a) The fraction misfolded form at different times of misfolding in 150 mM NaCl at pH 4, 37 °C of 10 μ M Y168A, F174A and Y217A moPrP (a) and of 10 μ M wt moPrP (b) are shown. The fraction misfolded form was calculated from the fractional change in the CD signal at 222 nm. The continuous lines through the data points in both panels a and b are fits to an exponential equation. (c) Plot of the relative observed rate constant of misfolding of the different moPrP variants in 150 mM NaCl at pH 4, at 37 °C *versus* the decrease in global stability of the mutant variants ($\Delta\Delta G_{NU}$) with respect to wt moPrP. The misfolding of the single aromatic mutant variants was studied at 10 μ M concentration, and that of the disease-linked mutant variants was studied at 100 μ M concentration. The continuous line through the data points are linear fits to the data points. For both sets of mutant variants, the misfolding rate constants were normalised to a value of 1 for the misfolding rate constant of 10 μ M and 100 μ M wt moPrP. Asterisks denote disease-linked mutant variants.

Table 3.1. Stabilities and misfolding rate constants of the different moPrP variants

moPrP variant	ΔG_{NU} (kcal mol ⁻¹)	Misfolding rate constant (h ⁻¹)
wt	4.2 ± 0.1	0.045 ± 0.01
Y168A	2.2 ± 0.2	0.73 ± 0.21
F174A	2.0 ± 0.1	2.13 ± 0.25
Y217A	1.8 ± 0.1	11.44 ± 0.72

The free energies of unfolding (ΔG_{NU}) were obtained from urea-induced equilibrium unfolding studies at pH 4, at 25 °C (Figure 2b). The m value was constrained to the same value (1.1 kcal mol⁻¹M⁻¹) for all the moPrP variants. The observed rate constants of misfolding were determined at 10 μM concentration in 150 mM NaCl at pH 4, 37 °C (Figure 3a, 3b). The error bars represent the standard deviations determined from three independent experiments.

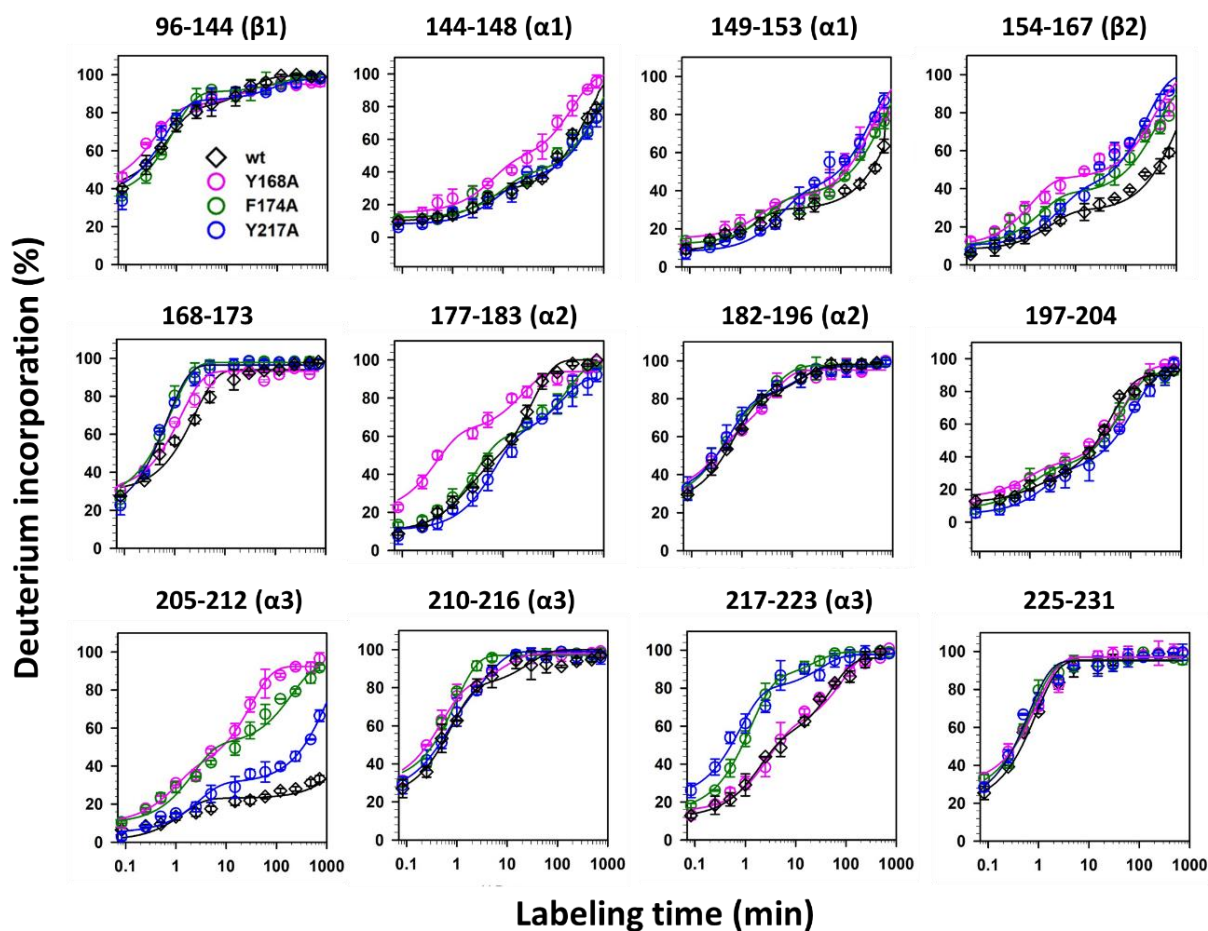


Figure 3.4. Effect of the mutations on the native state dynamics. The time courses of HDX into different sequence segments of the native states of the moPrP variants at pH 4, 25°C are shown. The differently colored circles represent the different moPrP variants, as indicated. The error bars represent the standard deviations determined from three independent experiments. The solid lines through the data represent fits to either monoexponential or biexponential equations

variants compared to that in wt moPrP (except sequence segment 177-183 in the case of Y168A moPrP). It should be noted that sequence segment 205-212 which showed significant increases in the observed rate constants of deuterium incorporation for all three mutant variants, is part of the hydrophobic core, and is the most stable sequence segment of the protein.

Figure 3.5 shows the ΔG_{op} values of the different sequence segments of the mutant variants studied here. It shows that the mutations principally decreased the ΔG_{op} values for the sequence segments of $\alpha 3$ (205-212, 210-216, and 217-223), along with the sequence segments of $\beta 1$ (96-144), the loop between $\alpha 1$ and $\beta 2$, and the $\beta 2$ (154-167), the loop between $\beta 2$ and $\alpha 2$ (168-173), the C-terminal end of $\alpha 2$ (182-196) and the C-terminal end of the protein (225-231). Since the conserved aromatic residues form a very stable network of aromatic interactions spanning the loop between $\alpha 1$ and $\beta 2$, $\beta 2$ (154-167) and the loop between $\beta 2$ and $\alpha 2$ (168-173) and the C-terminal end of $\alpha 3$ (210-216, 217-223) and 225-231 (Figure 3.1) their mutation decreased the local stabilities (ΔG_{op}) of these sequence segments (Figure 3.5). This suggests that the mutations principally affected the local stabilities of the sequence segments near the sites of the mutations. It is interesting that sequence segments 96-144 and 182-196 also showed reduced ΔG_{op} values upon mutation, even though they are far from the sites of the mutations.

Identification of partially unfolded forms in equilibrium with the N state

Previous HDX-NMR and HDX-MS measurements had identified two sparsely populated, partially unfolded forms (PUFs), PUF1 and PUF2, in dynamic equilibrium with the N state, in the case of both isolated CTD (Moulick et al., 2015a; Moulick and Udgaonkar, 2017) and full length wt moPrP (Pal and Udgaonkar, 2022a). PUF2 was more disordered than PUF1; it had more regions that had lost intramolecular hydrogen bonding and become fully solvent-exposed, and hence unprotected against HDX. In the case of wt moPrP, PUF1 was characterised by a local stability (ΔG_{op}) of 1.3 ± 0.2 kcal mol⁻¹, whereas PUF2 was characterised by a higher local stability (ΔG_{op}) of 3.3 ± 0.2 kcal mol⁻¹ (Moulick et al., 2015a; Pal and Udgaonkar, 2022a). The current study not only confirmed the existence of PUF1 and PUF2 in the case of wt moPrP, but also determined the effects of the mutations of the conserved aromatic residues on the stabilities of the two PUFs. These mutations were found to increase the sampling of both PUFs from the N state by decreasing the ΔG_{op} values of the sequence segments that exchange out in PUF1 and PUF2.

Identification of PUF2, a structurally distinct sub-population of PUF2**

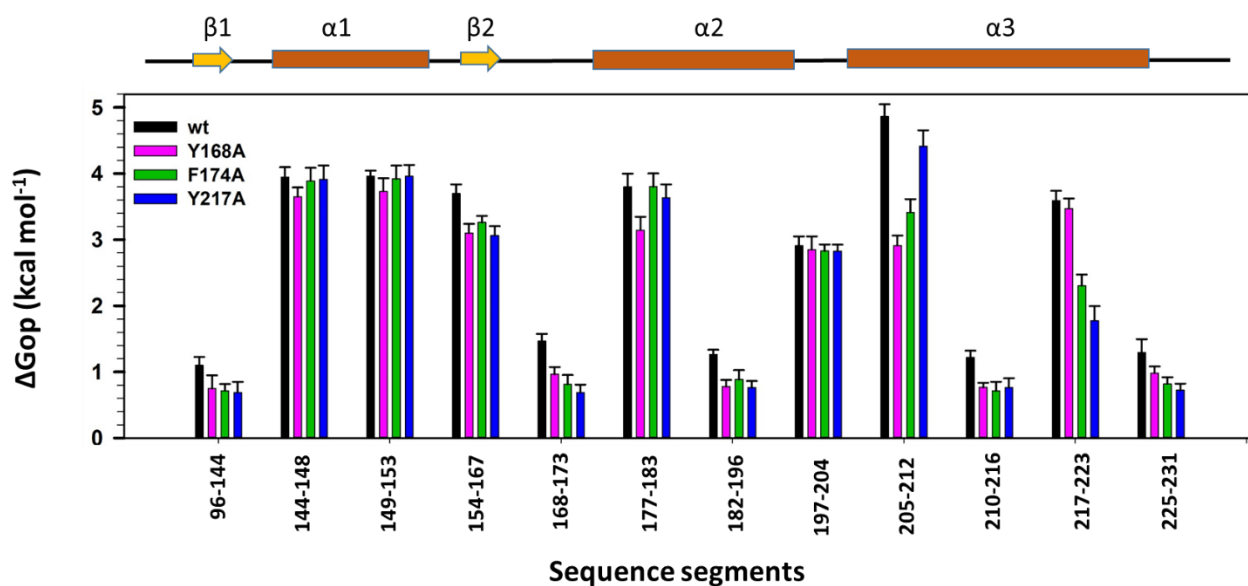


Figure 3.5. ΔG_{op} values of the different sequence segments of the aromatic mutant variants. The secondary structural elements coded for by the sequence are shown above the sequence segments (rectangular boxes represent the α -helices, and arrows represent the β -strands). The differently colored bars represent the different moPrP variants, as indicated. The error bars represent the standard deviations determined from three independent experiments.

When PUF1 and PUF 2 were first identified by HDX-NMR and HDX-MS studies of moPrP as two distinct states that differed in structure and energy, it was assumed that each PUF consisted of a structurally homogenous population of molecules (Moulick et al., 2015a), and not of iso-energetic sub-populations differing in their structures. The first evidence for conformational heterogeneity in PUF2 came from the HDX-MS characterization of *Pro* to *Ala* mutant variants of moPrP (Pal and Udgaonkar, 2022a), which identified a sub-population of PUF2, called PUF2*, which was disordered in only some of the sequence segments known to have become disordered in PUF2, but which had a local stability (ΔG_{op}) of 3.7 ± 0.4 kcal mol⁻¹ (Pal and Udgaonkar, 2022a). Of the sequence segments known to be disordered in PUF2, PUF2* had sequence segments 144-148, 149-153, 154-167 and 197-204 disordered, but not sequence segments 177-183 and 217-223. That study suggested that in addition to the sub-population PUF2*, PUF2 must consist of another isoenergetic sub-population in which sequence segments 177-183 and 217-223, and possibly other sequence segments, had become disordered.

Of the sequence segments known to be disordered in PUF2, only sequence segments 154-167 (the loop between $\alpha 1$ and $\beta 2$, and the $\beta 2$) and 217-223 (the C-terminal end of $\alpha 3$) became disordered upon mutation of the conserved aromatic residues (Figure 3.5). Importantly, the sequence segments known to be disordered in PUF2* (Pal and Udgaonkar, 2022a) were not affected, except for sequence segment 154-167. Hence, the effects of the mutations of the three aromatic residues suggested that there exists a second sub-population of PUF2, designated here as PUF2**, in which sequence segments 154-167 and 217-223 have become disordered (Figure 6). In the case of wt moPrP, the local stability (ΔG_{op}^{av}) of PUF2**, determined by averaging the ΔG_{op} values of sequence segments 154-167 and 217-223, is 3.7 ± 0.1 kcal mol⁻¹. Mutation of the aromatic residues also has a significant impact on sequence segments 96-144, 168-173, 182-196, 210-216 and 225-231, which were known to be disordered in PUF1, and it is likely that these segments are also disordered in PUF2**, as indicated in Figure 3.6 and discussed later.

Effect of mutation of the aromatic residues on the stabilities of the PUFs

The mutations of the conserved aromatic residues had a significant impact on the stability with respect to the unfolded (U) state of each PUF ($\Delta G_{PU} = \Delta G_{NU} - \Delta G_{op}$) for each protein variant. In the case of PUF1, the ΔG_{PU} value was found to be equal to about 0.7 ΔG_{NU} for wt moPrP, indicating that PUF1 retains approximately 70 % of the stabilizing interactions that were originally present in the native state of wt moPrP (Figure 3.7). Although the ΔG_{PU}

values for the three mutant variants are different from each other, they all fall in the range of 0.61 to 0.69 times the ΔG_{NU} value (Figure 3.7). Consequently, it can be concluded that, on averaging the ΔG_{PU} values for PUF1 of the three mutant variants, the stability of PUF1 in each of the mutant variants is about half the stability in the case of wt moPrP. Hence, it appears that PUF1 of the mutant variants, is stabilized by only about 45 % of the stabilising interactions that are present in the N state of wt moPrP.

In the case of PUF2**, the ΔG_{PU} value was found to be approximately 0.25 ΔG_{NU} for wt moPrP (Figure 3.7). This result suggested that, in the case of wt moPrP, PUF2** possesses only 25% of the stabilising interactions that are present in the N state. However, for the mutant variants, the ΔG_{op} values for PUF2** were higher than their corresponding ΔG_{NU} values (Figure 3.7), suggesting that PUF2** was a high energy intermediate form less stable than the U state. The data cannot distinguish between PUF2** being on or off the pathway from the N state to the U state. It would appear that the U state of the mutant variants is stabilised by residual structure that is absent in the U state of wt mPrP, as suggested for other proteins (Park and Marqusee, 2004; Wedemeyer et al., 2002; Wrabl and Shortle, 1999). Such stabilizing residual structure would be absent in PUF2**.

Effect of the mutations on the sequence segment not disordered in PUF2

In the case of wt moPrP, sequence segment 205-212 did not become disordered (open to HDX) in either PUF1 or PUF 2, and its ΔG_{op} value suggested that it becomes disordered only in the U state (Moulick et al., 2015a). While this sequence segment appeared to be impacted by all three mutations (Figure 5), the impact was found to be significant in the case of Y168A and F174A moPrP. For these two mutant variants, the ΔG_{op} value was lowered to a value equal to the ΔG_{op} value for PUF2 (Figure 3.5), suggesting that this segment may have lost structure in PUF2 itself. It could not, however, be determined whether this segment has lost structure in PUF2**.

Misfolding rate constants of the mutant variants correlate with their local stabilities

The logarithm of the observed rate constant of misfolding of the mutant variants was found to increase linearly with the decrease in the average of the ΔG_{op} values ($\Delta G_{\text{op}}^{\text{av}}$), for the sequence segments that exchange out in PUF1 (96-144, 168-173, 182-196, 210-216, and 225-231) as well as those that exchange out in PUF2** (154-167 and 217-223). As the logarithm of the observed misfolding rate constant correlates well with the equilibrium populations of

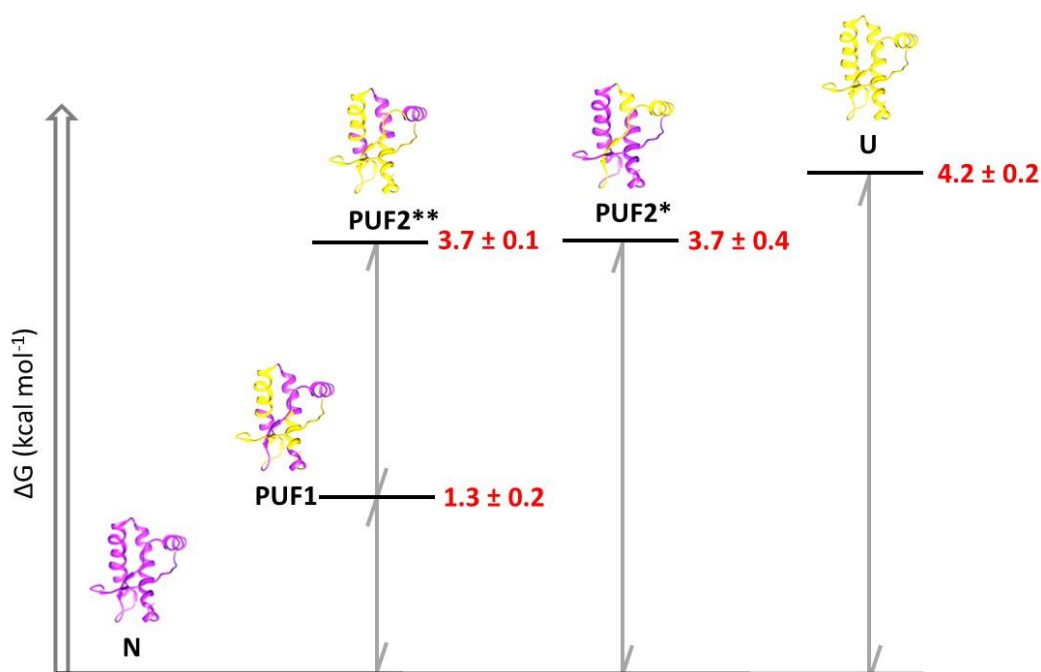


Figure 3.6. Stabilities (ΔG) of the native and partially unfolded forms of wt moPrP at pH 4 as identified by native state HDX-MS. PUF1 appears to be a homogenous population of molecules. PUF2 is shown to comprise of two isoenergetic sub-populations of molecules, PUF2* and PUF2**, which differ in structure. For each PUF, segments that are intact in structure are shown in purple, while those that have unfolded are shown in gold. The stabilities of the individual PUFs are shown in red. It appears that the perturbation of different elements of structure, $\alpha 1$ and the spatially adjacent N-terminal end of $\alpha 2$ in PUF2*, and the $\beta 2$ - $\alpha 2$ loop and the C-terminal end of $\alpha 3$ in PUF2**, can lead to the same extent of destabilization. The different forms (PUF1, PUF2*, PUF2** and U) in dynamic equilibrium with the N state will be populated in accordance with Boltzmann's Law. Hence, while it has been shown (see text) that misfolding can potentially commence from any of the three PUFs shown, it will occur predominantly from PUF1 which only is populated to a significant extent.

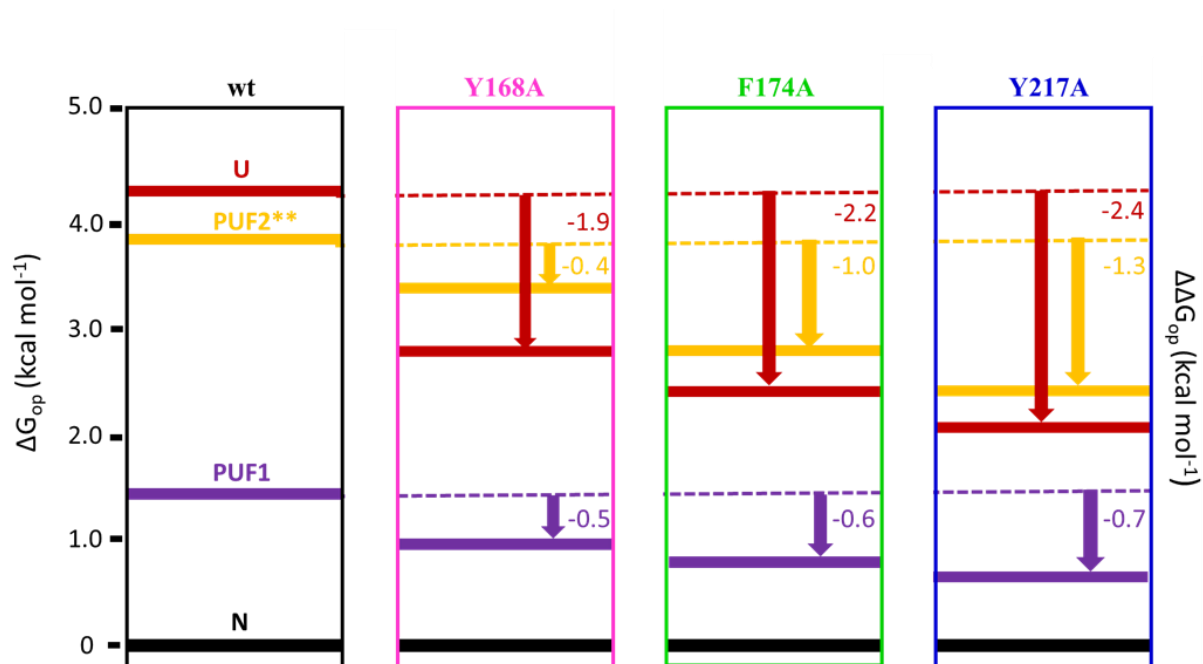


Figure 3.7. Effect of mutation of the aromatic residues on the stabilities of the PUFs. The free energy levels of the PUFs, produced from unfolding of specific sequence segments, have been placed on a free energy ladder. It should be noted that the figure is meant to only show the free energy levels, and not whether the PUFs formed sequentially or in parallel from the N state. The differently colored broken lines indicate the free energy levels of the different conformations in the case of wt moPrP.

PUF1 as well as of PUF2** (Figure 3.8a), it appears that both PUFs can act as precursors to misfolding.

The dependences of the logarithm of misfolding rate constant on ΔG_{op} for PUF1 and for PUF2** were a bit different (Figure 3.8a). Interestingly, when the logarithm of the observed rate constant of misfolding of the mutant variants was plotted against the fractional destabilisation (calculated as $[(\Delta G_{op}^{wt} - \Delta G_{op}^{mutant})/\Delta G_{op}^{wt}]$), of the sequence segments that are unstructured in PUF1 (sequence segments 96-144, 168-173, 182-196, 210-216, 225-231) as well as in PUF2** (sequence segments 154-167 and 217-223), the linear dependences were found to be same for both PUFs (Figure 3.8b).

Discussion

Mutation of the conserved aromatic amino acids destabilise the N state

The observation that the mutations of the conserved aromatic residue to Ala reduces the free energy of unfolding (ΔG_{NU}) can be rationalised to be the result of enthalpic destabilisation of the N state. The network of stabilising interactions by the three aromatic residues would be perturbed in the mutant variants. The side-chains of Phe174 and Tyr217 are 100% and 90% buried respectively, and are also part of the hydrophobic core of the protein, in addition to being part of the network of aromatic contacts. Mutation of Phe174 or Tyr217 to the smaller Ala is expected to create a cavity, and to perturb the packing of the core. The occurrence of cavities within the hydrophobic core of a protein is known to have a strong destabilising effect on the N state (Jackson et al., 1993). Not surprisingly, mutation of Tyr168, whose side chain is only 40% buried, has a weaker destabilising effect than do the mutations of Phe174 and Tyr217. However, it appears from NMR and MD simulation studies that the Tyr168 hydroxyl group and the Asp177 carboxyl group form a very stable H-bond (Caldarulo et al., 2017; Huang and Caflisch, 2015). The reduced stability of the Y168A mutant variant can be rationalised by invoking the loss of this stabilising interaction, as well as the loss of stabilising stacking interactions with Phe174. The combined effect of disruption of these stabilising interactions have a synergistic impact, causing the mutant variants studied here to have a markedly destabilised N state.

Heterogeneity in PUF2

The current study has identified a sub-population, PUF2**, in the PUF2 ensemble that is structurally distinct from a previously identified sub-population, PUF2* (Pal and Udgaonkar, 2022a). PUF2* is disordered in $\alpha 1$ (sequence segments 144-148, 149-153), the loop between

$\alpha 1$ and $\beta 2$ and $\beta 2$ (sequence segment 154–167), and the loop between $\alpha 2$ and $\alpha 3$ as well as the N-terminal end of $\alpha 3$ (sequence segment 197–204) (Figure 3.6). In contrast, PUF2** is not disordered in these regions except for the loop between $\alpha 1$ and $\beta 2$ and $\beta 2$ (sequence segment 154–167), and is disordered also at the C-terminal end of $\alpha 3$ (sequence segment 217–223) (Figure 6). Neither PUF2* nor PUF2** are disordered in sequence segment 177–183, which is known to be disordered in PUF2, suggesting that PUF2 must comprise of other sub-populations of distinct conformations.

Hence, the N state of moPrP is in dynamic equilibrium with at least two PUF2s, which have distinct conformations, but which nevertheless have similar stabilities. This result is supportive of energy landscape theories of protein folding which posit that completely different conformations may have similar energies (Eaton and Wolynes, 2017).

PUF1 and PUF2 may form either sequentially or in parallel from the N state**

It has been suggested that protein unfolding occurs by the sequential loss of blocks of secondary structure (Hughson et al., 1990a), and for several proteins, including apomyoglobin (Hughson et al., 1990a), cytochrome *c* (Hu et al., 2016b) and ribonuclease H (Hu et al., 2013), partially unfolded forms have been shown to be populated sparsely, in equilibrium with the N state. For cytochrome *c* and ribonuclease H, PUFs could be placed on a ladder of increasing disorder from the N to U state, as determined from their ΔG_{op} values and their exposures to solvent. In this way, PUFs were assumed to form on a sequential unfolding pathway from the N to U state, with regions unfolded in a PUF of lower ΔG_{op} value being also unfolded in a PUF of higher ΔG_{op} value. A PUF was assumed to be a homogeneous population of molecules, and not instead be a heterogeneous mix of sub-populations with different structures. In the case of moPrP, since the ΔG_{op} value of PUF2 was found to be higher than that of PUF1 (Moulick et al., 2015a; Pal and Udgaonkar, 2022a), it appeared that PUF1 was on the pathway of unfolding of the N state to PUF2, and, consequently that the sequence segments that were unfolded in PUF1 were also unfolded in PUF2.

In the case of the aromatic mutations studied here, each mutation significantly destabilises the N state as well as the PUFs. With respect to their stabilities in wt moPrP, the N state is destabilized the most, then PUF1, and PUF2 is destabilized the least, which is not surprising given that PUF2 would have less structure that can be destabilised. Unfortunately, while it appears that PUF1 and PUF2** are populated sequentially on the pathway of unfolding from the N to U state, it cannot be definitely concluded that this is the case. The observation that the dependence of the logarithm of the observed rate constant of misfolding on fractional

localized destabilisation (calculated by $[(\Delta G_{op}^{wt} - \Delta G_{op}^{mutant})/\Delta G_{op}^{wt}]$) of PUF1 is the same as that of PUF2** (Figure 3.8b) suggests that PUF2** may populate from the N state *via* PUF1. If this is indeed so, then PUF2** would have $\beta 1$ (96-144), the $\alpha 1$ - $\beta 2$ loop, $\beta 2$ (154-167), the $\beta 2$ - $\alpha 2$ loop (168-173), the C-terminal end of $\alpha 2$ (182-196) and the second half (210-216) and C-terminal end of $\alpha 3$ (217-223) and sequence segment 225-231 all disorder at the main chain level. In Figure 3.6, it is assumed that PUF1 is on the pathway of unfolding from the N state to PUF2**, but it cannot, however, be ruled out at the present time that PUF1 and PUF2** form independently and in parallel from the N state.

Misfolding can potentially commence from multiple PUFs

The observation that the logarithm of the observed rate constant of misfolding is linearly proportional to the free energy difference (ΔG_{op}) between the N state and PUF1 as well as PUF2** (Figure 7) suggests that misfolding can potentially commence from both these PUFs. An earlier study identified PUF2* as a precursor conformation from which misfolding begins in the case of full-length moPrP (Pal and Udgaonkar, 2022a). Hence, it appears that misfolding can potentially commence from either of the two isoenergetic but structurally distinct sub-populations, PUF2* and PUF2**. Mutation of the conserved Pro residues ⁴⁴ affects the stability of PUF2*, while mutation of the conserved aromatic residues affects the stability of PUF2**. In this way, misfolding of different mutant variants may commence from or proceed *via* either PUF2* or PUF2**.

Misfolding will, however, predominantly begin from PUF1, as it is considerably more stable than both PUF2* and PUF2**, and would be populated > 30-fold more than them at equilibrium. Hence, PUF1 would be the dominant precursor conformation from which misfolding commences. Misfolding can, however, begin, albeit to very small extents, from PUF2* and PUF2**, which have very different structures (Figure 3.6). If PUF1 is indeed populated on the pathways of unfolding from the N state to PUF2*, and from the N state to PUF2**, it is likely that it is the structure lost during the unfolding of the N state to PUF1, which makes all three PUFs competent to misfold. If PUF1 is not populated on the pathways of unfolding from the N state to PUF2*, and from the N state to PUF2**, then when misfolding commences from PUF1, additional structural loss and conformational conversion would occur only during the oligomerization process.

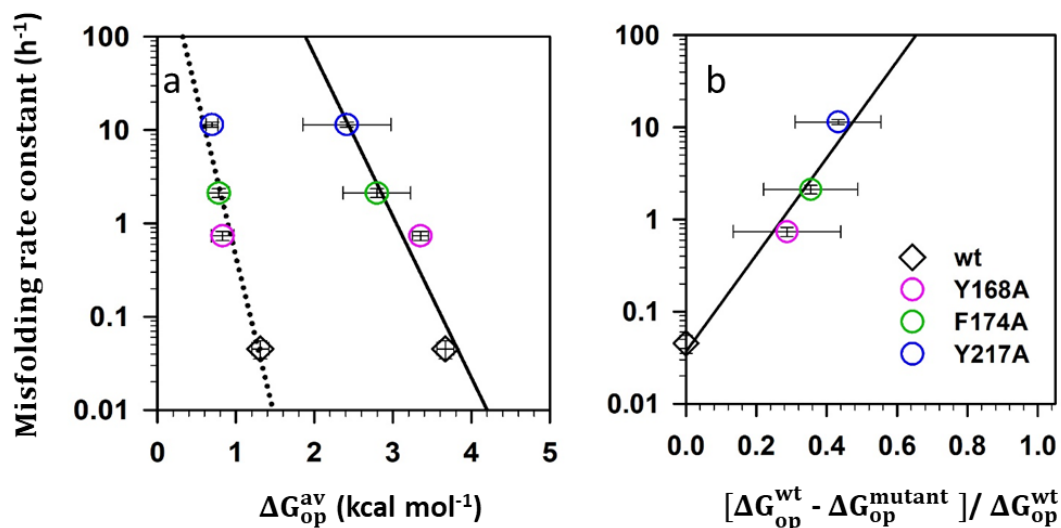


Figure 3.8. Dependence of the observed misfolding rate constant on ΔG_{op} for different sequence segments. (a) Plot of the dependence of the observed rate constant of misfolding of the aromatic mutant variants at 10 μ M concentration in 150 mM NaCl at pH 4, 37 $^{\circ}$ C on the stability (ΔG_{op}^{av}) of PUF1 (dotted line) and PUF2** (solid line). The stabilities (ΔG_{op}^{av}) of PUF1 and PUF2** were determined by averaging the ΔG_{op} values of the sequence segments 96-144, 168-173, 182-196, 210-216 and 225-231 for PUF1 and 154-167 and 217-223 for PUF2**. The continuous lines through the data points are linear fits. (b) Plot of the dependence of the observed rate constant of misfolding of the single aromatic mutant variants at 10 μ M concentration in 150 mM NaCl at pH 4, at 37 $^{\circ}$ C on the fractional destabilisation of the sequence segments 96-144, 154-167, 168-173, 182-196, 210-216, 217-223 and 225-231. Each data point represents the fractional destabilisation averaged over these seven sequence segments. The continuous lines through the data points are linear fits. The error bars represent the standard deviations determined from three independent experiments

Structure lost in PUF1 makes it prone to misfolding

It is important to determine what critical structure is lost in the PUFs which make them start to misfold. Misfolding rate constants will depend not only on the extent to which the PUFs are populated, but also on the nature of the structural perturbation that has occurred to them. A mutation might lead to a small increase in the population of a PUF by changing its stability only to a small extent, but might yet have altered its structure in such a way that drastically lowers the barrier to misfolding.

In the case of PUF1, the sequence segments covering $\beta 1$ (96-144), the $\alpha 1$ - $\beta 2$ loop, the $\beta 2$ - $\alpha 2$ loop (168-173), the C-terminal end of $\alpha 2$ (182-196), the second half of $\alpha 3$ (210-216), and the sequence segment 225-231 are found to have become disordered. An earlier NMR study of the temperature dependence of chemical shifts seen for wt moPrP (Bhate et al., 2021c) had indicated that in the absence of added salt, many residues in $\beta 1$ and the $\beta 1$ - $\alpha 1$ loop (in sequence segment 96-144), as well as Gln211 in the second half of $\alpha 3$ (in sequence segment 210-216), which is adjacent in structure, sample at least one alternative conformation. Both these sequence segments are disordered in PUF1 suggesting that it is the alternative conformation that is sampled.

It is not yet known how the addition of the salt (150 mM NaCl) affects the extents to which the PUFs are populated. The NMR study of the temperature dependence of chemical shifts (Bhate et al., 2021c), indicated that, upon the addition of the salt, the alternative conformation in which the residues in $\beta 1$ and the $\beta 1$ - $\alpha 1$ loop as well as Gln211 in the second half of $\alpha 3$ are perturbed (see above), becomes populated to a greater extent. This could mean that PUF1 becomes populated to a greater extent. Another NMR chemical shift study⁵⁰ also indicated that the salt bridge between Lys193 and Glu195 (in sequence segment 182-196 which is disordered in PUF1), as well as the local environment of Gln211, become structurally perturbed in the monomer upon the addition of the salt, before oligomerisation commences. The very early perturbation of the the salt bridge between Lys193 and Glu195 was also indicated in the burst phase change in the fluorescence of a Trp introduced at residue position 197, before misfolding commences. These previous results support the results of the current study that misfolding commences primarily from PUF1.

Of the sequence segments that have become disordered in PUF1, the C-terminal end of $\alpha 2$ (182-196) contains the sequence ¹⁸⁷TVTTTT¹⁹², which has a high propensity for β -sheet formation (Dima and Thirumalai, 2004), but is part of the helix $\alpha 2$. The $\beta 2$ - $\alpha 2$ loop (168-173) spans the sequence ¹⁶⁹SNQNNF¹⁷⁴, and a peptide with this sequence is known to be highly

amyloidogenic (Huang and Caflisch, 2015). When these sequence segments become disordered in PUF1, they would become available for potential intermolecular interactions that facilitate misfolding. Moreover, the tertiary interactions between the $\beta 2$ - $\alpha 2$ loop and the second half of $\alpha 3$, which are known to play a crucial role in prion misfolding, are also perturbed in PUF1, making it prone to misfolding.

The $\beta 2$ - $\alpha 2$ loop interacts also with residues at the C-terminal end of $\alpha 3$, which are disordered in PUF2**, and perturbation of these intramolecular interactions would appear to be required for the formation of the specific intermolecular contacts that are involved in PrP conversion (Bett et al., 2012; Kurt et al., 2014a; Meli et al., 2011b). It appears that mammals carrying a flexible $\beta 2$ - $\alpha 2$ loop are more easily infected than mammals with a rigid loop (Scialò et al., 2019; Zhang, 2011). *In vivo* as well as *in vitro* studies have revealed that several residues in the $\beta 2$ - $\alpha 2$ loop can regulate the formation of PrP^{Sc} by influencing the structural stability of this loop (Bett et al., 2012; Kurt et al., 2014a; Kurt et al., 2014b; Sigurdson et al., 2011). PrP^C structures that are characterised by a rigid $\beta 2$ - $\alpha 2$ loop, have a closer contact between the loop and the C-terminal half of $\alpha 3$ (Scialò et al., 2019). The spontaneous formation of PrP^{Sc} in the cases of the E200K (Meli et al., 2011b), Y217N (Jung Cheng and Daggett, 2014), V210I (Biljan et al., 2011), Q212P (Giachin et al., 2011), and Q226E (Slapšak et al., 2019a) mutant variants, which are associated with familial prion diseases, is thought to be caused by the disruption of the long range interactions between the $\beta 2$ - $\alpha 2$ loop and the C-terminal half of $\alpha 3$.

Sub-domain separation is facilitated by two different mechanisms

It has been proposed that during the misfolding of the prion protein, the separation of the $\alpha 2$ - $\alpha 3$ and the $\beta 1$ - $\alpha 1$ - $\beta 2$ subdomains must occur before conversion of $\alpha 2$ and $\alpha 3$ to the β -conformation later during the misfolding process (Goluguri et al., 2019a; Hadži et al., 2015a; Sengupta and Udgaonkar, 2019a). Locking of the $\alpha 2$ - $\alpha 3$ sub-domain to the $\beta 1$ - $\alpha 1$ - $\beta 2$ subdomain by binding to anti-prion drugs (Kamatari et al., 2013; Kuwata et al., 2007b), prevents misfolding and oligomerisation, presumably by stabilizing the N state with respect to an aggregation competent PUF. When a disulfide bond was introduced between the $\beta 2$ - $\alpha 2$ loop and the C-terminal half of $\alpha 3$, the formation of misfolded oligomers was significantly reduced, which suggested that the interactions between the two-subdomains guarded against misfolding (Prigent and Rezaei, 2011b). In the case of the mutant variants studied here, the perturbation of the aromatic and hydrophobic interactions between the $\beta 2$ - $\alpha 2$ loop and the C-terminal part of $\alpha 3$ appears to result in a decrease in the local stability of the $\beta 2$ - $\alpha 2$ loop. This loop serves as

a hinge loop connecting the two sub-domains, whose perturbation facilitates their separation. It seems that the decreases in the local stabilities (ΔG_{op}) of the sequence segments covering $\beta 1$ (96-144) and the C-terminal end of $\alpha 2$ (182-196), which are far from the site of mutations, is a consequence of the separation of the two subdomains. Several disease-linked mutations (Singh and Udgaonkar, 2015c), as well as mutations of the CTD Pro residues (Pal and Udgaonkar, 2022a), facilitate sub-domain separation by perturbing the electrostatic interaction network at one end of the protein. This electrostatic network is comprised of salt bridges formed by residues present on $\alpha 1$ of the $\beta 1$ - $\alpha 1$ - $\beta 2$ subdomain, and the loop between $\alpha 2$ and $\alpha 3$, and the N terminal end of $\alpha 3$ of the $\alpha 2$ - $\alpha 3$ subdomain (Figure 3.9a). In the case of the mutant variants studied here, this electrostatic interaction network is largely unaffected as the ΔG_{op} values of the sequence segments $\alpha 1$ (144-148, 149-153), and 197-204 (the loop between $\alpha 2$ and $\alpha 3$ and the N terminal end of $\alpha 3$) of the mutant variants were found to be same to those of wt moPrP. Nevertheless, sub-domain separation appears to be facilitated by the perturbation of aromatic and hydrophobic interactions present at the other end of the protein (Figure 3.9b). This result reveals that subdomain separation can be triggered by two different structural mechanisms in which PUF1 can potentially misfold *via* both PUF2* and PUF2**, in which electrostatic interactions at one end and aromatic interactions at the other end of the protein, respectively, are perturbed.

A previous study utilizing single-molecule force spectroscopy (Yu et al., 2012a) had identified multiple, off-pathway partially structured intermediates that fold from the U state, but no structural information could be obtained, nor was it shown that they lead to misfolding. In a previous study utilizing fluorescence correlation spectroscopy, the N state was shown to be in rapid dynamic equilibrium with two native-like states (Goluguri et al., 2019a), but, again, these states could not be characterized structurally. In this study, as well as in a previous study (Pal and Udgaonkar, 2022a), the structures of partially unfolded forms (PUF1, PUF2* and PUF2**) from which misfolding can commence has been obtained. Both PUF2* and PUF2** have the same stability and would be populated equally at equilibrium. Since PUF2* and PUF2** differ in the regions of the protein structure that have become disordered, they must form in parallel from N, on independent pathways. It is interesting that the loss of very different elements of secondary structure can destabilize a protein to very similar extents.

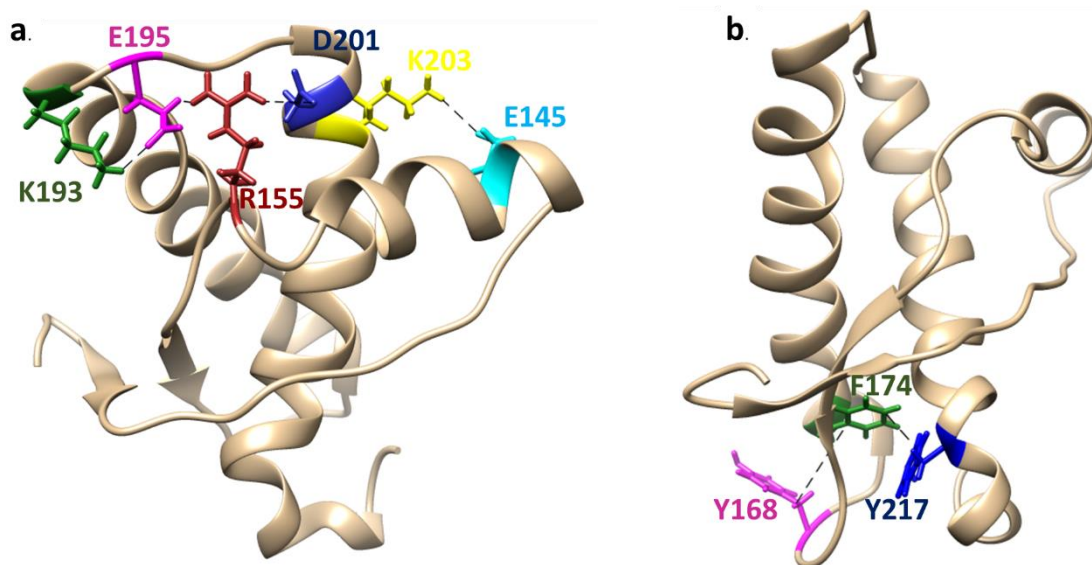


Figure 3.9. (a) Stabilizing electrostatic network formed between Arg155 (brown) and Glu195 (purple), Arg155 and Asp201 (blue), Lys193 (green) and Glu195, Lys203 (yellow) and Glu145 (cyan). This network of electrostatic interactions is destabilised in PUF2*. (b) Stabilizing aromatic network formed between Tyr168 (purple) and Phe174 (green), Phe174 and Tyr217 (blue). This network of aromatic interactions is destabilised in PUF2**. The figure was drawn using Chimera and the Protein Data Bank entry 1AG2.

Chapter 4

Slow misfolding of mutant variant of a prion protein variant into a β -rich dimer

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Introduction

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), include fatal neurodegenerative conditions such as scrapie in sheep, bovine spongiform encephalopathy in cattle, chronic wasting disease in cervids, as well as human disorders such as Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker disease, and fatal familial insomnia (Aguzzi and Polymenidou, 2004; Caughey, 2001; Collinge, 2001; Prusiner, 1982b; Prusiner, 1998). These diseases, whether occurring spontaneously, *via* genetic mutation, or through infection, are characterized by the conversion of the native cellular prion protein (PrP^C) into a misfolded form, PrP^{Sc} (Prusiner, 1982b; Prusiner, 1998). Mature PrP^C is a 208-residue glycoprotein consisting of an unstructured N-terminal region, and a structured globular C-terminal domain comprising three α -helices and two very short β -strands (Riek et al., 1996b). PrP^C and PrP^{Sc} exhibit significant differences in their biophysical characteristics. PrP^C is monomeric, soluble, sensitive to proteinase digestion, and predominantly α -helical in structure, while PrP^{Sc} is multimeric and highly insoluble, displays resistance to proteinase digestion, and possesses a substantial amount of β -sheet structure (Aguzzi and Polymenidou, 2004; Glynn et al., 2020; Manka et al., 2022a; Prusiner, 1998; Wang et al., 2020a).

The precise physiological role of the prion protein remains elusive. Nevertheless, potential functions such as maintaining copper levels (Vassallo and Herms, 2003), protecting against stress and neuronal damage (Bravard et al., 2015; Choi et al., 2007), as well as controlling cell proliferation (Limone et al., 2023), cell signaling (Benarroch, 2024; Haigh et al., 2010) and cell adhesion (Schmitt-Ulms et al., 2001), have been proposed. In the case of transgenic PrP-knocked-out mice, no significant abnormalities were observed, except age-related cognitive decline, implying a possible redundancy in the protein's function (Büeler et al., 1992; Steele et al., 2007b).

Although it is clear that prion diseases are associated with the misfolding of PrP, the final misfolded forms can be very heterogeneous, and the identity of the misfolded forms that lead to disease is not clear. Misfolding of PrP can lead to oligomer as well as fibril formation (Baskakov et al., 2002a; Jain and Udgaonkar, 2011b; Singh and Udgaonkar, 2015a). Small non-fibrillar β -rich soluble oligomers have been suggested to be the most efficient mediators of prion infectivity (Silveira et al., 2005a) and have been shown to exhibit more neurotoxicity than the fibrils, both *in vitro* and *in vivo* (Simoneau et al., 2007). Small β -rich oligomers have been implicated as the toxic species in other amyloid-related diseases as well (Bucciantini et al., 2004; Krishnan et al., 2012; Laganowsky et al., 2012; Tomic et al., 2009).

It is likely that the misfolding of PrP begins when it encounters a low pH in the endocytic pathway (Borchelt et al., 1992b). β -rich oligomers are formed at low pH, and these can trigger cellular apoptosis (Bucciantini et al., 2004), impede the functioning of the 26S proteasome (Kristiansen et al., 2007), and readily perturb lipid membranes (Caughey et al., 2009; Chich et al., 2010), any of which might account for their toxicity. It is important to note that the susceptibilities of different mammals to sporadic prion diseases correlate well with the ability of their PrP to misfold into β -rich oligomers at pH 4 (Khan et al., 2010a). *In vitro* studies have shown that the mouse prion protein readily undergoes misfolding and oligomerization at acidic pH when either His186 or Asp201 becomes protonated, with a transition midpoint at pH 4.7 (Singh and Udgaonkar, 2016f). Oligomerization occurs nearly fully at pH 4, and while it can be observed at one pH unit above the transition midpoint (Singh and Udgaonkar, 2016f), it cannot be studied at physiological pH, where only a miniscule fraction of protein molecules would have either His186 or Asp201 in their protonated form. While oligomer formation requires these residues to be protonated (Singh and Udgaonkar, 2016f; Van der Kamp and Daggett, 2010b), the presence of salt is also crucial (Bhate et al., 2021c; Sengupta et al., 2017a). In the absence of salt, misfolding and oligomerization of wt moPrP do not occur on an experimentally amenable time scale at pH 4, emphasizing the indispensable role of salt in these processes (Sengupta et al., 2017a). Salt is known to disrupt specific intramolecular interactions in the native protein, such as a crucial salt bridge between residues Lys193 and Glu195. Specific anion binding and salt-induced screening of electrostatic interactions in the monomeric protein emerge as critical factors in initiating misfolding and oligomerization (Bhate et al., 2021c; Sengupta et al., 2017a).

The key to understanding the mechanism of the spontaneous conversion of the α -helix-rich monomeric prion protein into misfolded β -rich oligomers is the identification of aggregation-prone intermediate conformations that are sparsely populated through native state fluctuations. Indeed, the unusually high specific heat capacity of the native state of the prion protein, compared to proteins of similar size, is indicative of it undergoing significant structural fluctuations (Moulick and Udgaonkar, 2014). HDX-MS/NMR measurements have shown that the native state of the prion protein at pH 4 exists in dynamic equilibrium with multiple partially unfolded intermediates (PUFs), which could serve as the precursor states from which misfolding begins (Moulick et al., 2015a; Pal and Udgaonkar, 2022a, 2023). It has also been shown that certain evolutionarily conserved residues act as 'gatekeeper residues', regulating the

extent to which PUFs are transiently populated, by modulating the energy difference between them and the native state (Pal and Udgaonkar, 2022a, 2023).

The molten globule (MG) form of a protein is a PUF that can be significantly populated at equilibrium. It is a partially folded, flexible form with exposed hydrophobic residues, significant secondary structure, and disrupted tertiary structure. MGs typically have a native-like topology and secondary structure but lack proper packing and tertiary interactions (Kuwajima, 1989). They are critical early intermediates in the misfolding of multiple proteins (Booth et al., 1997; Carrotta et al., 2001; Juneja et al., 2002; Khurana and Udgaonkar, 1994; Uversky et al., 2001a), including the prion protein (Honda et al., 2014a; Singh and Udgaonkar, 2016b). A capability to completely convert the N state of the mouse PrP (moPrP) to the MG form at equilibrium is expected to enable elucidation of the structural mechanism of misfolding and oligomerization of the latter, which has so far eluded characterization. For several proteins, it has been shown that partially unfolded forms, including MG forms, can be populated at equilibrium by perturbing tertiary interactions that are critical in stabilizing the native state (Nath and Udgaonkar, 1995; 51; 52; Skagg et al., 1998). In the case of mammalian prion proteins, aromatic interactions involving Tyr168, Phe174, and Tyr217 (mouse numbering has been used), which stabilize the interface of the β 2- α 2 loop with the C-terminal end of α 3 (54) (Figure 4.1), are amongst the most conserved long-range interactions. Their disruption is implicated in the spontaneous formation of PrP^{Sc} by disease-associated mutant variants (55-56). It seemed likely that a mutant variant lacking these critical tertiary interactions, might exist as a MG under conditions amenable to study its conversion to misfolded oligomer.

In this study, it is shown that the mutant variant 3A moPrP, created by the simultaneous mutation of Tyr168, Phe174, and Tyr217 to Ala, indeed exists in a MG conformation. Unlike wt moPrP, which requires the addition of 150 mM NaCl to initiate misfolding and oligomer formation at pH 4, 3A moPrP misfolds even in the absence of added salt, but to form a dimer. Upon the addition of 150 mM NaCl, the dimer forms an oligomer that is structurally similar to it. This study provides important insight into the structural mechanism of the misfolding of the prion protein, and into the role of misfolded dimer in oligomer formation.

Materials and Methods

Buffers and reagents

The reagents used in this study were of high purity grade, and were obtained from Sigma. Ultra-pure guanidine hydrochloride (GdnHCl), of the highest purity grade, was procured from United States Biochemicals.

Site-directed mutagenesis

The mutant variant of full-length moPrP, 3A moPrP, was generated using the Quickgene site-directed mutagenesis kit (Stratagene). Primers containing 3 nucleotide changes were obtained from Sigma. 3A moPrP contains the three mutations Y168A, F174A and Y217A. The mutations were confirmed by DNA sequencing.

Protein expression and purification

wt moPrP and 3A moPrP were expressed in *Escherichia coli* BL21(DE3) codon plus cells (Stratagene), that had been transformed with a pET17b plasmid containing the full-length sequence (23-231) of the moPrP gene. The variants were purified as described previously (Jain and Udgaonkar, 2008; Singh et al., 2014a). The purity and mass of each moPrP variant preparation were confirmed by SDS-PAGE and mass spectrometry using a Synapt G2 HD mass spectrometer (Waters Corporation).

Far-UV CD measurements

Far-UV CD spectra were recorded on a Jasco J-815 spectropolarimeter using a protein concentration of 10 μ M in a 1 mm cuvette, using a scan speed of 50 nm/min, a digital integration time of 2 s, and a bandwidth of 1 nm. The wavelength was scanned from 200 to 250 nm, and a total of 15 spectra were averaged. Far-UV CD spectra under native conditions were acquired in 10 mM sodium acetate buffer, at pH 4, 25 °C.

Urea-induced equilibrium unfolding studies

Urea-induced equilibrium unfolding studies were carried out in 10 mM sodium acetate buffer at pH 4, 25 °C. 10 μ M protein was incubated at different urea concentrations for 1 h, before the far-UV CD signal at 222 nm was measured. The data were fit to a two-state N $\leftrightarrow$ U equilibrium unfolding model (Agashe and Udgaonkar, 1995), and the thermodynamic parameters were obtained.

Misfolding studies at pH 4 in the presence of salt

Misfolding was initiated by diluting 100 μ M wt moPrP or 3A moPrP in 10 mM sodium acetate buffer (pH 4) two-fold with 2x misfolding buffer (10 mM sodium acetate buffer

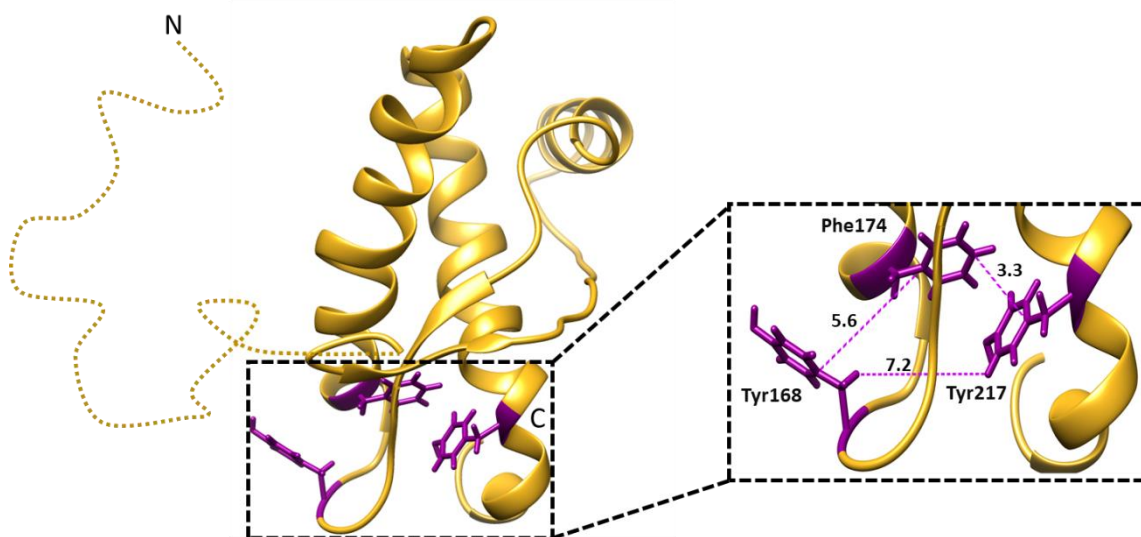


Figure 4.1. Structure of the C-terminal domain of the mouse prion protein showing the locations of the three evolutionary conserved aromatic residues, Tyr168, Phe174, and Tyr217. The N-terminal domain, shown as a dotted line, spans residues 23-120, and is unstructured in the full-length protein. The three conserved aromatic residues that were mutated in this study are colored in magenta. The side-chains of Tyr168, Phe174, and Tyr217 are buried to the extents of 40 %, 100 %, and 90 %, respectively. The interactions between Tyr168, Phe174, and Tyr217 are the most conserved aromatic-aromatic interactions observed in the structures of different mammalian prion proteins. The distances between the side-chains are shown in Å. The figure was drawn using Chimera and Protein Data Bank entry [1AG2](#).

containing 300 mM NaCl, pH 4). The samples were then incubated at 37 °C. At different times of misfolding, aliquots of the incubated sample were withdrawn and diluted to 10 µM in 1x misfolding buffer (10 mM sodium acetate buffer containing 150 mM NaCl, pH 4). Far-UV CD spectra were acquired using the parameters mentioned above. The fraction misfolded form (f_m) was calculated using the following equation:

$$f_m = \frac{\Theta_t - \Theta_0}{\Theta_{100} - \Theta_0}$$

Here, Θ_t and Θ_0 are the ellipticities at 222 nm at time t and at time $t = 0$ of the misfolding process, respectively, and Θ_{100} is the ellipticity at 222 nm of the completely misfolded form.

To study the kinetics of misfolding of 3A moPrP by measurement of θ_{222} , a 10 mm quartz cuvette maintained at 25 °C was used. The reaction was started by mixing 2 µM moPrP solution with an equal volume of 2x misfolding buffer. The time taken from the mixing of the protein solution with 2x misfolding buffer to the first reading was 15 s.

Misfolding studies at pH 4 in the absence of salt

100 µM 3A moPrP in 10 mM sodium acetate buffer (pH 4) was incubated at 37 °C. At different times of misfolding, aliquots were withdrawn and diluted to 10 µM in 10 mM sodium acetate buffer pH 4. Far-UV CD spectra were acquired for analysis using the parameters mentioned above. The fraction misfolded form (f_m) was calculated using equation 1.

Dynamic and static light scattering measurements

Dynamic Light Scattering (DLS) measurements were carried out on a DynaPro-99 instrument (Wyatt Technology Corp.). All the buffers were filtered through 0.02 µm filters (Whatman). Samples were centrifuged at 15,000 g for 30 min before acquisition. The scattering intensity at 90 °, and its autocorrelation, were acquired simultaneously using a laser at 829.4 nm to illuminate the sample. 50 acquisitions were collected in each experiment. The acquisition time was set at 5 s, the signal-to-noise ratio threshold at 2.5, the temperature at 25 °C, and the sensitivity at 80%. Data with uneven autocorrelation functions were excluded. The data were resolved into a Gaussian distribution using DynaLS (Protein Solutions Ltd.)

Measurements of static light scattering were performed using a DAWN 8+, eight-angle light scattering instrument (Wyatt Technology Corp., Santa Barbara, CA). Each sample was diluted to 50 µM, centrifuged at 15,000g for 30 min, and then run through a 0.02-µm filter at a constant rate into the light scattering fused silica flow cell for determining its mass. Data were

analyzed using the software Astra. Normalization of the scattering intensity was done using a solution of bovine serum albumin.

Fibril formation at pH 7

Fibrillation experiments at pH 7 were carried out using a Fluoroskan Ascent plate reader in a 96-well plate. To initiate fibrillation, 50 μ M protein in a 10 mM sodium acetate buffer (pH 4) was diluted by half with 2 $\times$ aggregation buffer [100 mM Tris-HCl and 4 M GdnHCl, pH 7.4], resulting in a final protein solution in 1 $\times$ aggregation buffer [50 mM Tris-HCl and 2 M GdnHCl, pH 7]. The final protein concentration for all experiments was 25 μ M, and 50 μ M ThT was added to each reaction sample. Subsequently, the protein in 1 $\times$ aggregation buffer was transferred into different wells of a 96-well plate in 300 μ L volumes, followed by incubation at 37 °C with agitation at 480 rpm. ThT fluorescence was then measured at 475 nm upon excitation at 445 nm, with ThT readings collected at 10-min intervals.

Seed formation and seeding reactions

At a time corresponding to $3 \times t_{50}$ of the fibrillation reaction (t_{50} was the time at which the fluorescence intensity reached 50% of its final value), the aggregated protein solution was centrifuged at 20,000g for 30 min. The pellet containing the aggregated protein was resuspended in 1 $\times$ aggregation buffer [50 mM Tris-HCl and 2 M GdnHCl, pH 7], and sonicated as described previously (Kumar and Udgaonkar, 2018). This sonicated solution was used to seed a fresh fibrillation reaction, using a fixed concentration of seed (10% v/v). Four types of seeded fibrillation reactions were carried out: wt moPrP monomer with wt moPrP seeds (wt [wt]), wt moPrP monomer with 3A moPrP seeds (wt [3A]), 3A moPrP monomer with wt moPrP seeds (3A [wt]), 3A moPrP monomer with 3A moPrP seeds (3A [3A]). The final ThT fluorescence intensity was found to be similar in each case.

HDX-MS measurements

For all HDX labeling experiments, the labeling buffer was 10 mM sodium acetate buffer ($\pm$ 150 mM NaCl) in D₂O at pH 4, corrected for the isotope effect.

Monomers: The peptide map of the moPrP variants was generated as described previously (Pal and Udgaonkar, 2022a). To initiate deuterium labeling, 100 μ M protein was diluted into labeling buffer, so that HDX occurred in 95% D₂O, at 25 °C. At different times, a 50 μ L aliquot was mixed with 50 μ L ice-cold 20 mM glycine-HCl buffer (pH 2.5) to quench the labeling reaction. Online pepsin digestion was carried out by injecting the samples immediately into an immobilized pepsin cartridge (Applied Biosystems) at a flow rate of 40 μ L/ min of water (0.05% formic acid) which was placed in a HDX module (Waters Corporation) coupled to a

nanoAcquity UPLC. The peptides eluting from the pepsin column were collected using a trap column, washed to remove salt, and eluted over 12 min on an analytical C18 reversed-phase chromatography column using a gradient of 3 to 40% acetonitrile (0.1% formic acid) at a flow rate of 40 μ l/ min. All columns were kept at 4 °C to minimize back exchange. The mass spectrometer parameters were set as follows: source temperature, 35 °C; desolvation temperature, 100 °C; capillary voltage, 3.0 kV.

Peptide masses were calculated using MassLynx from the centroid of the isotopic envelope. The shifts in the masses of labeled peptides relative to unlabeled peptides yielded the extent of deuterium incorporation at each time point of HDX. Since the sample was in 95% D₂O during labeling, and exposed to H₂O during quenching, control experiments were carried out to correct for back exchange. For this, the protein was incubated in 10 mM sodium acetate, pH 4 (in 95% D₂O), and deuterated by unfolding at 65 °C for 15 min, followed by refolding on ice. CD spectroscopy and thermal equilibrium unfolding studies showed that the refolded protein behaved like the native protein. The 95% deuterated moPrP samples were processed in the same way as the labeling reaction samples. The extent of deuterium incorporation in each peptide fragment, % D, was calculated using the equation:

$$\%D = \frac{m(t) - m(0\%)}{m(95\%) - m(0\%)} \times 100$$

where $m(t)$ is the measured centroid mass at time t , $m(0\%)$ is the measured mass of the undeuterated reference peptide, and $m(95\%)$ is the measured mass of a 95% deuterated reference peptide.

To calculate the free energy of opening (ΔG_{op}) of a sequence segment, the observed rate constant of deuterium incorporation (k_{obs}) was first obtained by fitting the kinetic curve of HDX to either a single exponential or a two-exponential equation, depending upon the goodness of fit. For each sequence segment, k_{obs} was compared to the rate constant (k_{int}) that would be observed in the corresponding peptide fragment in a random coil state (Bai et al., 1993b; Nguyen et al., 2018). The quantity $P_f = k_{int}/k_{obs}$ is the protection factor that slows down HDX into the sequence segment. The protection factor determined for each peptide fragment was used to calculate the local stability of the corresponding sequence segment in the protein, $\Delta G_{op} = RT \ln P_f$. For each sequence segment, ΔG_{op} is, therefore, the free energy of opening of the N state to form a HDX-competent partially unfolded form.

Oligomers and dimers: Oligomers prepared by incubation of 100 μ M protein in 150 mM NaCl, 10 mM sodium acetate, pH 4, at 37 °C (for a time corresponding to 5 time constants of the misfolding reaction) were concentrated to 500 μ M by centrifugation (10000 g) using a 10 kDa molecular mass cut-off filter. Deuterium labeling was initiated by diluting 5 μ l of oligomers into 45 μ l of 1x misfolding buffer prepared in D₂O. Labeling was allowed to occur at 25 °C for 300 s or 3000 s. Then, 450 μ l of ice-cold quenching buffer (8 M GdnHCl, 100 mM glycine, pH 2.5) was added to stop the labeling reaction and dissolve the oligomers. After 2 min of incubation on ice, the samples were desalted using a Sephadex G-25 HiTrap desalting column pre-equilibrated with water at pH 2.5, in conjunction with an AKTA Basic HPLC. The desalted samples were injected into the HDX module coupled with the nanoAcquity UPLC, for online pepsin digestion (see above). Exchange experiments of native moPrP were also carried out in a similar way after 10-fold dilution of the protein in labeling buffer containing buffer. To mimic the digestion conditions used for oligomers, 50 μ l of a deuterium-labeled sample of native moPrP was mixed with 450 μ l of ice-cold quenching buffer containing 8 M GdnHCl, 100 mM glycine, pH 2.5, and processed in the same way as were the oligomers.

Dimers were prepared by incubation of 100 μ M protein in 10 mM sodium acetate, pH 4, at 37 °C, for 30 days. Deuterium labeling was initiated by diluting 10 μ l of dimer into 90 μ l of labeling buffer. Labeling was allowed to occur at 25 °C for 300 s or 3000 s. Then 200 μ l of ice-cold quenching buffer (8 M GdnHCl, 100 mM glycine, pH 2.5) was added to stop the labeling reaction and dissolve the dimers. Samples were then processed and injected into the HDX module, as was done in the case of oligomers. Exchange experiments of native 3A moPrP were also carried out in a similar way after 10-fold dilution of the protein in 10 mM sodium acetate into D₂O containing buffer (pH 4, corrected for the isotope effect). To mimic the digestion conditions used for dimers, 100 μ l of the deuterium-labeled sample of native 3A moPrP were mixed with 200 μ l of ice-cold quenching buffer containing 8 M GdnHCl, 100 mM glycine, pH 2.5, and processed in the same way as were the dimers.

Peptide masses were calculated from the centroids of the isotopic envelopes, using the MassLynx software, and the shift in the mass of a labeled peptide relative to that of the unlabeled peptide was used to determine the extent of deuterium labeling at 300 s and 3000 s of HDX. As the sample was in 90% D₂O during labeling and exposed to H₂O after dissolution in GdnHCl, control experiments were carried out to correct for back exchange. To this end, the protein was incubated in 10 mM sodium acetate, pH 4 (in 90% D₂O), and deuterated by unfolding at 65 °C for 15 min, followed by refolding on ice. The 90% deuterated samples were

processed in the same way as the labeling reaction samples. The extent of deuterium incorporation in each peptide, % D, was calculated using equation 2.

HDX-MS monitored dimerization kinetics of 3A moPrP

100 μ M 3A moPrP in 10 mM sodium acetate buffer at pH 4 was incubated at 37 °C. Pulses of HDX labeling were given at different times of misfolding. At each time, a 10 μ l aliquot was withdrawn and diluted into 90 μ L of labeling buffer. After labeling for 300 s, 100 μ L samples were mixed with 200 μ L of ice-cold 8 M GdnHCl in 100 mM glycine buffer (pH 2.5) to dissolve the dimers. Samples were then processed and injected into the HDX module, as described above. Further processing of the sample for mass determination using a Waters Synapt G2 mass spectrometer was carried out as described above. In order to determine the rate constants of structural change in different sequence segments during dimerization, the kinetic traces of deuterium incorporation ($D(t)$) were fit to either equation 3a or equation 3b.

$$D(t) = D_0 + D_1(1 - e^{-k_1 t}) \quad (3a)$$

$$D(t) = D_0 + D_1(1 - e^{-k_1 t}) + D_2(1 - e^{-k_2 t}) \quad (3b)$$

Here, D_0 is the % deuterium incorporation at 0 min, and D_1 and D_2 are the amplitudes of the fast and slow phases, whose rate constants are given by k_1 and k_2 , respectively. The amplitude-averaged rate constant, k_{app} , is given by

$$k_{app} = \frac{1}{\left(\frac{\alpha_1}{k_1}\right) + \left(\frac{\alpha_2}{k_2}\right)} \quad (3c)$$

In equation (3c), α_1 and α_2 are the fractional amplitudes of the fast and slow phases, calculated as

$$\alpha_1 = \frac{D_1}{D_1 + D_2} \text{ and } \alpha_2 = \frac{D_2}{D_1 + D_2}$$

Results

Mutation of the three conserved aromatic residues induces changes in both secondary and tertiary structure

The far-UV CD spectrum of 3A moPrP showed characteristics of a helical protein, as seen in Figure 4.2a. However, a significant difference was observed in the shape of its spectrum, along with a reduction in the mean residual ellipticity, specifically at 222 nm, when compared to the corresponding far-UV CD spectrum of wt moPrP (Figure 4.2a). The observed changes indicated that 3A moPrP possesses less secondary structure than wt moPrP.

Dynamic light scattering (DLS) measurements yielded hydrodynamic radii (R_H) of 2.8 ± 0.2 nm and 4.0 ± 0.3 nm, for wt and 3A moPrP, respectively (Figure 4.2b). The values of R_H indicated that both wt and 3A moPrP do not form oligomers (which have been reported to have a R_H of 12 ± 1 nm) but remain as monomers. The larger R_H observed for 3A moPrP suggested that it might exist as a molten globule. To confirm that 3A moPrP exists as a monomer, multi-angle light scattering (MALS) experiments were carried out. The mass (molecular weight) obtained for 3A moPrP was 25.1 ± 1.4 kDa, and for wt moPrP was 23.4 ± 0.8 kDa (data not shown), confirming that both protein variants exist as monomers.

The fluorescence characteristics of the dye 8-anilinonaphthalene-1-sulfonic acid (ANS) provide valuable insights into the exposed hydrophobic regions of proteins. ANS preferentially binds to hydrophobic surfaces of proteins, undergoing a substantial increase in its fluorescence intensity as well as a blue shift in its wavelength of maximum emission (Semisotnov et al., 1991). The presence of wt moPrP did not increase the ANS fluorescence intensity, although a ~ 7 nm blue shift in the wavelength of maximum emission was observed (Figure 4.2c). In contrast, the presence of 3A moPrP increased the ANS fluorescence intensity significantly (~ 3.2 -fold), as well as resulted in a ~ 28 nm blue shift in the wavelength of maximum emission (Figure 4.2c). Hence, 3A moPrP has substantially more solvent-exposed hydrophobic surface than does wt moPrP.

Mutation of the three aromatic residues results in drastic destabilization and loss of cooperativity of the unfolding transition

Denaturant-induced unfolding studies revealed that the thermodynamic stability of 3A moPrP is significantly lower than that of wt moPrP at pH 4 (Figure 4.2d). Importantly, 3A moPrP showed a loss of cooperativity of the unfolding transition (Figure 4.2d), suggesting that its structural integrity was compromised compared to that of wt moPrP. Furthermore, the denaturant-induced unfolding transition of the 3A moPrP was observed to be notably broader than that of wt moPrP (Figure 4.2d). Broad, non-cooperative unfolding transitions are characteristic of MG forms of proteins (Kuwajima, 1989).

Effect of mutation on protein dynamics

It was important to check how the mutation of the three conserved aromatic residues impacts the structural dynamics of the protein, as 3A moPrP exhibited an altered secondary structure as well as a significant decrease in both thermodynamic stability and cooperativity. Hydrogen-deuterium exchange studies coupled with mass spectrometry (HDX-MS) were carried out to gather sequence-specific insights on the structural dynamics. The HDX

experiments were carried out at pH 4, where the intrinsic rate constant of HDX is slow (Bai et al., 1993b). This allowed HDX to be monitored at amide hydrogen sites spread over all the secondary structural elements of the protein. Figure 4.3 shows the kinetics of deuterium incorporation into different sequence segments of the CTD of moPrP, for both wt and 3A moPrP. The deuterium incorporation rate constants observed for 3A moPrP were significantly faster than the rate constants observed for wt moPrP (Figure 4.3). This indicated that all the sequence segments of 3A moPrP had increased structural dynamics, as manifested by lower protection factors against HDX, compared to those seen for wt moPrP. The largest mutation-induced changes were seen in sequence segments 144-167 and 205-212. Sequence segment 144-167 encompasses helix 1 and the loop between $\alpha 1$ and $\beta 2$, and is away from the sites of mutation. Sequence segment 205-212 is part of the hydrophobic core and is the most stable sequence segment of the protein. The large increase in the observed rate constant of deuterium incorporation suggested that the mutations indirectly disrupt the hydrophobic core.

The values of ΔG_{op} for the different sequence segments of wt moPrP and 3A moPrP are compared in Figure 4.4. It is seen that mutation of the three conserved aromatic residues decreased the values of ΔG_{op} for sequence segments spread over all the secondary structural units of the protein. This suggests that the three mutations not only affected the local stabilities of the sequence segments near the site of mutations, but also of distant sequence segments.

3A moPrP misfolds to a dimer in the absence of salt

The soluble oligomers formed at pH 4 in the presence of 150 mM NaCl, have been shown to be β -rich, with a hydrodynamic radius (R_H) of 12 ± 1 nm. While in the absence of salt, wt moPrP does not misfold and oligomerize at pH 4 on an experimentally tractable time scale, 3A moPrP was found to misfold into a β -rich conformation (Figure 4.5a). The misfolding of 3A moPrP in the absence of salt was, however, slow (Figure 4.5a and b). The rate constant of misfolding was found to be dependent on protein concentration, as expected when misfolding is accompanied by protein association to form a dimer or higher oligomer. A plot of the logarithm of the observed rate constant of misfolding *versus* the logarithm of protein concentration was found to have a slope of 2.6 (data not shown), indicating that the product of misfolding was a dimer. Interestingly, the misfolded β -rich conformation of 3A moPrP formed in the absence of salt at pH 4 was found to have a much smaller hydrodynamic radius ($R_H \sim 5.6$ nm) (Figure 4.5c) than the oligomers of wt moPrP formed in the presence of salt. The molar mass of the misfolded conformation of 3A moPrP formed in the absence of salt was determined by MALS measurements. It was determined that 3A moPrP formed a misfolded β -rich dimer

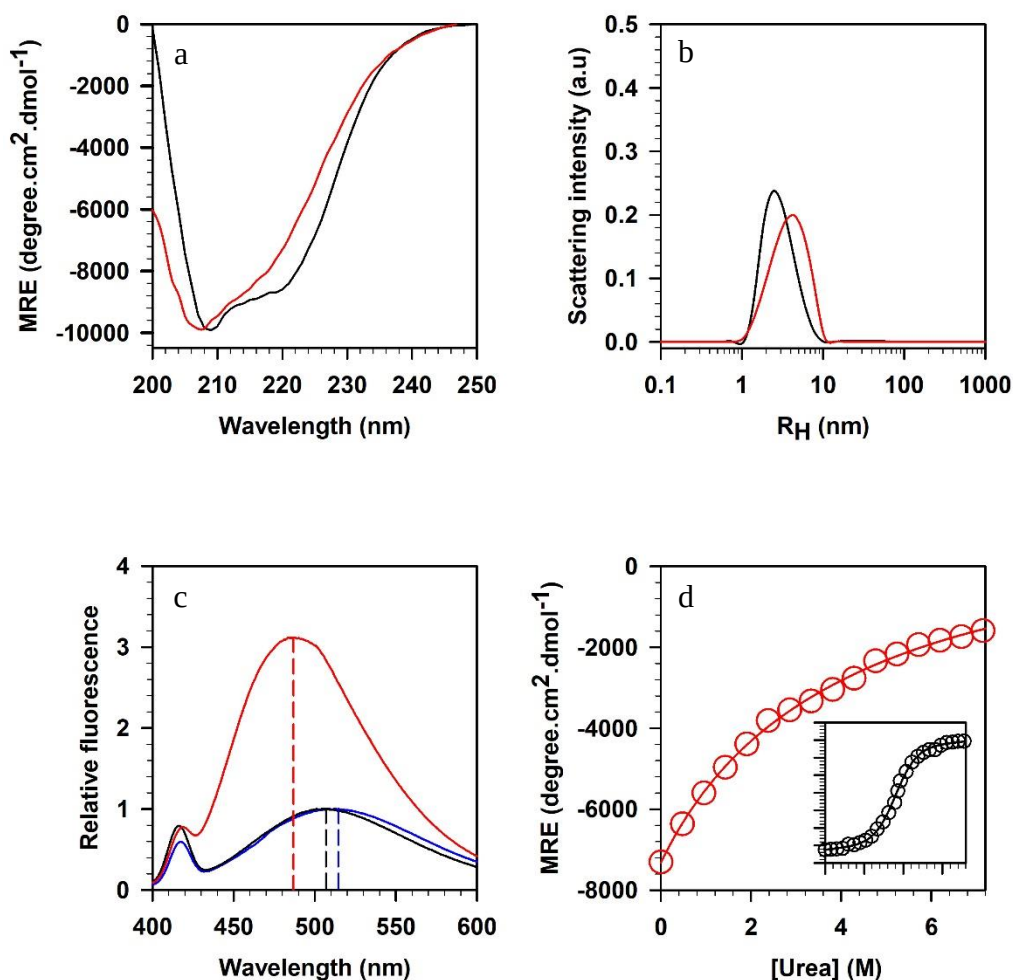


Figure 4.2. Structure and stability of native monomeric wt and 3A moPrP at pH 4, 25 °C. (a) Far-UV CD spectra of the native monomeric forms of the moPrP (23-231) variants. (b) Hydrodynamic radii (R_H) of wt and 3A moPrP, as determined from dynamic light scattering measurements. (c) Relative fluorescence of 10 μ M ANS in the absence (blue spectrum) and presence of 1 μ M wt (black spectrum) and 3A (red spectrum) moPrP at pH 4, 25 °C. The signals are normalized with respect to that of 10 μ M ANS. ANS emission maxima in the absence and presence of wt and 3A moPrP are indicated by the dashed vertical lines. (d) Urea-induced equilibrium unfolding transition of 3A moPrP, as monitored by far-UV CD at 222 nm. The inset shows the urea-induced equilibrium unfolding transition of wt moPrP under the same conditions. The x- and y-axis scales in the inset are the same as in the main Figure. In all panels, data for wt moPrP is shown in black, and data for 3A moPrP is shown in red.

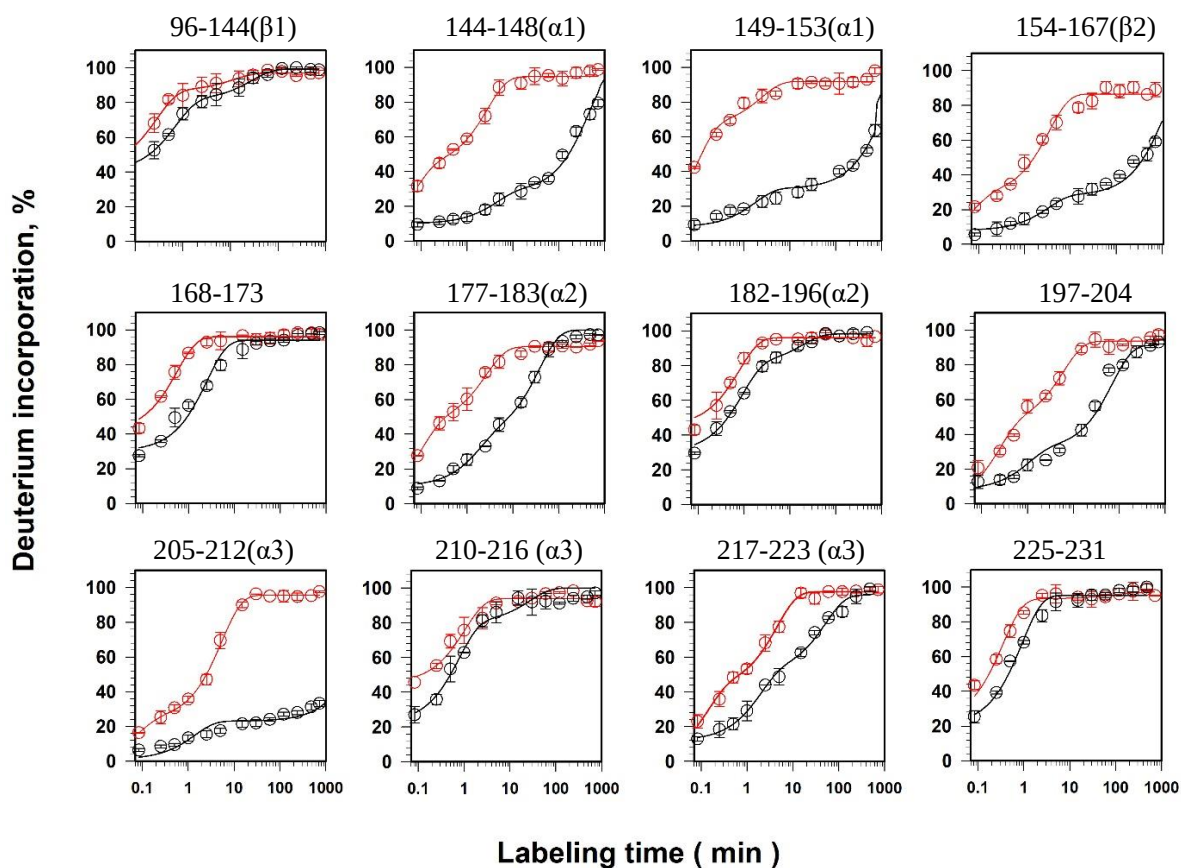


Figure 4.3. Kinetics of HDX into 3A moPrP compared to wt moPrP. The time course of HDX into different sequence segments of the native monomeric forms at pH 4, 25 °C was monitored. Data for wt moPrP and 3A moPrP are shown in black and red, respectively. The error bars represent the standard deviations determined from three independent experiments. The solid lines through the data represent fits to either monoexponential or biexponential equations. The data for wt moPrP is reproduced from Pal and Udgaonkar, 2023.

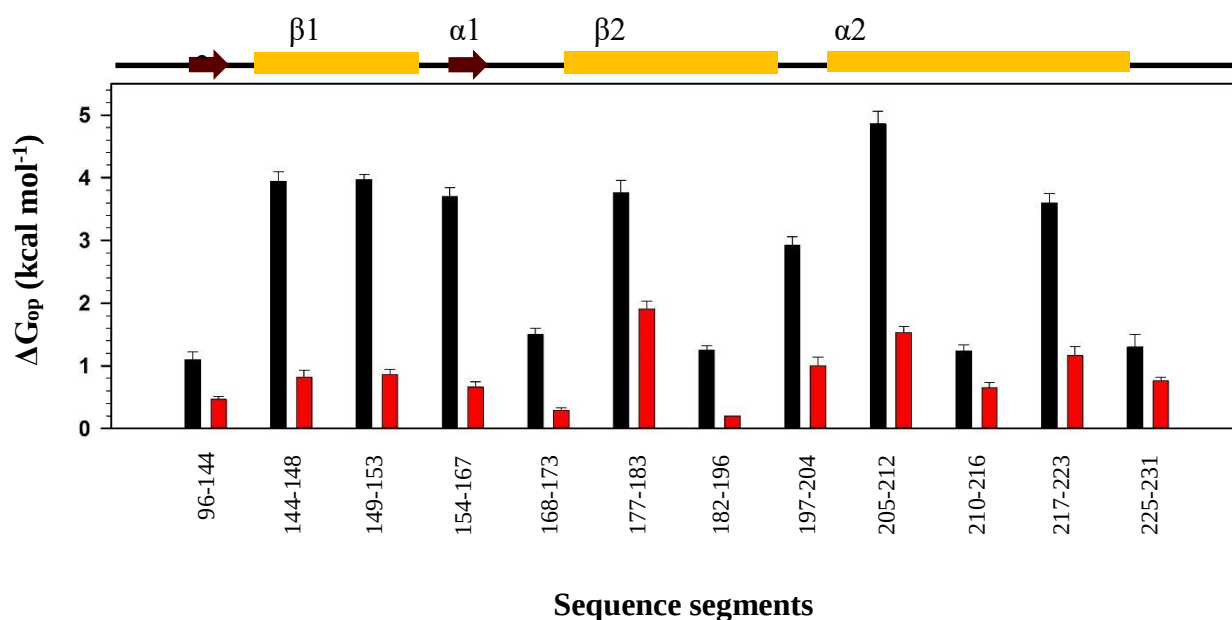


Figure 4.4. ΔG_{op} values of the different sequence segments of wt and 3A moPrP. The secondary structural elements are shown above the sequences. (rectangular boxes represent the α -helices, and arrows represent the β - strands). Data for wt moPrP and 3A moPrP are shown in black and red, respectively. The error bars represent the standard deviations determined from three independent experiments.

in the absence of salt, with a molar mass of 54.3 ± 8 kDa (Table 4.1). Interestingly, DLS measurements also revealed that during its slow misfolding in the absence of salt, 3A moPrP dimerized at an early stage (Figure 4.5d), indicating that the process of overall conformational conversion occurred much more slowly than the formation of dimers.

Dimer formation leads to structure formation in multiple regions

To understand the kinetics of structural changes in different segments of 3A moPrP during dimerization, and to identify the onset of conformational conversion, HDX labeling pulses of 300 s duration were applied at different times of misfolding. Sequence segments 96-144, 159-166, 168-173, 182-196, 197-204, and 210-216 became more protected during dimerization (Figure 4.6). Sequence segments 159-166, 168-173, and 182-196 displayed biexponential kinetics of gain in protection (Figure. 4.6), while segments 96-144, 197-204, and 210-216 displayed monoexponential kinetics (Figure. 4.6). Sequence segment 177-183 showed monoexponential kinetics of loss in protection (Figure 4.6). The faster rate constant observed for segments showing biexponential kinetics, was found to be similar to the rate constant of gain in protection for segment 96-144 (Table 4.2). Considering that DLS measurements indicated early dimerization of 3A moPrP in the absence of salt (Figure. 4.5d), the fast rate constant could be reporting on a gain in protection due to dimerization. The slow rate constants observed for sequence segments 197-204 and 210-216, displaying single exponential kinetics, were found to be similar to the rate constant of misfolding determined by CD measurement, and could be attributed to dimerization-induced conformational conversion.

Rapid misfolding of 3A moPrP in the presence of salt

The misfolding of 100 μ M wt moPrP at pH 4 was studied in the presence of a physiological concentration of salt (150 mM) at 37 °C, as it is extremely slow ($\sim 10^{-4}$ h⁻¹) in the absence of salt (Sengupta et al., 2017a). The kinetics of misfolding monitored by far-UV CD appeared monophasic, and fit well to a single exponential equation. It should be noted that a previous study (ref) had shown that the far-UV-monitored kinetics of misfolding of wt moPrP is the same whether measured at 222 nm or at 216 nm. The misfolding of wt moPrP at pH 4 is accompanied by oligomerization (Sabareesan and Udgaonkar, 2016b). Under similar conditions, the misfolding of 3A moPrP was found to be too fast to be monitored (Figure 4.7a) by CD. The mutant protein variant misfolded completely into a β -rich conformation within 15 s after the addition of 150 mM NaCl (Figure 4.7a). However, at a lower temperature (25 °C), and at a lower protein concentration (1 μ M), the misfolding of 3A moPrP in the presence of

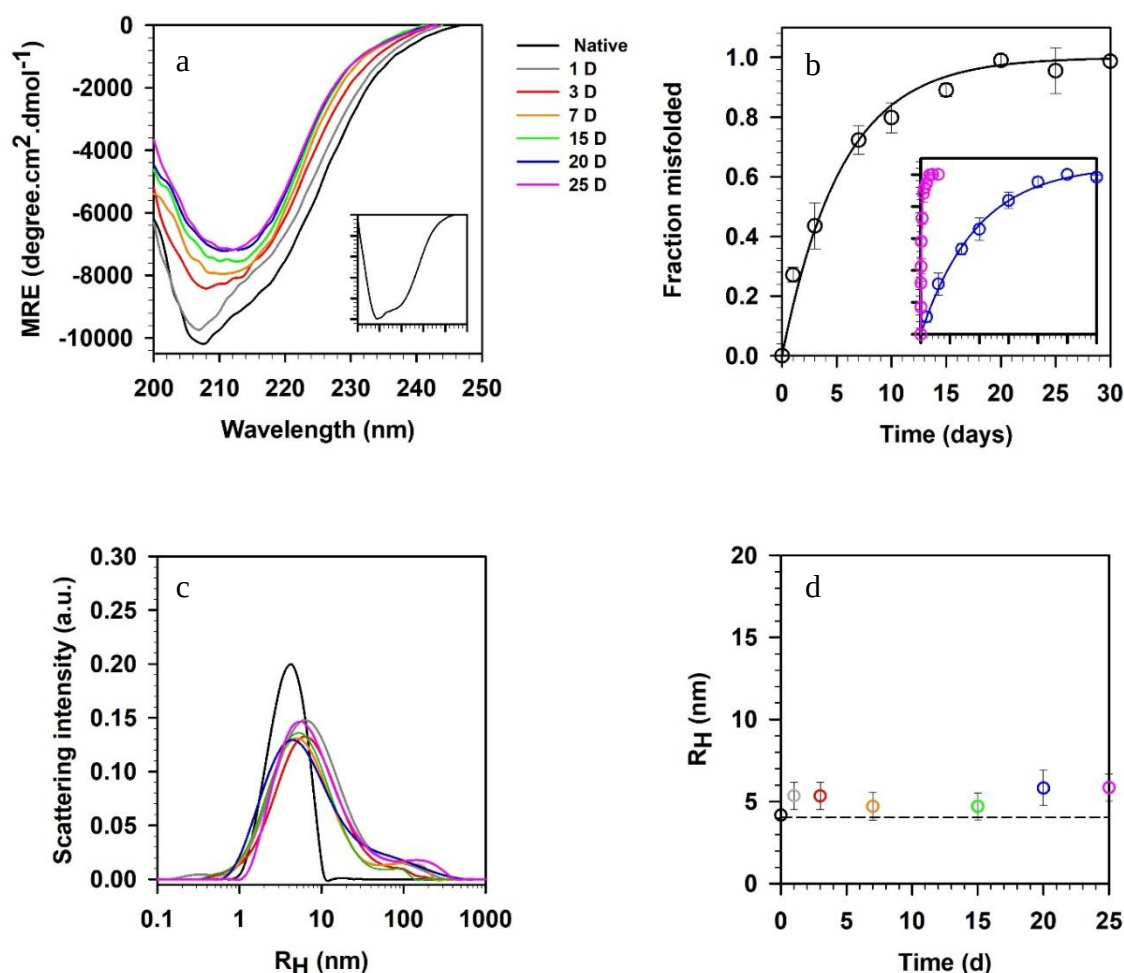


Figure 4.5. Misfolding of 3A moPrP in the absence of salt at pH 4, 37 °C. (a) Time course of conformational conversion of 100 μM 3A moPrP, as probed by far- UV CD spectra. The inset shows the far-UV CD spectrum of wt moPrP after 25 days in the same conditions, which indicates that no conformational conversion of wt moPrP occurs in 25 days. The x- and y-axis scales in the inset are the same as in the main figure. (b) The kinetics of misfolding of 100 μM 3A moPrP was monitored using far-UV CD at 222 nm. The inset shows the kinetics of misfolding of 50 (blue) and 200 (pink) μM 3A moPrP. The x- and y-axis scales in the inset are the same as in the main figure. The solid lines through the data represent fits to a single exponential equation. (c) DLS profiles at different times of misfolding of 100 μM 3A moPrP in the absence of salt. (d) R_H values at different times of misfolding of 100 μM 3A moPrP. The

Table 4.1: Weight-averaged molar mass (M_w)

Protein	M_w (kDa)
BSA (control)	67.1 ± 0.5
wt moPrP monomer	23.4 ± 0.8
3A moPrP monomer	25.1 ± 1.4
3A moPrP dimer	54.3 ± 8.0

3A moPrP Dimers were prepared by incubating 100 μ M protein in 10 mM sodium acetate (pH 4) at 37°C for 30 days. The samples were then diluted to 50 μ M, centrifuged at 15,000g for 30 min, and filtered through a 0.02- μ m filter at a constant rate into a light scattering fused silica flow cell for mass determination. The mean and standard deviation was calculated from the weight-averaged molar mass values obtained from three independent experiments.

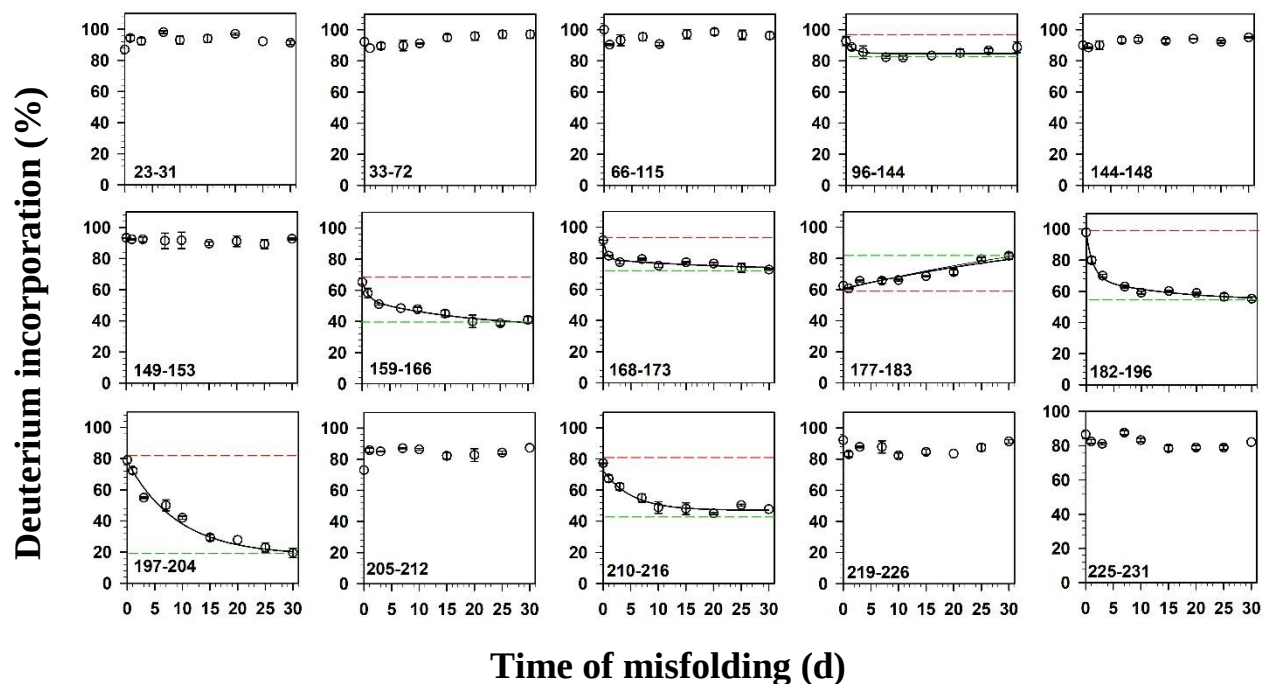


Figure 4.6. Kinetics of pulsed HDX into different sequence segments of 3A moPrP during misfolding. The time courses of deuterium incorporation into different sequence segments upon the application of 300 s HDX-labeling pulses at different times of misfolding in the absence of salt at pH 4, 37 °C, are shown. The red dashed line and the green dashed line represent the initial and final percentage of deuterium incorporation into each sequence segment, respectively. The error bars represent the standard deviations determined from three independent experiments.

Table 4.2: Rate constants of structural change in different sequence segments of 3A moPrP during misfolding in the absence of salt

Sequence segment	k_{obs} (d^{-1})	Amplitude averaged rate constant (d^{-1})
96-144	0.78 ± 0.12	0.78 ± 0.12
159-166	0.92 ± 0.12 (59%) 0.05 ± 0.01 (41%)	0.11 ± 0.02
168-173	1.58 ± 0.20 (62%) 0.04 ± 0.01 (38%)	0.13 ± 0.03
177-183	0.02 ± 0.005	0.02 ± 0.005
182-196	0.78 ± 0.14 (66%) 0.07 ± 0.01 (34%)	0.17 ± 0.03
197-204	0.11 ± 0.01	0.11 ± 0.01
210-216	0.19 ± 0.03	0.19 ± 0.03

Misfolding of 100 μM 3A moPrP was carried out at pH 4, 37 °C. The rate constants for the misfolding of different sequence segments were obtained from the data shown in Figure 4.6. The errors represent the standard deviations determined from three independent experiments.

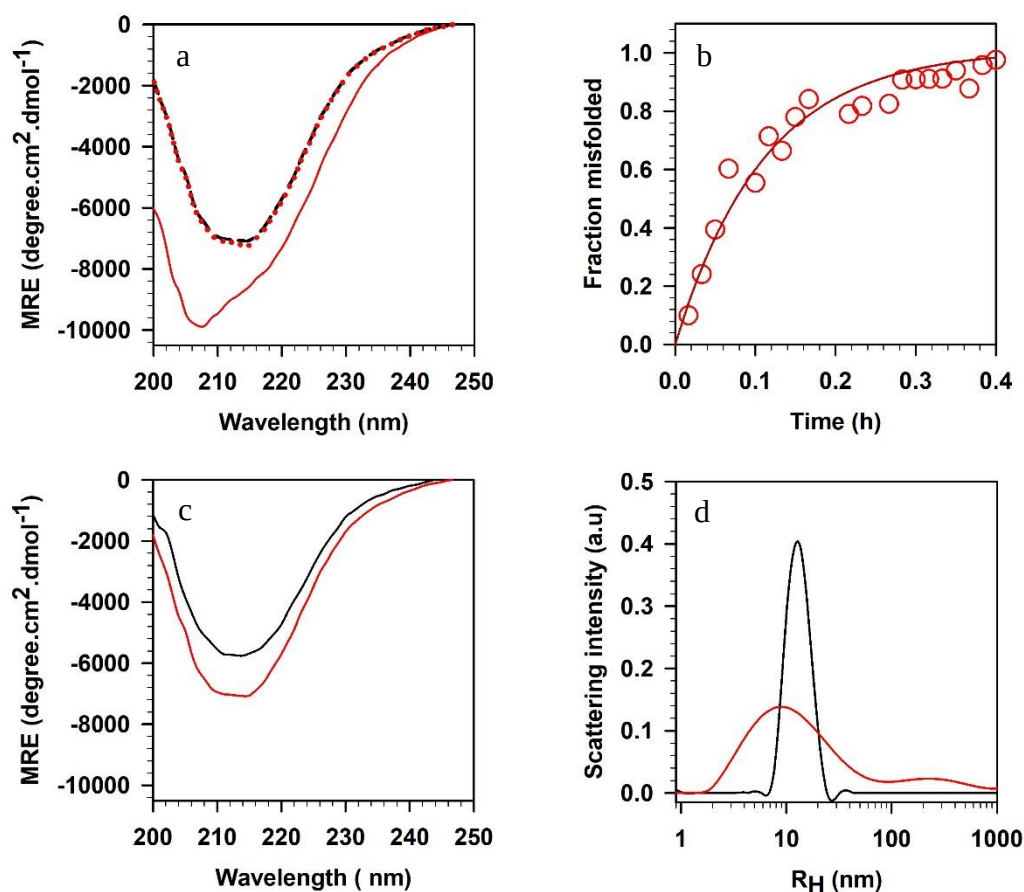


Figure 4.7. Misfolding of 3A moPrP in the presence of 150 mM NaCl at pH 4, 37 °C. (a) Time course of conformational conversion of 100 μ M 3A moPrP, as probed by far-UV CD spectra. The spectrum shown in red is that of the native monomeric protein before the addition of salt. The spectra shown by the black dashed line and the red dotted line are spectra at 15 s and 2 h after addition of 150 mM NaCl. (b) The kinetics of misfolding of 1 μ M 3A moPrP in the presence of 150 mM NaCl at pH 4, at 25 °C monitored using far-UV CD at 222 nm. The solid line is a non-linear least-squares fit to the data, which yields a value for the rate constant of misfolding of 8.6 h⁻¹. (c) Far UV CD spectra and (d) DLS profiles of oligomers of wt and 3A moPrP formed from 100 μ M protein. In panels c and d, the data shown in black and red are the data for wt moPrP and 3A moPrP, respectively.

150 mM NaCl, could be monitored (Figure 4.7b). Far UV CD measurements showed that the oligomers formed by 3A moPrP at pH 4, 37 °C in the presence of 150 mM NaCl, are rich in its β -sheet. Figure 4.7c shows a difference in the far-UV CD spectra between the oligomers formed by wt moPrP and the oligomers formed by 3A moPrP in the presence of 150 mM NaCl. These spectra, which provided a qualitative measure of β -sheet content, indicated more β -sheet content in the oligomers of wt moPrP. Nevertheless, the hydrodynamic radius of the oligomers formed by 3A moPrP ($R_H \sim 10\text{nm}$) were found to be similar to that of the oligomers formed by wt moPrP (Figure 4.7d).

HDX–MS characterization of the misfolded forms of the moPrP variants at pH 4

Figure 4.8 shows the extent of deuterium incorporation in different sequence segments of oligomers formed by wt moPrP and 3A moPrP with 150 mM NaCl during 300 s and 3000 s labeling pulses. It also displays deuterium incorporation in the 3A moPrP dimer formed without salt. The core regions, expected to be the most structured, protect against HDX. In wt moPrP oligomers, residues 182-212 incorporated less than 30% deuterium after a 300 s HDX pulse, indicating they form the core. This segment was more protected in wt moPrP oligomers than in 3A moPrP oligomers, suggesting a more stable core in wt moPrP. In the case of 3A moPrP dimers formed in the absence of salt, only residues 197-204 had less than 30% deuterium incorporation after 300 s, indicating a smaller core. However, residues 210-216 showed less deuterium incorporation in dimers than in oligomers, suggesting this segment is more protected in 3A moPrP dimers. These differences in core structures likely lead to the different sizes of the misfolded forms of moPrP variants.

3A moPrP monomer converts into amyloid fibrils faster than wt moPrP monomer

To understand whether mutation of conserved aromatic residues affects the formation of amyloid fibrils by moPrP, fibrillation experiments were carried out (Figure 4.9). It is known that wt moPrP forms long straight amyloid fibrils at pH 7 when agitated in the presence of denaturants (Singh and Udgaonkar, 2013a). Both wt moPrP and the 3A moPrP converted into fibrils with typical sigmoidal kinetics. wt moPrP exhibited a lag phase of approximately ~8 h, whereas 3A moPrP converted into fibrils faster, with a lag phase lasting around ~2.5 h (Figures 4.9 a and b). Since the characteristic time for elongation was about the same for wt moPrP and 3A moPrP, mutation of the three conserved aromatic residues resulted in overall acceleration of the formation of amyloid fibrils. The ThT fluorescence signal was the same for both moPrP variants, indicating that they both formed the same amount of fibrils.

Pathological fibrils of moPrP are known to act as catalysts to convert soluble monomeric protein into fibrils in a template-dependent manner (Stöhr et al., 2008). To check whether the three evolutionarily conserved aromatic residues play a role in template-driven conformational conversion, fibrillation reactions were carried out in the presence of wt moPrP and 3A moPrP seeds. Seeds of both wt moPrP and 3A moPrP were found capable of seeding monomers of wt moPrP as well as 3A moPrP (Figure 4.9). The addition of 10% v/v 3A moPrP seeds abolished the lag phase in the fibrillation process of both wt moPrP and 3A moPrP monomers (Figure 4.9). However, the addition of 10% v/v wt moPrP seeds did not completely abolish the lag phase in the fibrillation process of the 3A moPrP monomer (Figure 4.9b).

Discussion

Mutation of the three conserved aromatic residues results in a MG form at pH 4.0

3A moPrP exhibits several characteristic structural features of a MG: (1) It has a perturbed secondary structure compared to wt moPrP. (2) It is swollen in its dimensions compared to wt moPrP. (3) It has more exposed hydrophobic patches to which ANS can bind, than does wt moPrP. (4) Protection factors against hydrogen exchange are low, between 2 and 8, throughout the structure, as also seen for the MGs formed by other proteins (Hughson et al., 1990b; Kobashigawa et al., 2000; Morozova et al., 1995). The exceptions are sequence segments adjacent to Cys178 and Cys213, which form a disulfide bond. These segments have high protection factors in wt moPrP, but have protection factors of only ~ 20 in 3A moPrP. (5) The HSQC-NMR spectrum of 3A moPrP shows peaks only from residues in the unstructured NTD and not from the CTD (data not shown). It is well known that peaks in the NMR spectrum of a MG are broadened due to conformational fluctuations (Redfield, 2004) and, hence, are usually not observed (McParland et al., 2002). In addition, 3A moPrP possesses an important thermodynamic feature characteristic of MG forms. Its denaturant-induced unfolding transition is broad and not sigmoidal in nature, indicating that its unfolding transition is not a cooperative transition as is seen for a native protein. MG forms of proteins are known to undergo highly non-cooperative unfolding transitions (Kuwajima, 1989; McParland et al., 2002).

The MG formed by 3A moPrP has structural similarities to the MG formed by the mutant variant T182A moPrP (Singh and Udgaonkar, 2016b), the MG formed upon methionine oxidation of PrP (Younan et al., 2012), and the MG formed at pH 2 by the isolated CTD of moPrP (Honda et al., 2014a). All these MG forms maintain a native-like tertiary structure around the disulfide bond within the $\alpha 2$ - $\alpha 3$ subdomain, while the $\beta 1$ - $\alpha 1$ - $\beta 2$ subdomain remains

unfolded. This is consistent with the suggestion that the formation of a partially unfolded form, with separated subdomains and an exposed hydrophobic core, may be an initial step in the PrP misfolding pathway that eventually results in β -rich oligomers (Singh and Udgaonkar, 2015a).

Mutation of the three conserved aromatic residues results in significant population of multiple aggregation-prone PUFs

Earlier HDX-MS/NMR studies had revealed that the N state of the isolated CTD (Moulick et al., 2015a), as well as full-length wt moPrP (35,36), is in dynamic equilibrium with two sparsely populated PUFs, referred to as PUF1 and PUF2, with distinct segments of the protein having undergone unfolding in each form. Since the free energy of opening of the N state to PUF2 ($\Delta G_{op} = 3.7 \pm 0.4 \text{ kcal mol}^{-1}$) was higher than that of opening of PUF1 ($\Delta G_{op} = 1.3 \pm 0.2 \text{ kcal mol}^{-1}$), it seemed likely that sequence segments that are unfolded in PUF1 are also unfolded in PUF2. HDX-MS characterization of mutant variants of moPrP, in which the evolutionarily conserved residues in the CTD were mutated individually to Ala, further revealed that PUF2 is an ensemble of multiple iso-energetic conformations (Pal and Udgaonkar, 2022a, 2023). Studies of the mutant variants identified PUF2* and PUF2** as two sub-populations of PUF2. In PUF2*, sequence segments 144–148, 149–153 ($\alpha 1$), 154–167 (the loop between $\alpha 1$ and $\beta 2$, the $\beta 2$), and 197–204 (the loop between $\alpha 2$ and $\alpha 3$ as well as the N-terminal end of $\alpha 3$), are unfolded. PUF2* has a local stability (ΔG_{op}) of $3.7 \pm 0.4 \text{ kcal mol}^{-1}$ in the case of wt moPrP (Pal and Udgaonkar, 2022a). In PUF2**, sequence segments 154–167 and 217–223 (C terminal end of $\alpha 3$) are unfolded. Sequence segment 177–183, which had been found to be unfolded in PUF2, was found to be folded in both PUF2* and PUF2**, suggesting that PUF2 consists of at least one other iso-energetic sub-population in which this sequence segment is unfolded.

The observation that the logarithm of the apparent rate constant of misfolding of moPrP is linearly proportional to the free energy difference (ΔG_{op}) between the N state and PUF1 as well as that between the N state and both PUF2* and PUF2**, suggested that misfolding can potentially commence from either of these PUFs in the case of wt moPrP (Pal and Udgaonkar, 2022a, 2023). Mutation of the evolutionarily conserved residues may affect the stability of only PUF2*, or of both PUF1 and PUF2**. Several disease-linked mutations have been shown to

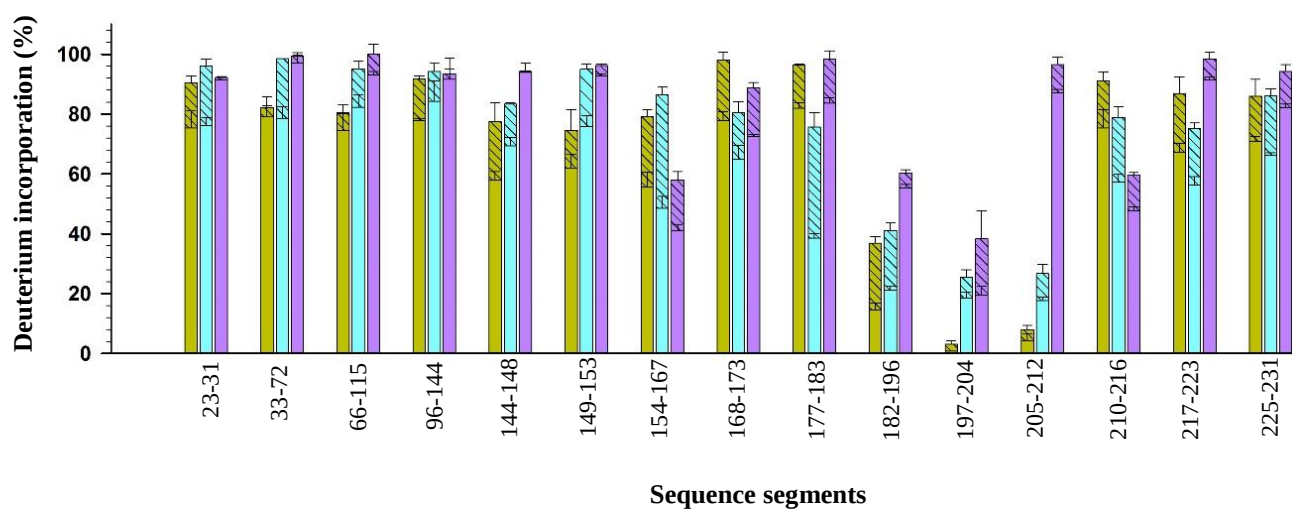


Figure 4.8. HDX-MS characterization of the misfolded forms of the moPrP variants.

Levels of deuterium incorporation into different sequence segments of the misfolded forms of the moPrP variants are shown. wt moPrP oligomers (gold) and 3A moPrP oligomers (cyan) were formed from 100 μ M protein in the presence of 150 mM NaCl at pH 4, 37 $^{\circ}$ C, and the 3A moPrP dimer (purple) was formed from 100 μ M protein in the absence of salt at pH 4, 37 $^{\circ}$ C. The empty and striped bars show the extent of deuterium incorporation after 300 s and 3000 s of pulse-labelling, respectively. The error bars represent the standard deviations determined from three independent experiments.

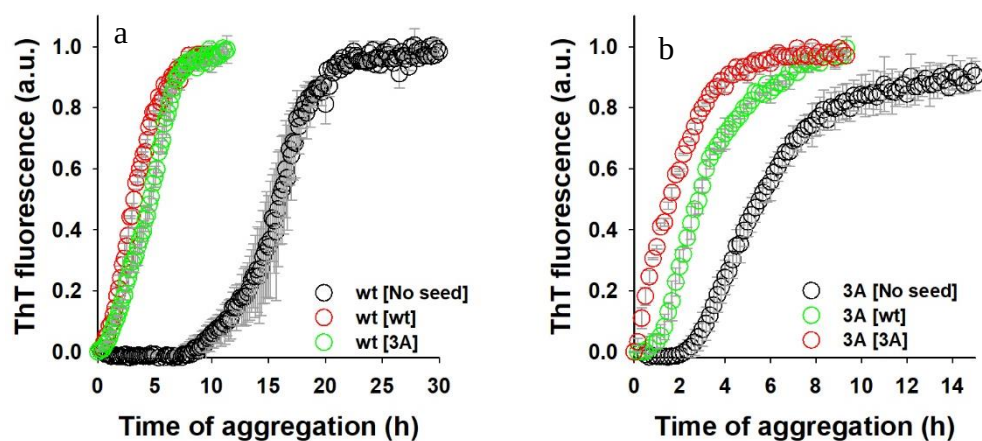


Figure 4.9. Fibril formation. (a) ThT fluorescence-monitored kinetics of fibril formation by wt moPrP in the absence and presence of 10% seed at pH 7, 37 °C. (b) ThT fluorescence-monitored kinetics of fibril formation by 3A moPrP in the absence and presence of 10% seed at pH 7. The nature of the seed is shown inside the square brackets. The error bars represent the standard deviations determined from three independent experiments.

increasing the population of the PUF2*, thereby accelerating misfolding (Pal and Udgaonkar, 2022a; Singh and Udgaonkar, 2015c). It has also been shown that several aggregation-prone regions become unfolded in the PUFs, making them highly prone to misfold (Pal and Udgaonkar, 2023). Misfolding primarily originates from PUF1 since it is more stable than both PUF2* and PUF2** and, hence, would be significantly more populated than them at equilibrium (Figure 4.10). Consequently, PUF1 would be the primary precursor conformation initiating misfolding, although misfolding could also occur from PUF2* and PUF2**, albeit to a smaller extent.

In the case of 3A moPrP, mutations of the three conserved aromatic residues have a significant impact on the stabilities (ΔG_{op}) of all three PUFs (Figure 4.10). 3A moPrP exists as a MG, having lost stabilizing interactions involving the three conserved aromatic residues. Hence, it would be destabilized with respect to the N state of wt moPrP. Since, the energy gap between the MG state and the PUFs is reduced, all three PUFs in the absence of added salt are populated significantly at equilibrium (Figure 4.10). The rapid misfolding of 3A moPrP in the presence of salt can be rationalized by the increased population of the PUFs at equilibrium, and, importantly, on the consequent lowering of the free energy barrier to misfolding.

Initiation of misfolding in the absence and presence of salt

Previous studies had shown that the mechanism of initiation of misfolding and oligomerization involves multiple steps (Goluguri et al., 2019b; Sengupta and Udgaonkar, 2019b). In the first step, the N state partially unfolds to form a PUF, which then misfolds to β -rich oligomer (O) (Figure 4.11). In such a sequential $N \leftrightarrow PUF \leftrightarrow O$ mechanism, the observed rate constant of conformational change depends on the extent to which the PUF is populated, and on the free energy barrier slowing down misfolding of the PUF. When the extent to which reduced, the free energy barrier to misfolding will also reduce to at least the same extent (Figure 4.11), for a sequential mechanism. The observed rate constant is determined by the free energy barrier to misfolding.

In an earlier NMR study of wt moPrP, salt was found to disrupt native electrostatic interactions in segments 96–144 ($\beta 1$ and $\beta 1$ - $\alpha 1$ loop) and 210–216 (C-terminal end of $\alpha 3$), and alter the curvature of helix $\alpha 3$, potentially affecting $\alpha 2$ - $\alpha 3$ interactions (Bhate et al., 2021c). In the absence of salt, the populations of the PUFs populated in the case of 3A moPrP are increased substantially, but yet misfolding is very slow. This implies that a significant component of the free energy barrier are destabilizing (repulsive) electrostatic interactions that

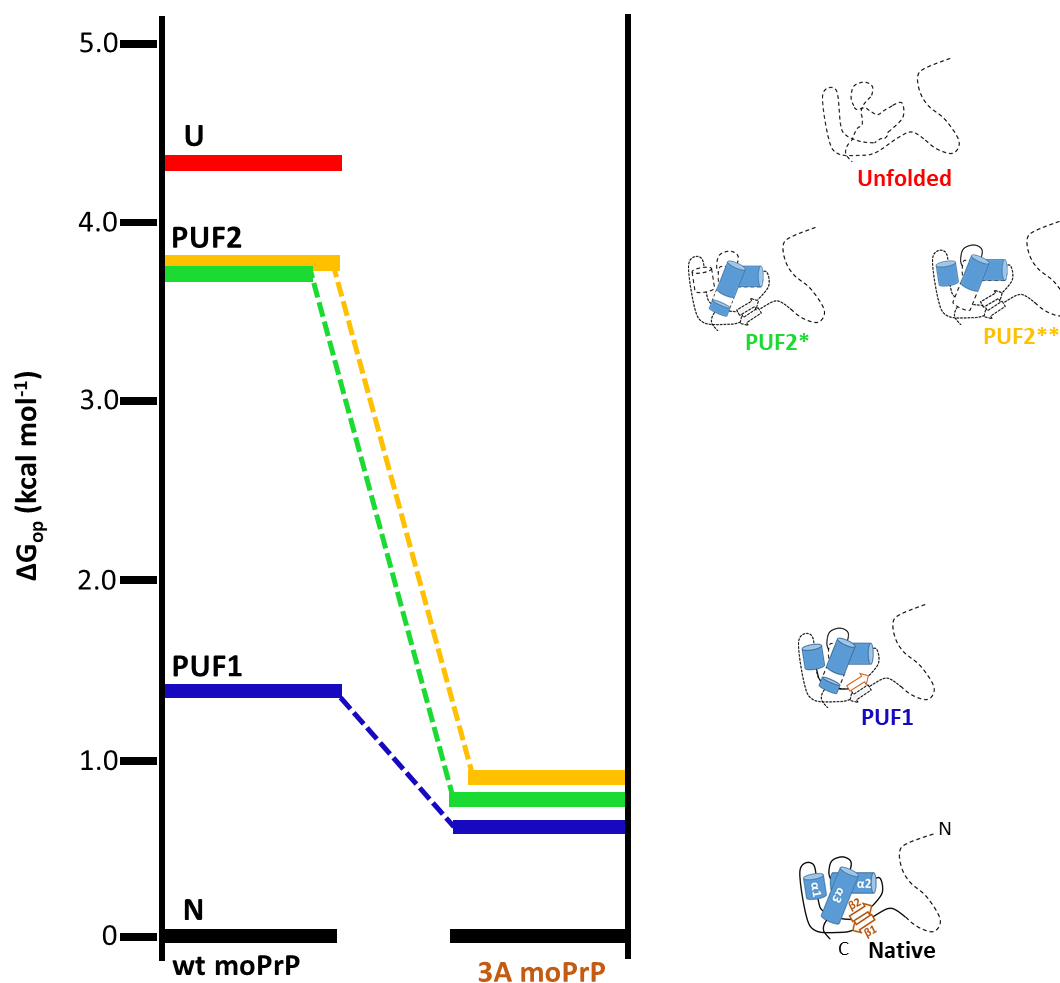


Figure 4.10. Effect of mutation of the three conserved aromatic residues on the stabilities of the PUFs. The free energy levels of the PUFs, produced from the unfolding of specific sequence segments, have been placed on a free energy ladder. PUF2 has been shown to comprise of two isoenergetic sub-populations of molecules, PUF2*(yellow) and PUF2** (green), which differ in structure (Pal and Udgaonkar, 2023). For each PUF, segments that have unfolded are shown in broken lines. The unstructured N-terminal domain (23-120) is shown as a dashed line. It should be noted that the Figure is meant only to show the free energy levels and not whether the PUFs are formed sequentially or in parallel, from the N state. The values of ΔG_{op} indicate that the equilibrium populations of N, PUF1, PUF2*, and PUF2** are 89%, 9%, $\approx 1\%$, and $\approx 1\%$, respectively, in the case wt moPrP, and 53%, 22%, 12%, and 14 %, respectively, for 3A moPrP.

must be first broken, before misfolding can proceed, as also observed during the oligomerization of peptibody A (Gokarn et al., 2009). In the presence of 150 mM NaCl, the destabilizing (repulsive) electrostatic interactions are screened, and the barrier is lowered. Consequently, misfolding becomes much faster in the presence of salt. It is known that polyanionic phospholipids trigger the misfolding of human PrP into β -rich oligomers, presumably because adsorption at the lipid bilayer/water interface disrupts the electrostatic interactions and reduces the free energy barrier to misfolding (Lührs et al., 2006). Other polyanions, such as nucleic acids (Cordeiro et al., 2001; Silva et al., 2008) and glucosaminoglycans (Caughey and Raymond, 1993), are also known to modulate the misfolding of PrP, presumably in a similar way.

Assembly of misfolded dimer

The observation that the reaction order of the misfolding reaction of 3A moPrP is 2.6 is not surprising, given that the product is a dimer. Interestingly, the reaction order of the misfolding reaction of wt moPrP in the presence of 150 mM NaCl, leading to the formation of the oligomer, was reported to be ~ 2.6 (Sengupta et al., 2017a). It seems that the formation of oligomers begins with misfolded dimer formation and possibly proceeds by accretion of dimers. An earlier study of oligomer growth of Syrian hamster PrP had reported a reaction order of 3 (Sokolowski et al., 2003), while an AFM-based study of the misfolding of human PrP had reported β -rich oligomers composed of 3-4 monomers as the initial stable species (Cho et al., 2011). Misfolding of human PrP in the presence of lipids, leading to the formation of β -rich oligomers, was also shown to have a reaction order of 2.0 ± 0.1 , indicating dimerization is indeed the rate-limiting step in PrP oligomerization (Lührs et al., 2006).

Structural change during dimer formation

The gain in protection against HDX in some sequence segments of 3A moPrP occurs in two kinetic phases. Rate constants observed for the faster kinetic phase fall in the range of 0.8-1.6 d⁻¹, and those observed for the slower kinetic phase fall in the range of 0.02-0.2 d⁻¹. The observation that sequence segment 96-144 gains protective structure only in the fast kinetic phase, suggests that part of it may form the interface between two monomeric units as they associate to form the dimer. The part that forms the interface may be the conserved palindromic sequence ¹¹¹AGAAAAGA¹²⁰, which is the most conserved sequence element in mammalian and non-mammalian prion proteins (Wopfner et al., 1999), and is also known to have a role in

the interaction between PrP^C and PrP^{Sc} (Norstrom and Mastrianni, 2005). Replica exchange molecular dynamics simulations studies suggest that this segment has a strong tendency to adopt β -hairpin structures, which promote misfolding (Ning et al., 2015). It is known that the pathogenic mutations G113V and A116V accelerate misfolding by enhancing monomer-monomer interaction at the dimer interface, as revealed by increased initial HDX protection in sequence segment 89-132 during oligomer formation in the presence of 150 mM NaCl (Sabareesan and Udgaonkar, 2016b). It appears that the initially formed interface between two monomers during dimer formation in the absence or presence of added salt may be similar.

The observation that sequence segments 159-166 (β 2), 168-173 (β 2- α 2 loop), and 182-196 (C-terminal end of α 2) gain protection in both the fast and slow kinetic phases suggests that parts of these segments may be part of the monomer-monomer interface that forms during dimer formation. Alternatively, it is possible that parts of these segments gain their own protective structure independently of, but concomitant with dimer formation, while other parts gain protective structure later in the slow kinetic phase along with the protective structure that forms in sequence segments 197-204 (α 2- α 3 loop) and 210-216 (C-terminal end of α 3). It is remarkable that the sequence segments which gain protection are unfolded in the PUFs identified earlier: sequence segments 96-144, 168-173, 182-196, and 210-216 are unfolded in PUF1; sequence segment 197-204 is unfolded in PUF2*, and sequence segment 159-166 is unfolded in both PUF2* and PUF2**. It seems that the native structure that undergoes conformational conversion during misfolding is lost initially during the formation of the PUFs. It is this loss of critical native structure that facilitates the formation of dimers, and subsequent misfolding into β -rich structure.

The dimer formed by misfolding in the absence of salt has a structure similar to oligomer formed in the presence of salt

The observation that the far-UV CD spectrum of the dimer formed by 3A moPrP in the absence of salt overlaps that of the oligomer formed in the presence of 150 mM NaCl (Figure 4.13) suggests that their β -sheet contents are similar. The similarity in β -sheet content is also evident in the extent of deuterium labeling of different sequence segments in HX-MS experiments. The extents of deuterium incorporation in the dimer formed in the absence of salt and the oligomer formed in the presence of 150 mM NaCl are similar for all sequence segments except 205-212, which seems to be unfolded in the dimer. The observation that sequence segment 205-212 becomes protected in both wt and 3A moPrP oligomers, compared to the dimers, could be because a part of the segment, or the entire segment, is the site for dimer-

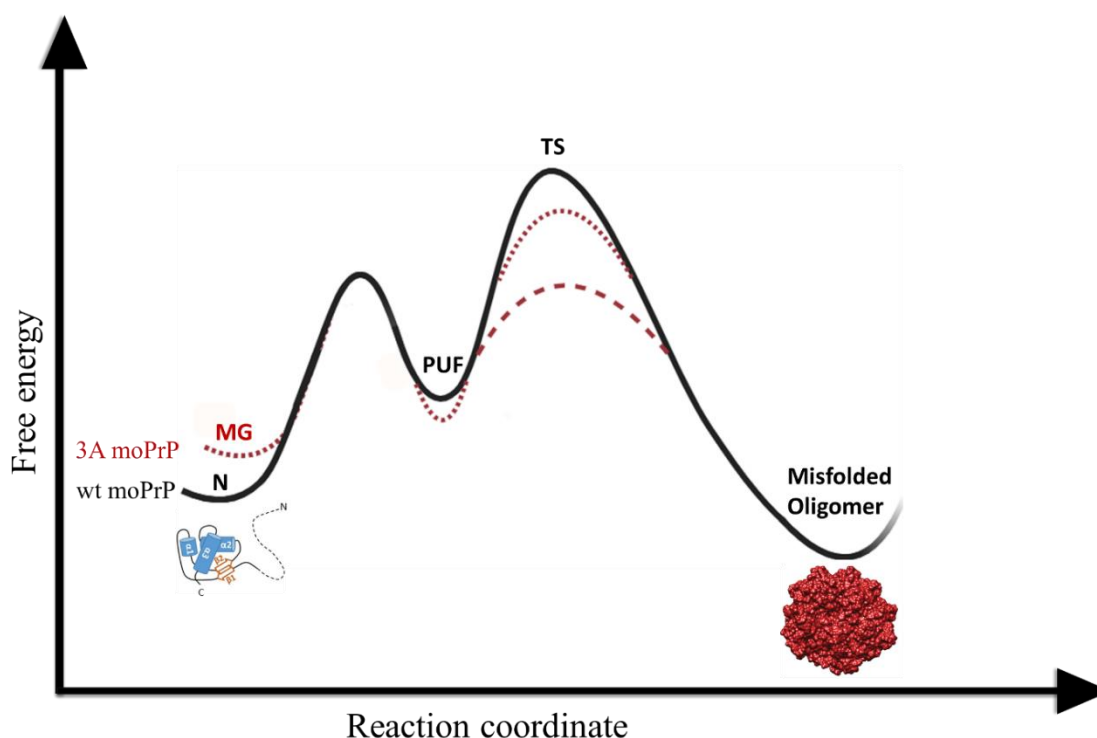


Figure 4.11. Schematic representation of the mechanism of misfolding and oligomerization of the prion protein at low pH. In the sequential $N \leftrightarrow PUF \leftrightarrow O$ mechanism, the N state undergoes partial unfolding to generate a PUF, which then proceeds to misfold into a β -rich oligomer (O). The red dotted and red dashed lines indicate the barrier to misfolding and the oligomerization of 3A moPrP in the absence and presence of salt, respectively. The observed rate constant of misfolding and oligomerization depends on the free energy barrier that impedes misfolding, which corresponds to the free energy difference between TS and N. 3A moPrP exists as a MG, and is expected to be destabilized with respect to the N state of wt moPrP. Hence, the energy difference between the MG and the PUF is reduced, and the equilibrium population of the PUF is increased. This leads to an increase in the rate constant of oligomerization, because reducing the energy difference between N and the PUF also reduces the energy barrier to misfolding, given the sequential nature of the mechanism. Even though the PUF is significantly populated, misfolding occurs slowly in an environment lacking salt. However, in the presence of salt, destabilizing (repulsive) electrostatic interactions are also broken, and the energy barrier is reduced. Consequently, misfolding occurs rapidly. The structural core of the oligomer appears to be more protected for wt moPrP than for 3A moPrP.

dimer interaction within the oligomer as the oligomer grows by assembly of dimers. The sequence segment 205-212 is highly hydrophobic and is part of the hydrophobic core of the protein. Indeed, multiple pathological mutations located in this region have been reported to disrupt the core and accelerate misfolding (Lee et al., 2019b). These results indicate that hydrophobic interactions between exposed hydrophobic patches of the dimers promote the formation of stable oligomers.

Prion dimers have been detected in brain samples from humans with inherited prion diseases (Gao et al., 2019) as well as in a murine neuroblastoma cell line expressing hamster PrP (Priola et al., 1995). However, the role played by dimer in prion disease has so far appeared uncertain, as PrP dimers have also been observed in the brains of non-infected hamsters and mice (Bendheim and Bolton, 1986; Sklaviadis et al., 1989). It should, however, be noted that prion assemblies extracted from brains of transgenic mice expressing prion proteins of different mammalian species exhibit the intrinsic property of spontaneously disassociating into dimers (Bohl et al., 2023). This observation is consistent with the results of the current study which suggest the misfolded oligomers grow by assembly of dimers.

The three conserved aromatic residues are crucial for optimal packing of the structural core of the β -rich PrP aggregates

Misfolded moPrP can form oligomers (Singh and Udgaonkar, 2015c), worm-like fibrils (Jain and Udgaonkar, 2008), and straight fibrils (Wang et al., 2020a), all featuring β -rich structures that offer some protection against hydrogen exchange. Earlier hydrogen exchange studies identified a core region, most protected against HDX, within these aggregates, spanning residues 168 to 223 in worm-like (Singh et al., 2012a) and straight (Singh and Udgaonkar, 2013a) fibrils. Oligomers showed weaker protection in their core region. In this study, it was found that the structural core of oligomers was more protected for wt moPrP than for 3A moPrP, indicating that the absence of the three conserved aromatic residues weakens the protection of the structural core. Interactions between aromatic residues, particularly Phe and Tyr, are known to promote amyloid formation and to stabilize the resulting aggregates (Bemporad et al., 2006; Gazit, 2002). The three conserved aromatic residues mutated in this study (Tyr 168, Phe 174, and Tyr 217), are located at crucial regions of the protein, and are in contact in the structure of prion aggregates derived from diseased mouse (Manka et al., 2022a) and hamster (Kraus et al., 2021a) brain. It seems that, the interactions between these conserved residues may be crucial for maintaining aggregate structure.

The $\beta 2$ - $\alpha 2$ loop, spanning sequence segment ¹⁶⁸YSNQNNFV¹⁷⁵, has been shown to form amyloid-like steric zippers in microcrystals of peptide fragments spanning it (Glynn et al., 2022; Kurt et al., 2014b; Sawaya et al., 2007). It has been shown that substitutions of the aromatic residues, especially of Tyr168, have a strong impact on the side-chain interactions of residues in the sequence segment 167-175, which occur within a β -sheet of the steric zipper model. The model is consistent with the experimental data reported here, in which the presence of aromatic residues at positions 168 and 174 contributes to optimal packing of the core of the misfolded β -rich oligomers. Bulky aromatic residues (Phe, Tyr, and Trp) at positions 168 and 174 could provide maximum hydrophobic contacts and tighter interfaces, whereas residues with smaller hydrophobic side chains would offer fewer residue contacts and result in poorer packing interfaces. This suggests that the three conserved aromatic residues might be crucial for optimal packing of the structural core of not only the amyloid fibrils, but also of the β -rich oligomers. Unfortunately, detailed structural characterization of prion oligomers still remains elusive.

Chapter 5

Rigidifying the $\beta 2$ - $\alpha 2$ loop in the mouse prion protein slows down formation of misfolded oligomers

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Introduction

The cellular prion protein (PrP^C) is a monomeric, α -helix-rich and protease-sensitive protein. It is expressed ubiquitously in various tissues, but is most abundantly found in the central nervous system (Bendheim et al., 1992b). Studies involving PrP^C-knockout mice models have suggested that protein is involved in regulating the circadian rhythm (Tobler et al., 1996), peripheral myelin maintenance, and synaptic function conservation (Collinge et al., 1994b; Steele et al., 2007b). In addition, PrP^C has appears to have a role in metal ion homeostasis (Vassallo and Herms, 2003), cell signaling (Haigh et al., 2010), cell adhesion (Málaga-Trillo et al., 2009), and protection from oxidative stress (Wong et al., 2001). The conformational conversion of PrP^C into an insoluble, misfolded, multimeric, β -rich, protease-resistant isoform, PrP^{Sc}, is the fundamental pathological event in prion diseases (Caughey et al., 1991; Pan et al., 1993a; Safar et al., 1993). These fatal neurodegenerative diseases include Creutzfeldt-Jacob disease (CJD), fatal familial insomnia (FFI), Gerstmann Straussler-Scheinker syndrome (GSSS) and kuru in humans, scrapie in sheep and goat, bovine spongiform encephalopathies (BSE) in cattle, and chronic wasting disease (CWD) in elk and deer (Aguzzi et al., 2008a; Caughey and Baron, 2006; Collinge, 2001; Prusiner, 1982a, 1998; Weissmann, 2004).

Prion diseases can be categorized into three main categories: genetic, sporadic, and acquired (Collinge, 2001). More than 30 mutations associated with CJD, FFI, and GSSS have been identified in the human *PRNP* gene, which encodes PrP^C (Apetri et al., 2005; Prusiner, 1998). While 10-15 % of human prion diseases have a genetic origin, around 85 % of them are sporadic in nature, and are associated with the spontaneous misfolding of PrP (Mackenzie and Will, 2017). The mechanism by which PrP spontaneously misfolds is not completely understood. Misfolding requires the protonation of two key residues (Singh and Udgaonkar, 2016f), and it has been proposed that PrP begins to misfold when it encounters a low pH in lysosomes as it traffics through the endocytic pathway (Borchelt et al., 1992b). Notably, the susceptibilities of different mammals to sporadic prion diseases correlate well with the propensities of their PrPs to misfold into β -rich oligomers formed at pH 4 (Khan et al., 2010a).

Healthy individuals may also develop Transmissible spongiform Encephalopathies (TSE) following infection with biological material containing PrP^{Sc} (Weissmann et al., 2002). The transmission of PrP^{Sc} is very efficient within the same species. However,

there are species barriers that restrict the inter-species transmission of PrP^{Sc}, and which result in incomplete transmission rates, and in extended incubation periods being required for clinical symptoms to manifest themselves (Bruce et al., 1994). However, this barrier can be breached, as seen by the emergence of variant Creutzfeldt-Jakob disease (vCJD) in humans due to the transmission of BSE PrP^{Sc} from cattle (Hill et al., 1997). The previously perceived species barrier of CWD between deer and humans also may not be as formidable as once believed (Barria et al., 2011). Thus, understanding the factors that dictate species barriers is crucial for assessing the risk to humans of prion disease.

Earlier studies suggested that the species barrier exists because of differences in the amino acid sequences of the prion proteins of the host and donor species (Kurt et al., 2015; Sweeting et al., 2010). The PrP sequence is highly conserved among mammalian species, suggesting that changes in only a few amino acid residues may significantly impact transmission efficiency (van Rheede et al., 2003b). The structures of the prion proteins of different species have been determined by NMR, and found to be very similar (Billeter et al., 1997a). All mammalian prion proteins consist of an unstructured N-terminal domain (residues 23–123) [mouse numbering has been used] and a structured C-terminal domain (residues 125–231) (Riek et al., 1996b). The C-terminal domain (CTD) contains three α -helices, spanning residues 144–153 (α 1), 172–193 (α 2), and 200–225 (α 3), and two short anti-parallel β -strands, comprising residues 127–130 (β 1) and 160–163 (β 2) (Billeter et al., 1997a; Riek et al., 1996b).

Although the amino acid sequence of PrP is highly conserved among mammals, sequence diversity and structural differences are observed at the interface of the β 2- α 2 loop and the C-terminal end of α 3 (Billeter et al., 1997a). The β 2- α 2 loop, spanning residues 164–175, is ordered in the PrP of elk and deer, but is disordered in the PrP from several other species, including human, cattle, sheep, and mouse, and is partially ordered in the case of Syrian hamster PrP (Billeter et al., 1997a; Gossert et al., 2005; Sigurdson et al., 2010). This special structural feature of the PrP of elk and deer has been attributed to the amino acid residues at positions 169 and 173 being Asn and Thr, respectively (Figure. 5.1). When Ser169 and Asn173 in moPrP are substituted with Asn and Thr, respectively, the resultant β 2- α 2 loop becomes ordered (Gossert et al., 2005). More importantly, prion transmissibility between different species is found to be dependent on the β 2- α 2 loop conformation (Sigurdson et al., 2010). Inoculation with

PrP^{Sc} from a species with a similar $\beta 2-\alpha 2$ loop structure results in efficient transmission of prion disease in transgenic mice expressing mammalian prion proteins (Christen et al., 2013). On the other hand, large prion transmission barriers exist between species having dissimilar $\beta 2-\alpha 2$ loop structures. Amino acid changes in the $\beta 2-\alpha 2$ loop at residues 169 and 173, which lead to a rigid loop in the mouse, exhibit resistance to transmission when challenged with normal brain homogenates (Sigurdson et al., 2010). It has also been suggested that the $\beta 2-\alpha 2$ loop is part of a recognition site for an unknown binding partner, that would regulate the conversion from PrP^C to PrP^{Sc} (Kaneko et al., 1997). Understanding the structural integrity of the $\beta 2-\alpha 2$ loop and its interactions with adjacent domains, could provide deeper insights into the transmission of prion diseases.

Materials and Methods

Buffers and Reagents

Reagents used in this study were purchased from Sigma. Ultra-pure GdnHCl, of the highest purity grade, was procured from United States Biochemicals.

Site-directed mutagenesis

The mutant variants of full-length moPrP were generated using the Quickgene site-directed mutagenesis kit (Stratagene). Primers containing three nucleotide changes were obtained from Sigma. Two single mutant variants, S169N moPrP and N173T moPrP, and one double mutant variant [S169N, N173T] moPrP were prepared. The mutations were confirmed by DNA sequencing.

Protein Expression and Purification

wt moPrP and the mutant variants were expressed in *E. coli* BL21(DE3) codon plus cells (Stratagene), transformed with a pET17b plasmid containing the full-length sequence (23- 231) of the moPrP gene. The variants were purified as described previously (Jain and Udgaonkar, 2008; Singh et al., 2014a). The purity and mass of each moPrP variant preparation were confirmed by mass spectrometry using a Synapt G2 HD mass spectrometer (Waters Corporation).

Far-UV CD measurements

A Jasco J-815 spectropolarimeter was used to record far-UV CD spectra. A protein concentration of 10 μ M in a 1 mm cuvette, a scan speed of 50 nm/min, a digital integration time of 2 s, and a bandwidth of 1 nm were used. A total of 15 spectra, with

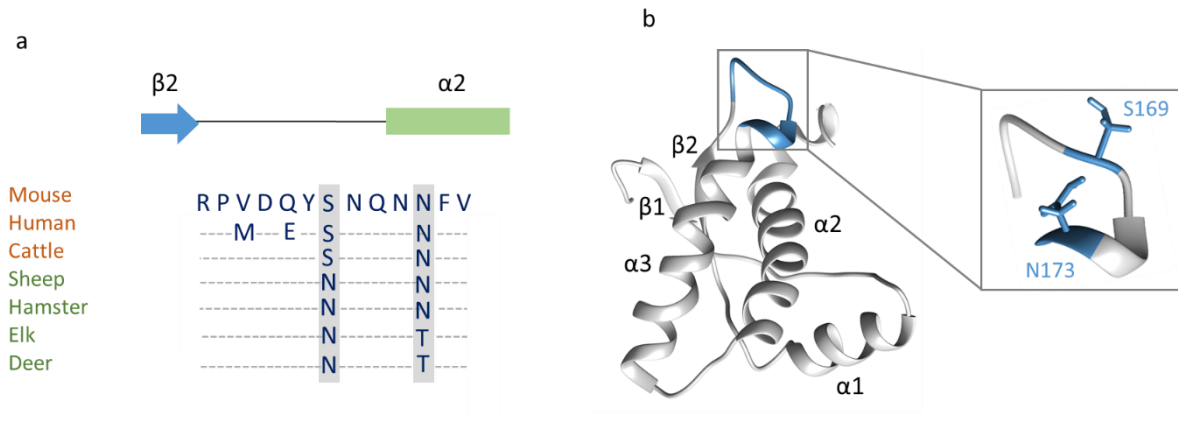


Figure 5.1. Sequence and structure of the $\beta 2$ - $\alpha 2$ loop. (a) Amino acid sequence alignment at the $\beta 2$ - $\alpha 2$ loop region of the prion proteins of different mammals. In mice, humans, and cattle, this region is highly flexible, whereas it appears to be more rigid in sheep, hamsters, elk, and deer. The key difference between proteins with a flexible *versus* a rigid loop structure lies in the presence of Asn at position 169 and Thr at position 173 in the latter, as opposed to Ser at 169 and Asn at 173 in the former (highlighted residues). Identical amino acid residues are indicated by dashes. At the top, the regular secondary structure elements are shown. (b) The structured C-terminal domain of the mouse prion protein. The N-terminal domain, spanning residues 23-120, is unstructured and not shown. The $\beta 2$ - $\alpha 2$ loop region is highlighted, and the residues mutated in this study are shown on the top right. The figure was drawn using Chimera and Protein Data Bank entry [1AG2](#)

wavelength scans from 200 to 250 nm for native monomeric and oligomers, and from 210 to 250 nm for urea-denatured monomeric state, were averaged. Far-UV CD spectra under native conditions were acquired in 10 mM sodium acetate, pH 4, at 25 °C, and under denaturing conditions were acquired in the presence of 8 M urea in 10 mM sodium acetate, pH 4, at 25 °C.

Urea-induced equilibrium unfolding studies

Urea-induced equilibrium unfolding studies were carried out in 10 mM sodium acetate buffer at pH 4, 25 °C. 10 μ M protein was incubated in different urea concentrations for 1 h, and the far-UV CD signal at 222 nm was measured. The data were fit to an equilibrium two-state N $\leftrightarrow$ U unfolding model (Agashe and Udgaonkar, 1995), and the thermodynamic parameters were obtained.

Misfolding studies at pH 4 in the presence of salt

To initiate misfolding, the protein in 10 mM sodium acetate buffer (pH 4) was diluted two-fold into 2x misfolding buffer (10 mM sodium acetate buffer containing 300 mM NaCl, pH 4), resulting in a final protein solution in 1x misfolding buffer (10 mM sodium acetate buffer containing 150 mM NaCl, pH 4). The final protein concentration for all experiments was 100 μ M. The samples were then incubated at 37 °C. At different times of misfolding, aliquots of the incubated sample were withdrawn and diluted to 10 μ M in 1x misfolding buffer (10 mM sodium acetate buffer containing 150 mM NaCl, pH 4). The far-UV CD spectra were acquired using the parameters

$$f_m = \frac{\Theta_t - \Theta_0}{\Theta_{100} - \Theta_0}$$

mentioned above. The fraction misfolded form (f_m) was calculated using the following equation:

Here Θ_t and Θ_0 are the ellipticities at 222 nm at time t and at time t = 0 of misfolding, respectively, and Θ_{100} is the ellipticity at 222 nm of the completely misfolded form.

Fibril Formation at pH 7

To initiate fibrillation, 50 μ M protein in 10 mM sodium acetate buffer (pH 4) was diluted two-fold into 2x fibrillation buffer (100 mM Tris-HCl and 4 M GdnHCl (pH 7.4)), resulting in a final protein solution in 1x fibrillation buffer [50 mM Tris-HCl and

2 M GdnHCl (pH 7)]. The final protein concentration for all experiments was 25 μ M, and 50 μ M thioflavin T (ThT) was added to each reaction sample. Subsequently, the protein in fibrillation buffer was transferred into different wells of the 96-well plate in 300 μ L volumes, followed by incubation at 37°C with agitation at 480 rpm using a Fluoroskan Ascent plate reader. ThT fluorescence was then measured at 475 nm upon excitation at 445 nm.

Seed formation and seeding reactions

At a time corresponding to $3 \times t_{50}$ of the fibrillation reaction (t_{50} was the time at which the fluorescence intensity reached 50% of its final value), the solution containing aggregated protein was centrifuged at 20,000g for 30 min at 25 °C. The pellet containing the aggregated protein was resuspended in aggregation buffer (50 mM Tris-HCl and 2 M GdnHCl (pH 7)), and sonicated as described previously (38). This sonicated solution was used to seed a fresh fibrillation reaction. All the seeding reactions were carried out using a fixed concentration of seed (10% v/v). Four types of seeded fibrillation reactions were carried out: wt moPrP monomer with wt moPrP seeds (wt), wt moPrP monomer with [S169N, N173T] moPrP seeds (wt [S16N, N173T]), [S169N, N173T] moPrP monomer with wt moPrP seeds ([S16N, N173T] (Newton)), [S169N, N173T] moPrP monomer with [S169N, N173T] moPrP seeds ([S16N, N173T] [S169N, N173T]). The final ThT fluorescence intensity was found to be similar in each case.

Dynamic Light Scattering measurements

Dynamic Light Scattering (DLS) measurements were carried out on a DynaPro-99 instrument (Wyatt Technology Corp.). All the buffers were filtered through 0.02 μ m filters (Whatman). Samples were centrifuged at 15,000 g for 30 min before acquisition. The scattering intensity at 90°, and its autocorrelation were acquired simultaneously using a laser at 829.4 nm to illuminate the sample. 50 acquisitions were collected in each experiment. The acquisition time was set at 5 s, the signal-to-noise ratio threshold at 2.5, the temperature at 25 °C, and the sensitivity at 80%. Data with uneven autocorrelation functions were excluded. The data were resolved into a Gaussian distribution using DynaLS (Protein Solutions Ltd.)

HDX-MS measurements

The peptide map of the moPrP variants was generated as described previously (Pal and Udgaonkar, 2022a). To initiate deuterium labeling, 100 μ M protein was diluted into

a labeling buffer (10 mM sodium acetate buffer in D₂O at pH 4, corrected for the isotope effect), so that HDX occurred in 95% D₂O, at 25 °C. At a variable time, a 50 µl aliquot was mixed with 50 µl ice-cold 20 mM glycine-HCl buffer (pH 2.5) to quench the labeling reaction. Online pepsin digestion was carried out by injecting the samples immediately into an immobilized pepsin cartridge (Applied Biosystems) at a flow rate of 40 µl/ min of water (0.05% formic acid), which was placed in a HDX module (Waters Corporation) coupled to a nanoAcquity UPLC. The peptides eluting from the pepsin column were collected using a trap column, washed to remove salt, and eluted over 12 min on an analytical C18 reversed-phase chromatography column using a gradient of 3 to 40% acetonitrile (0.1% formic acid) at a flow rate of 40 µl/ min. All columns were kept at 4 °C to minimize back exchange. The mass spectrometer parameters were set as follows: source temperature, 35°C; desolvation temperature, 100 °C; capillary voltage, 3.0 kV.

The MassLynx software was used to calculate peptide masses from the centroid of the isotopic envelope. The shifts in the masses of labeled peptides relative to unlabeled peptides were used to determine the extent of deuterium incorporation at each time point of HDX. Control experiments were carried out to correct for back exchange since the sample was in 95% D₂O during labeling and exposed to H₂O during quenching. For this, the protein was incubated in 10 mM sodium acetate, pH 4 (in 95% D₂O), and deuterated by unfolding at 65 °C for 15 min, followed by refolding on ice. CD spectroscopy and thermal equilibrium unfolding studies showed that the refolded protein behaved like the native protein. The 95 % deuterated moPrP samples were processed in the same way as the labeling reaction samples. The extent of deuterium incorporation in each peptide fragment, % D, was calculated using the following equation:

where $m(t)$ is the measured centroid mass at time t , $m(0\%)$ is the measured mass of the undeuterated reference peptide, and $m(95\%)$ is the measured mass of a 95% deuterated reference peptide.

$$\%D = \frac{m(t) - m(0\%)}{m(95\%) - m(0\%)} \times 100$$

deuterated reference peptide.

In the current study, native state HDX occurred in the EX2 limit (Pal and Udgaonkar, 2022a), allowing the stabilities of the partially unfolded forms in which HDX occurs to be determined. For calculation of the free energy of opening (ΔG_{op}) of

a sequence segment, the observed rate constant of deuterium incorporation (k_{obs}) was first obtained by fitting the kinetic curve of HDX to either a single exponential or a two-exponential equation, depending upon the goodness of fit. For each of the sequence segments, the observed rate constant (k_{obs}) was compared to the rate constant (k_{int}) that would be observed in the corresponding peptide fragment in a random coil state (Bai et al., 1993b). The quantity $P_f = k_{\text{int}}/k_{\text{obs}}$ is the protection factor that slows down HDX into the sequence segment. The protection factor determined for each peptide fragment was used to calculate the local stability (ΔG_{op}) of the corresponding sequence segment in the protein, $\Delta G_{\text{op}} = RT \ln P_f$. For each sequence segment, ΔG_{op} is, therefore, the free energy of opening of the N state to form a HDX-competent partially unfolded form.

Results

Introducing a rigid loop into mouse prion protein did not alter the secondary structure and the stability of the protein

Among the two single mutant variants, N173T moPrP is known to have a disordered $\beta 2$ - $\alpha 2$ loop, similar to that in wt moPrP, while S169N moPrP has a partially ordered $\beta 2$ - $\alpha 2$ loop (Gorfe and Caflisch, 2007). On the other hand, the NMR studies suggested that the $\beta 2$ - $\alpha 2$ loop of the double mutant variant, [S169N, N173T] moPrP exactly mimics the structured rigid $\beta 2$ - $\alpha 2$ loop of elk/deer PrP (Gossert et al., 2005). All three mutant variants showed far-UV CD spectra that were very similar to that of wt moPrP (Figure 5.2a), indicating that altering the conformation of the $\beta 2$ - $\alpha 2$ loop did not affect the global secondary structure of the protein. Urea-induced equilibrium studies showed that these mutations do not lead to any significant change in the stability of the N state (Figure 5.2b and

Table 5.1), suggesting that it does not depend on the conformation of the $\beta 2$ - $\alpha 2$ loop.

Introduction of a rigid loop slowed down the formation of misfolded oligomers

The monomeric form of moPrP, which is predominantly α -helical, misfolds into β -rich oligomers in a pH-dependent manner (Singh and Udgaonkar, 2016f; Van der Kamp and Daggett, 2010b). This pH-induced conformational conversion has an apparent pKa value of 4.7 (Singh and Udgaonkar, 2016f). Hence, even at physiological pH, moPrP has the potential to misfold into oligomers, albeit to a negligible extent. The addition of salt is crucial for initiating misfolding. Salt disrupts native electrostatic interactions in the monomeric protein, playing a critical role in triggering misfolding

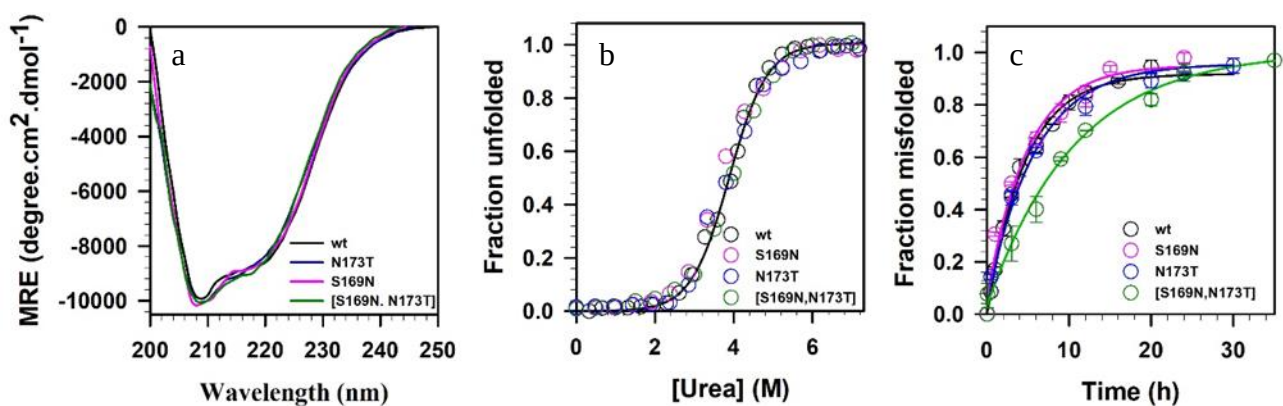


Figure 5.2. Effect of the rigid loop substitutions on secondary structure, stability, and misfolding. (a) Far-UV CD spectra of the native monomeric forms of the moPrP variants acquired at pH 4, at 25 °C. (b) Urea-induced equilibrium unfolding transitions of the moPrP variants at pH 4, at 25 °C, as monitored by measurement of the far-UV CD signal at 222 nm. The signal change was normalized to obtain the fraction unfolded. The solid line through the data points for all protein variants is a fit to an equation describing a two-state unfolding transition, and yields $\Delta G_u = 4.2$ kcal/mol and $C_m = 4.1$ M. (c) The kinetics of misfolding of 100 μ M protein in the presence of 150 mM NaCl at pH 4, at 37 °C, is shown. The colors of the lines represent the different moPrP variants as indicated. The error bars represent the standard deviations determined from three independent experiments.

Table 5.1. Stabilities and misfolding rate constants of the different moPrP variants

moPrP variant	ΔG_u (kcal mol⁻¹)	Misfolding rate constant (h⁻¹)
wt	4.2 ±0.2	0.17 ±0.03
S169N	4.0 ±0.3	0.21 ±0.02
N173T	4.2 ±0.1	0.16 ±0.01
[S169N,N173T]	4.3 ±0.2	0.09 ±0.02

The free energy of unfolding (ΔG_u), was obtained from urea-induced equilibrium unfolding studies at pH 4, 25 °C (Figure 5.2b). The m value was constrained to the same value (1.02 kcal mol⁻¹M⁻¹) for all the moPrP variants. The observed rate constant of misfolding was determined at 100 µM protein concentration in the presence of 150 mM NaCl at pH 4, 37 °C (Figure 5.2c).

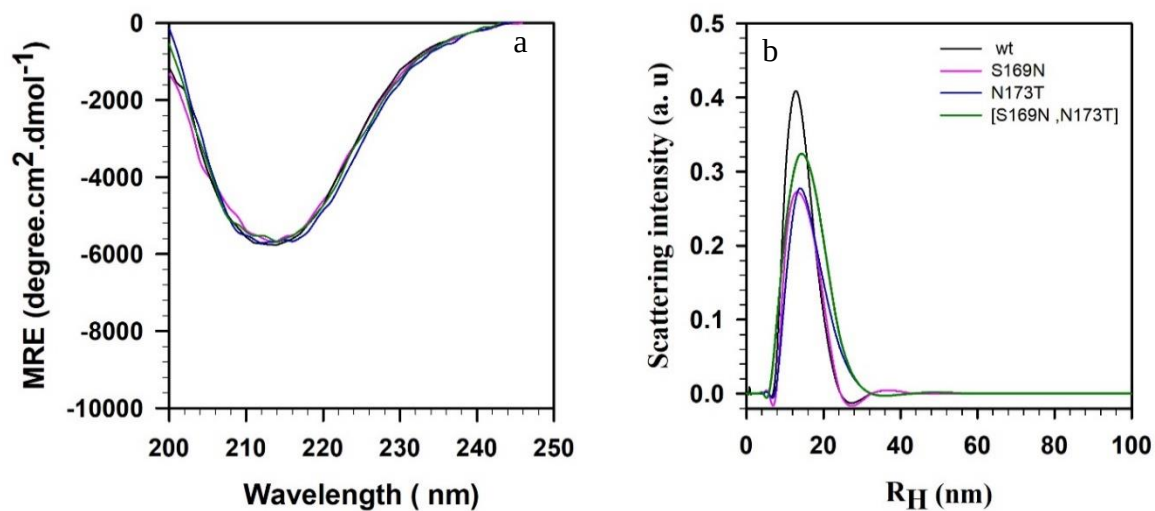


Figure 5.3. Secondary structure and size of the moPrP oligomers. (a) Far-UV CD spectra. (b) Hydrodynamic radii (R_H) of the matured misfolded oligomers of the different moPrP variants. The oligomers were prepared by incubation of 100 μ M protein in 150 mM NaCl, 10 mM sodium acetate, pH 4, at 37 °C, for a time corresponding to 5 time constants of the misfolding reaction. The differently colored lines represent the different moPrP variants as indicated.

and subsequent oligomerization (Sengupta et al., 2017a). Earlier studies had shown that the kinetics of CD-monitored misfolding and the kinetics of oligomerization monitored by size exclusion chromatography were similar for wt moPrP, suggesting that both processes occur concurrently (Sabareesan and Udgaonkar, 2016b). Consequently, in the present study, misfolding of moPrP was studied at pH 4, in the presence of 150 mM NaCl at 37 °C. The three moPrP variants exhibited kinetics of misfolding that fit well with a single exponential equation. N173T moPrP had a misfolding rate constant very similar to that of wt moPrP, while S169N moPrP misfolded very marginally faster (Figure 5.2c and Table 5.1). On the other hand, [S169N, N173T] moPrP exhibited a misfolding rate constant two-fold slower than that of wt moPrP (Figure 5.2c and Table 5.1).

Far-UV CD spectra showed that the oligomers formed by the different mutant variants studied here, and those formed by wt moPrP, have similar β -sheet contents (Figure 5.3a). The hydrodynamic radii (R_H) of the oligomers formed by the different moPrP variants were also very similar ($R_H = 12 \pm 1$ nm), as measured by DLS (Figure 5.3b).

Effect of introduction of the rigid loop on protein dynamics

To understand why [S169N, N173T] moPrP with a rigid $\beta 2$ - $\alpha 2$ loop misfolded slower than wt moPrP, it was important to determine the extent to which the introduction of the rigid loop influences the structural dynamics of the protein. Hydrogen-deuterium exchange (HDX) coupled with mass spectrometry (MS) studies were carried out to obtain information about sequence-specific structural dynamics. In HDX studies, structured and buried regions of the protein are protected from deuteration, while unstructured and solvent-exposed regions become deuterium-labeled. These labeled segments gain mass, and can be identified through peptic digestion at low pH, under which condition the exchange reaction is quenched (Bai et al., 1993b). This method provides localized information on the structural dynamics of different parts of the protein. It is advantageous to carry out HDX studies at pH 4 because intrinsic HDX rates are low at this pH, allowing the monitoring of deuterium incorporation at amide hydrogen sites distributed throughout all secondary structural elements of the protein (Bai et al., 1993b). The rate constants of deuterium incorporation into different sequence segments of the single mutant variants were found to be similar to those observed for wt moPrP (Figure 5.4). This indicated that in most of the secondary structural regions, the structural dynamics in the mutant variants were similar to those seen for wt moPrP. The kinetics of deuterium incorporation in

sequence segment 168-173, spanning the $\beta 2$ - $\alpha 2$ loop, was found to be slower in the case of S169N moPrP (Figure 5.4). In the case of [S169N, N173T] moPrP, the sequence segment 168-173, straddling the $\beta 2$ - $\alpha 2$ loop, as well as the sequence segments 210-216, 217-223, 225-231, straddling the C-terminal end of $\alpha 3$ were found to undergo exchange significantly slower than in wt moPrP (Figure 5.5)

For both S169N and N173T moPrP, most of the sequence segments showed ΔG_{op} values (Figure 5.6) similar to those for wt moPrP. In the case of S169N moPrP, the sequence segment straddling the $\beta 2$ - $\alpha 2$ loop (168-173) showed a slight increase in its ΔG_{op} value (Figure 5.6). In the case of [S169N, N173T] moPrP, the sequence segment 168-173, spanning the $\beta 2$ - $\alpha 2$ loop, as well as the sequence segments 210-216, 217-223 and 225-231, spanning the C-terminal end of $\alpha 3$ showed a significant increase in ΔG_{op} (Figure 5.7). This indicated that introducing a rigid $\beta 2$ - $\alpha 2$ loop into moPrP increased the local stabilities of the sequence segments spanning the $\beta 2$ - $\alpha 2$ loop as well as the adjacent C-terminal end of $\alpha 3$.

Effect of introduction of the rigid loop on the stability of partially unfolded forms of PrP

Previous HDX-NMR and HDX-MS studies detected two sparsely populated partially unfolded forms (PUFs), referred to as PUF1 and PUF2, which exist in dynamic equilibrium with the N state of moPrP at pH 4 (Figure 5) (Moulick et al., 2015a; Pal and Udgaonkar, 2022a, 2023). The free energy required to transition from the N state to PUF2 ($\Delta G_{op} = 3.7 \pm 0.4$ kcal mol⁻¹) is higher than that to PUF1 ($\Delta G_{op} = 1.3 \pm 0.2$ kcal mol⁻¹), suggesting that the segments unfolded in PUF1 are also likely unfolded in PUF2 (Pal and Udgaonkar, 2023). Further characterization of moPrP mutant variants in which evolutionarily conserved residues in the CTD were mutated to Ala, revealed that PUF2 is composed of at least two iso-energetic conformations, PUF2* and PUF2** (Pal and Udgaonkar, 2023). In PUF2*, sequence segments 144–148, 149–153 ($\alpha 1$), 154–167 (the loop between $\alpha 1$ and $\beta 2$, $\beta 2$), and 197–204 (the loop between $\alpha 2$ and $\alpha 3$, and the N-terminal end of $\alpha 3$) are unfolded (Pal and Udgaonkar, 2022a). In PUF2**, sequence segments 154–167 and 217–223 (C-terminal end of $\alpha 3$) are unfolded (Pal and Udgaonkar, 2023).

In the current study, in the case of [S169N, N173T] moPrP, the ΔG_{op} values of the sequence segments covering the $\beta 2$ - $\alpha 2$ loop (168-173) and the adjacent C-terminal end of $\alpha 3$ (210-216, 217- 223, 225-231) were found to be significantly higher than those of wt moPrP (Table S3). Of the sequence segments in [S169N, N173T] moPrP that have higher local stability than in wt moPrP (see above and Table S3), sequence segments 168-173, 210-216, and 225-231 were

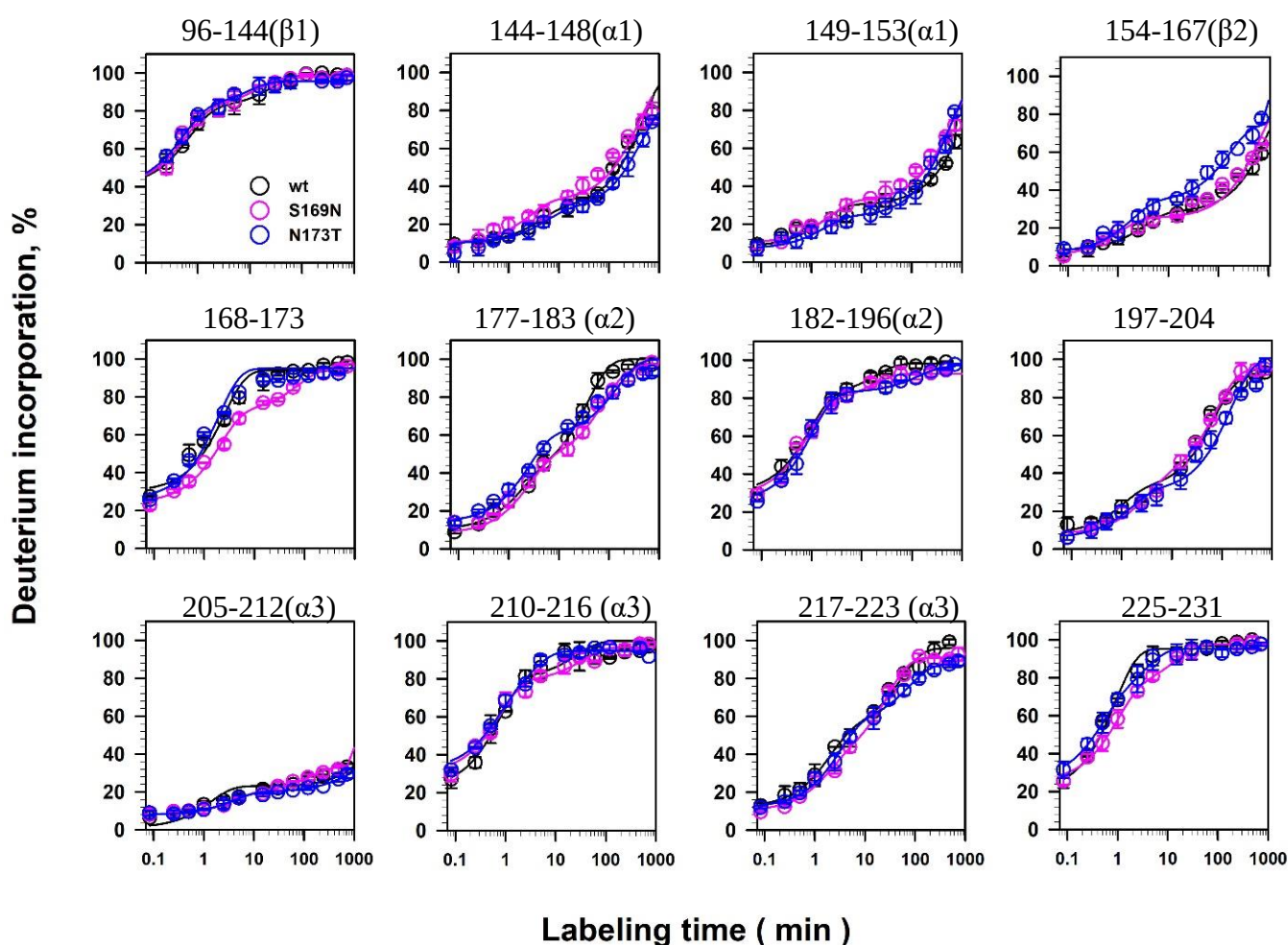


Figure 5.4 Effect of the single mutations, S169N and N173T, on the native state dynamics.

The time courses of HDX into different sequence segments of the native monomeric form at pH 4, 25 °C are shown. The differently colored circles represent the different moPrP variants, as indicated. The error bars represent the standard deviations determined from three independent experiments. The solid lines through the data represent fits to either monoexponential or biexponential equations. The data for wt moPrP is reproduced from Pal and Udgoankar, 2023.

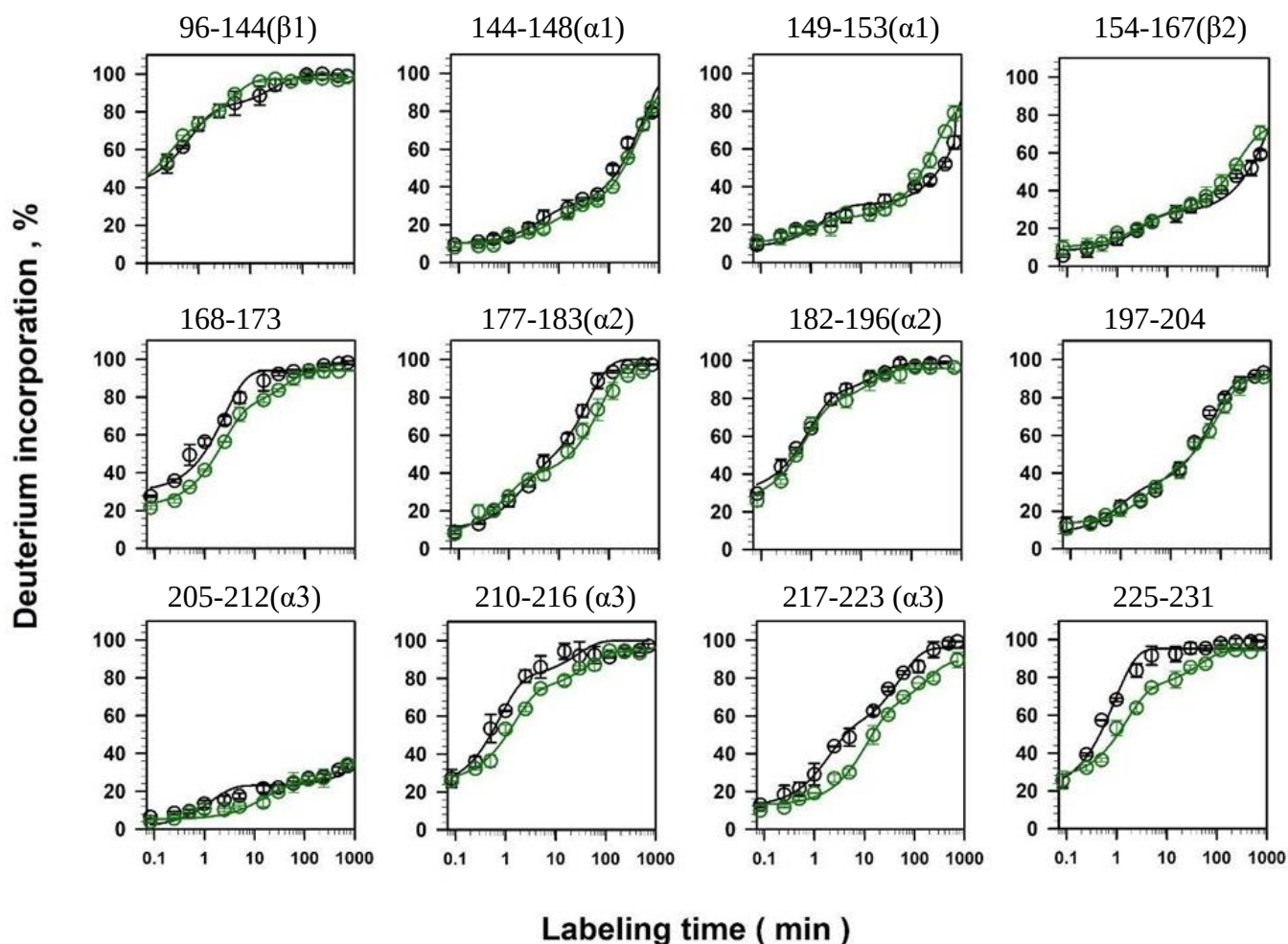


Figure 5.5. Effect of the [S169N, N173T] mutations on the native state dynamics. The time courses of HDX into different sequence segments of the native monomeric forms at pH 4, 25°C are shown. Data for wt moPrP and [S169N, N173T] moPrP are shown in black and green, respectively. The solid lines through the data represent fits to either monoexponential or biexponential equations. The error bars represent the standard deviations determined from three independent experiments. The data for wt moPrP is reproduced from Pal and Udgaonkar, 2023.

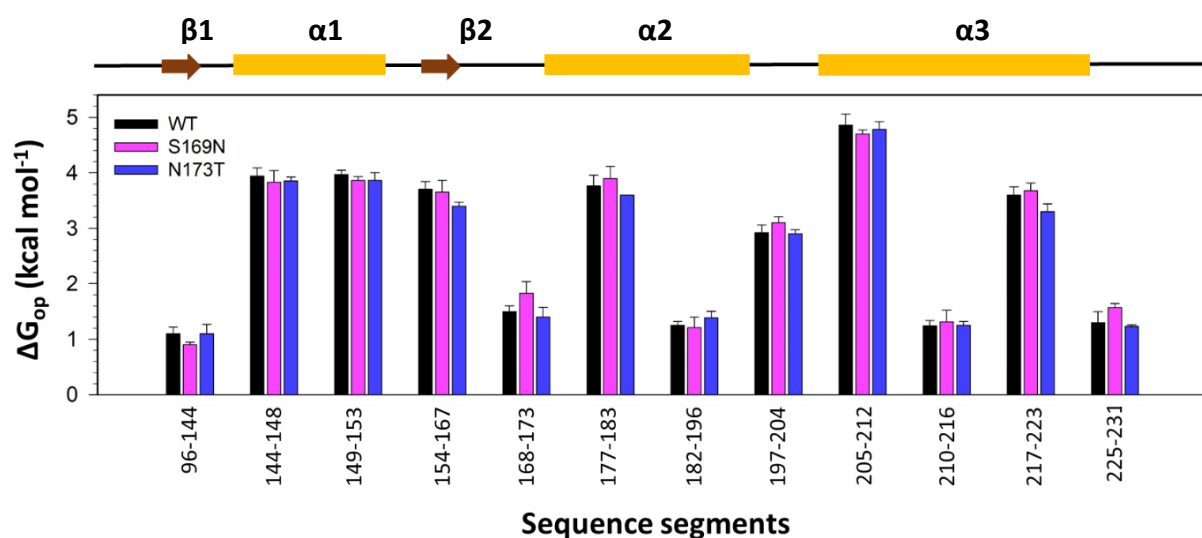


Figure 5.6. ΔG_{op} values of the different sequence segments of the wt and single mutant variants of moPrP. The secondary structural elements are shown above the sequences (rectangular boxes represent the α -helices, and arrows represent the β - strands). The differently colored bars represent the different moPrP variants, as indicated. The error bars represent the standard deviations determined from three independent experiments.

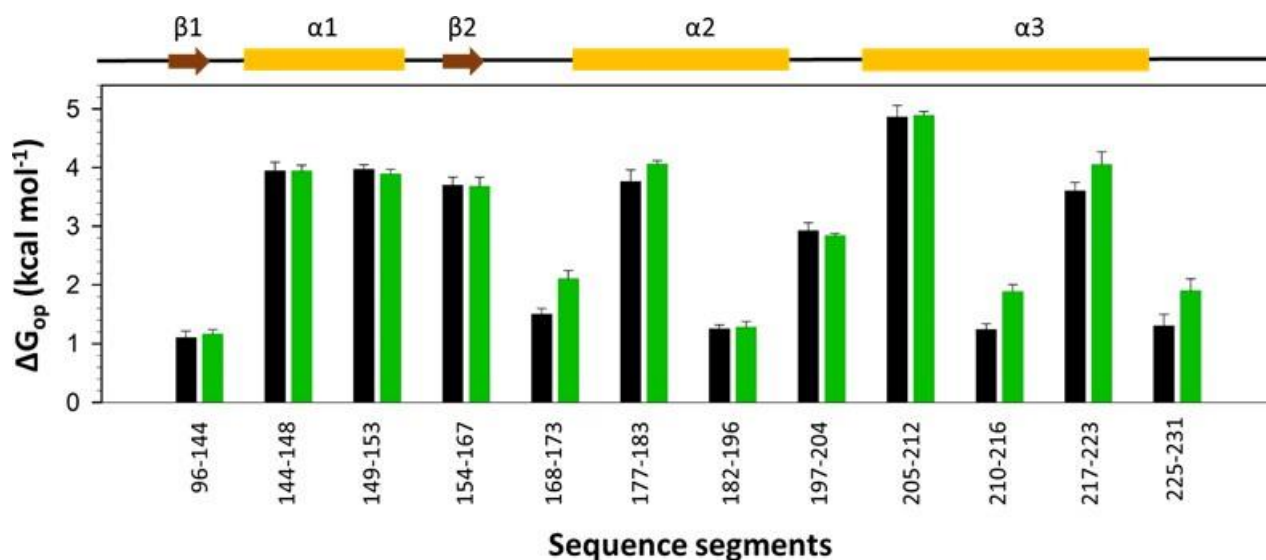


Figure 5.7. ΔG_{op} values of the different sequence segments of wt and [S169N, N173T] **moPrP**. The secondary structural elements are shown above the sequences. (rectangular boxes represent the α -helices, and arrows represent the β -strands). Data for wt moPrP and [S169N, N173T] moPrP are shown in black and green, respectively. The error bars represent the standard deviations determined from three independent experiments.

found to be unfolded in PUF1, and sequence segment 217-223 was found to be unfolded in PUF2**. This suggested that introducing a rigid $\beta 2$ - $\alpha 2$ loop into moPrP significantly impacts the local stabilities of the sequence segments that are known to become unfolded in the previously identified PUFs. The free energy of opening of the N state to PUF1 and PUF2** in the case of [S169N, N173T] moPrP was 1.8 ± 0.3 and 4.05 ± 0.2 kcal mol⁻¹, respectively, suggesting that the [S169N, N173T] mutations significantly increased the local stabilities of PUF1 and PUF2** (Figure 5.8).

Dependence of the rate constants of misfolding on the stability of PUFs

In earlier HDX-MS studies, a close correspondence was found between the observed rate constants of misfolding of mutant variants and the extent to which the PUFs were populated, indicating that the PUFs were the initial monomeric states from which misfolding commences (Pal and Udgaonkar, 2022a, 2023). Mutation of the conserved Pro residues in the CTD, as well as several disease-linked mutations, reduced ΔG_{op} between the N state and PUF2*, and increased the population of PUF2*, thereby accelerating the misfolding process (Pal and Udgaonkar, 2022a; Singh and Udgaonkar, 2015c). For both sets of mutant variants, the logarithm of the observed misfolding rate constants correlated linearly with the equilibrium population of PUF2* (Pal and Udgaonkar, 2022a). On the other hand, mutation of three conserved aromatic residues (Tyr168, Phe174, and Tyr217) present at the interface of the $\beta 2$ - $\alpha 2$ loop and the C-terminal end of $\alpha 3$ caused rapid misfolding of moPrP under similar conditions. For these mutant variants too, a linear dependence of the logarithm of the observed rate constant of misfolding on ΔG_{op} between the N state and PUF1, and PUF2**, had been observed (Pal and Udgaonkar, 2023). Since PUF1 is more stable than both PUF2* and PUF2**, it would be more populated at equilibrium and would be the primary precursor conformation initiating misfolding. Figure 5.9 compares the dependence of the observed rate constant of misfolding of [S169N, N173T] moPrP on the stability of PUF1 and PUF2**, with that of the aromatic mutant variants. Interestingly, the observed rate constants of misfolding of both sets of mutant variants were found to have very similar dependences on the stability of PUF1 and PUF2**.

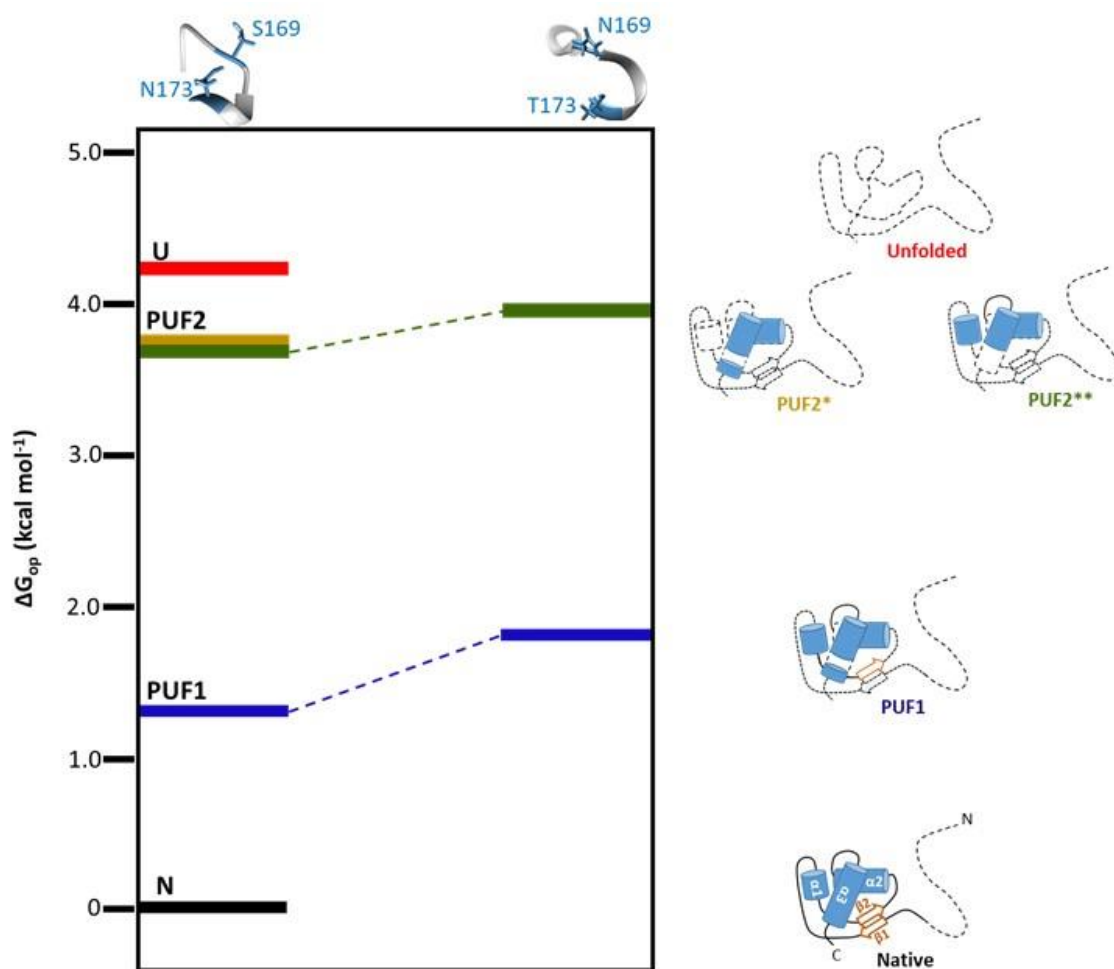


Figure 5.8. Effect of rigid loop substitutions on the stabilities of the PUFs. The free energy levels of the PUFs, produced from the unfolding of specific sequence segments, have been placed on a free energy ladder. PUF2 has been shown to comprise two iso-energetic sub-populations of molecules, PUF2* (gold) and PUF2** (green), which differ in structure (Pal and Udgaonkar, 2023). The structures of the PUFs are shown on the right. For each PUF, the unfolded segments are shown as dotted lines. The unstructured N-terminal domain (23-120) is shown as a dashed line. Substitutions of the two amino acids (S169N and N173T) in moPrP change the loop structure from a flexible into a rigid structure, resulting in the increase of the free energy levels (and decrease in the equilibrium populations) of the aggregation-prone PUF1 and PUF2**, as indicated by differently colored broken lines.

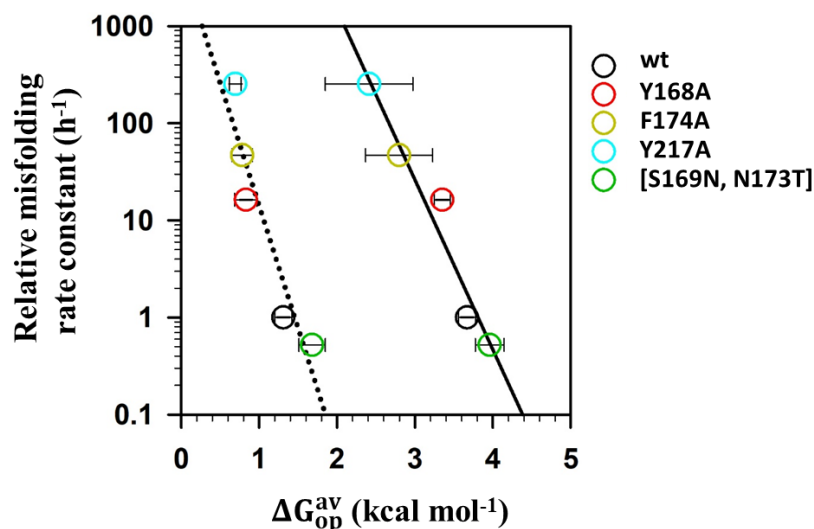


Figure 5.9. Dependence of the observed rate constant of misfolding on the stability of the PUFs. The plot shows the dependence of the observed rate constant of misfolding of the aromatic mutant variants, Y168A, F174A, and Y217A moPrP, and [S169N, N173T] moPrP in the presence of 150 mM NaCl at pH 4, 37 °C, on the stability (ΔG_{op}^{av}) of PUF1 (dotted line) and PUF2** (solid line). The stabilities (ΔG_{op}^{av}) of PUF1 and PUF2** were determined by averaging the ΔG_{op} values of the sequence segments 96-144, 168-173, 182-196, 210-216, and 225-231 for PUF1**, and 154-167 and 217-223 for PUF2**. The continuous line through the data points is a linear fit to the data points. The differently colored circles represent the different moPrP variants, as indicated. The error bars represent the standard deviations from three independent experiments. The data for wt moPrP and the aromatic mutant variants are taken from Pal and Udgaonkar, 2023

Introducing a rigid loop does not affect the lag phase of unseeded fibrillation reaction

To determine whether the [S169N, N173T] mutations affected the formation of amyloid fibrils, fibrillation experiments were carried out. In previous studies, fibrillation was carried out under partially denaturing conditions to induce fibril formation (Singh and Udgaonkar, 2013a). Under these conditions, moPrP has been shown to form straight amyloid fibrils (Singh and Udgaonkar, 2013a). In the study presented here, fibrillation experiments at pH 7 were also carried out in the presence of 2 M GdnHCl. The kinetics of fibril formation were very similar for wt moPrP, N173T moPrP and [S169N, N173T] moPrP, while S169N moPrP had a shorter lag phase (Figure 5.10 and Table 5.2). Thus, the decreased structural dynamics at the interface of the β 2- α 2 loop and the C-terminal end of α 3, caused by the [S169N, N173T] mutations, did not significantly affect the lag phase of unseeded fibrillation under the mild denaturing conditions studied here. For all three mutant variants, the final ThT fluorescence signal was the same as that observed for wt moPrP, indicating that the extent of fibril formation was the same.

Introducing a rigid loop slows down the elongation rates of seeded fibrillation reactions

Pathological fibrils can drive the conversion of soluble monomeric protein into fibrils in a template-dependent manner (Esler et al., 2000; Singh and Udgaonkar, 2013a). To check the involvement of the β 2- α 2 loop in this process, fibrillation experiments were carried out in the presence of wt moPrP and [S169N, N173T] moPrP seeds. Interestingly, both wt and [S169N, N173T] mutant seeds were found capable of seeding both wt moPrP and [S169N, N173T] moPrP monomers (Figure 5.11). However the elongation rate constants for the seeded fibrillation reactions of wt moPrP were 1.6 times faster than those for the seeded fibrillation reactions of [S169N, N173T] moPrP, regardless of the seeds selected (Table 5.3).

Discussion

Native state stability does not depend on the conformation of the β 2- α 2 loop

Understanding the factors influencing the stability of the prion protein is important, as thermodynamic stabilization has been shown to affect both conformational conversion *in vitro*, and the propagation of prion infection *in vivo* (Kong et al., 2013). The β 2- α 2 loop region has been studied much, given its role in prion conversion (Danzhi and Amedeo, 2015; Kurt et al., 2014a). This study shows that rigidifying the β 2- α 2 loop by introducing [S169N, N173T] mutations, does not affect the overall secondary structure as well as the global stability of the

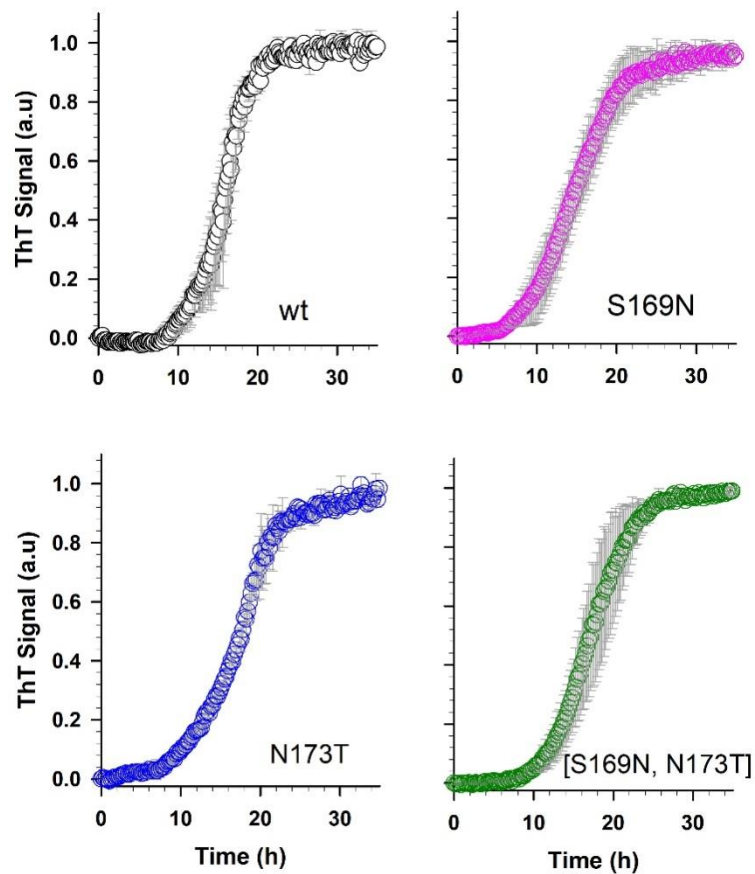


Figure 5.10. Kinetics of moPrP fibril formation. ThT fluorescence-monitored aggregation kinetics of 25 μ M wt moPrP (black), S169N moPrP (pink), N173T moPrP (blue), [S169N, N173T] moPrP (green) at pH 7. The error bars represent the standard deviations from three independent experiments. The data for wt moPrP is reproduced from Pal and Udgaonkar, 2024.

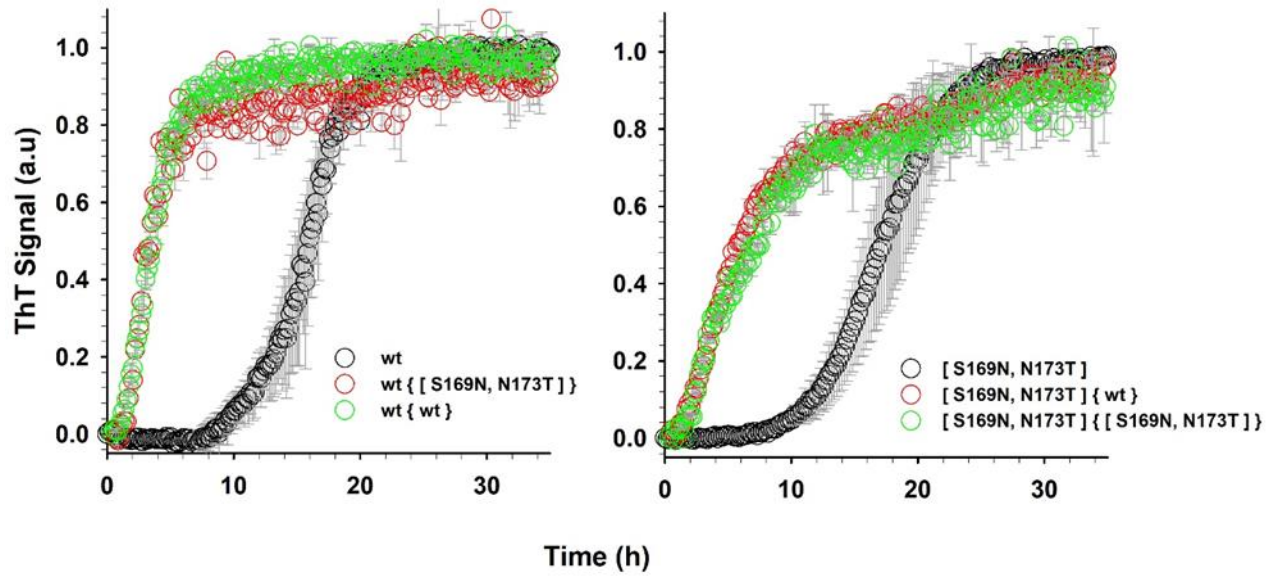


Figure 5.11. ThT fluorescence-monitored kinetics of fibril formation by moPrP variants in the absence and presence of 10% seed at pH 7. The nature of the seed is shown inside the brackets. The error bars represent the standard deviations from three independent experiments. The data for wt moPrP is reproduced from Pal and Udgaonkar, 2024

Table 5.2. Lag phases of fibrillation of the different moPrP variants shown in Figure 5.10

moPrP variant	Lag phase (h)
wt	11.6 ± 0.9
S169N	9.6 ± 0.5
N173T	11.5 ± 0.3
[S169N,N173T]	12.1 ± 0.7

The lag time for fibril formation reactions were determined by fitting the kinetic curve to the following equation:

$$S_t = S_0 + \frac{a}{1 + e^{\left(\frac{t - t_{50}}{\tau}\right)}}$$

where S_t and S_0 are the ThT fluorescence intensities at time t , and zero respectively, a is the amplitude of the signal, t_{50} is the time at which the fluorescence intensity is 50%, and τ is a time constant. The lag time (t_{lag}) was calculated as $t_{lag} = t_{50} - 2\tau$.

Table 5.3. Rate constants of fibril elongation of the different moPrP variants shown in Figure 5.11

moPrP variant	Rate constant of fibril elongation (h ⁻¹)
wt	0.14 ± 0.022
wt	0.26± 0.024
wt [S169N, N173T]	0.23± 0.015
[S169N,N173T]	0.11 ± 0.010
[S169N,N173T]	0.17± 0.021
[S169N,N173T] [S169N, N173T]	0.15± 0.016

The nature of the seed is shown inside the brackets. In the case of both the unseeded and seeded fibrillation reactions, the apparent rate constant of fibril elongation was determined by fitting the kinetic data points, to the following single equation

$$S_t = S_0 + a(1 - e^{-kt})$$

where S_t and S_0 are the ThT fluorescence signals at times t , and zero, a is the amplitude of the signal and k is the rate constant of fibril elongation. In all cases, the initial 20% of the curve was excluded for the fit. The error bars represent the standard deviations from three independent experiments.

protein, even though they decrease the rate constant of misfolding. This suggests that the global stability of the protein may not be the key factor in determining the misfolding propensity.

NMR studies have shown that the $\beta 2$ - $\alpha 2$ loop of wt moPrP exists in a conformational equilibrium between two distinct forms: a 3_{10} helix turn and a type-I β turn (Christen et al., 2013). In [S16N, N173T] moPrP, the 3_{10} helix turn form is dominant (Christen et al., 2013). The $\beta 2$ - $\alpha 2$ loop is structurally ordered and stabilized in the 3_{10} helix turn conformation, by a favorable stacking interaction between Tyr168 and Phe174 within the $\beta 2$ - $\alpha 2$ loop (Figure 5.12). Additionally, the 3_{10} helix turn enables Tyr168 to form a stable hydrogen bond with Asp177 in $\alpha 2$ (Figure 5.12). The well-defined conformation of the $\beta 2$ - $\alpha 2$ loop also appears to stabilize the otherwise weak hydrophobic interactions of the side-chain of Val165 with Tyr217, Ser221 and Tyr224 at the C-terminal end of $\alpha 3$ (Figure 5.13). In contrast, in the type-I β turn conformation, the $\beta 2$ - $\alpha 2$ loop is structurally disordered; consequently, Tyr168 cannot engage in stable hydrogen bonding with Asp177 or in a stacking interaction with Phe174 (Figure S6). The interactions between Val165 and Tyr217, Ser221 and Tyr224 would also be weaker (Figure 5.13) compared to the N state of [S169N, N173T] moPrP.

Hence, the N state of [S169N, N173T] moPrP is enthalpically stabilized by favorable stacking and hydrogen bonding interactions of the $\beta 2$ - $\alpha 2$ loop, but it is entropically destabilized due to restrictions on the number of potential conformations that the loop can adopt. $\alpha 3$ is also better defined in the NMR spectrum of the N state of [S169N, N173T] moPrP than in that of wt moPrP (32), indicating that it is less dynamic, and hence, is entropically destabilized. This likely explains why the [S169N, N173T] mutations do not impact the overall global stability of the protein.

Long-range interactions between the $\beta 2$ - $\alpha 2$ loop and the C-terminal end of $\alpha 3$ determine the stabilities of the PUFs

The spontaneous formation of PrP^{Sc} in the cases of the disease-associated E200K, Y217N, V210I, Q212P, and Q226E mutant variants, which are associated with familial prion diseases, is thought to be caused by the disruption of the long-range interactions between the $\beta 2$ - $\alpha 2$ loop and the C-terminal end of $\alpha 3$ (Biljan et al., 2012b; Biljan et al., 2011; Giachin et al., 2011; Jung Cheng and Daggett, 2014; Meli et al., 2011b; Slapšak et al., 2019a). Such disruption would destabilize the N states of these mutant variants, but not the PUFs in which the interactions are absent. The resultant decrease in ΔG_{op} between the N state and PUF1 as well as PUF2** would increase the probability of misfolding to occur. Clearly, the long-range intramolecular interactions between the $\beta 2$ - $\alpha 2$ loop and the C-terminal end of $\alpha 3$ play an

important role in determining the ΔG_{op} values between the N state and PUF1, and PUF2**, which act as precursors for spontaneous misfolding.

Heterogeneity in PUF2

The ΔG_{op} value of sequence segment 177-183 ($\Delta G_{op} = 3.76 \pm 0.20$ kcal mol⁻¹) would suggest that it should be unfolded in PUF2 in the case of wt moPrP (Pal and Udgaonkar, 2022a, 2023). Interestingly, in both sub-populations of the PUF2 ensemble, PUF2* and PUF2**, this segment retains its native structure, indicating the presence of an additional iso-energetic subpopulation within PUF2, in which it is unfolded (Pal and Udgaonkar, 2022a, 2023). In the case of [S169N, N173T] moPrP, an increase in the ΔG_{op} value was observed for sequence segment 177-183 ($\Delta G_{op} = 4.06 \pm 0.05$ kcal mol⁻¹). Hence, introducing a rigid $\beta 2$ - $\alpha 2$ loop increases the local stability of this specific sub-population. This finding confirms the existence of another sub-population within the PUF2 ensemble, in which the 177-183 segment has become unfolded. These results highlight the nuanced structural dynamics within the PUF2 ensemble, with multiple sub-populations with distinct structural characteristics but with similar stabilities.

The conformation of the $\beta 2$ - $\alpha 2$ loop modulates the separation of the $\beta 1$ - $\alpha 1$ - $\beta 2$ sub-domain from the $\alpha 2$ - $\alpha 3$ sub-domain

It has been proposed that separation of the $\beta 1$ - $\alpha 1$ - $\beta 2$ sub-domain from the $\alpha 2$ - $\alpha 3$ sub-domain must occur before conformational conversion to misfolded β -rich-oligomers (Goluguri et al., 2019a; Hadži et al., 2015a; Sengupta and Udgaonkar, 2019a). Locking these two subdomains by binding to anti-prion drugs can prevent misfolding and oligomerization (Kamatari et al., 2013; Kuwata et al., 2007b). Earlier studies had shown that sub-domain separation can be triggered by two different mechanisms in which PUF1 misfolds *via* both PUF2* and PUF2**. (Pal and Udgaonkar, 2023). When PUF2* is the precursor to misfolding, the perturbation of the electrostatic network comprised of salt bridges formed by multiple charged residues present at the interface of the $\alpha 1$ and the $\alpha 2$ - $\alpha 3$ loop facilitates sub-domain separation (Pal and Udgaonkar, 2022a). On the other hand, when PUF2** is the precursor for misfolding, the electrostatic network is unaffected, but the disruption of aromatic and hydrophobic interactions present at the opposite end of the protein induces the sub-domain separation (Pal and Udgaonkar, 2023). Not surprisingly, increasing the stability of the $\beta 2$ - $\alpha 2$ loop through a disulfide bond between the $\beta 2$ - $\alpha 2$ loop and the C-terminal

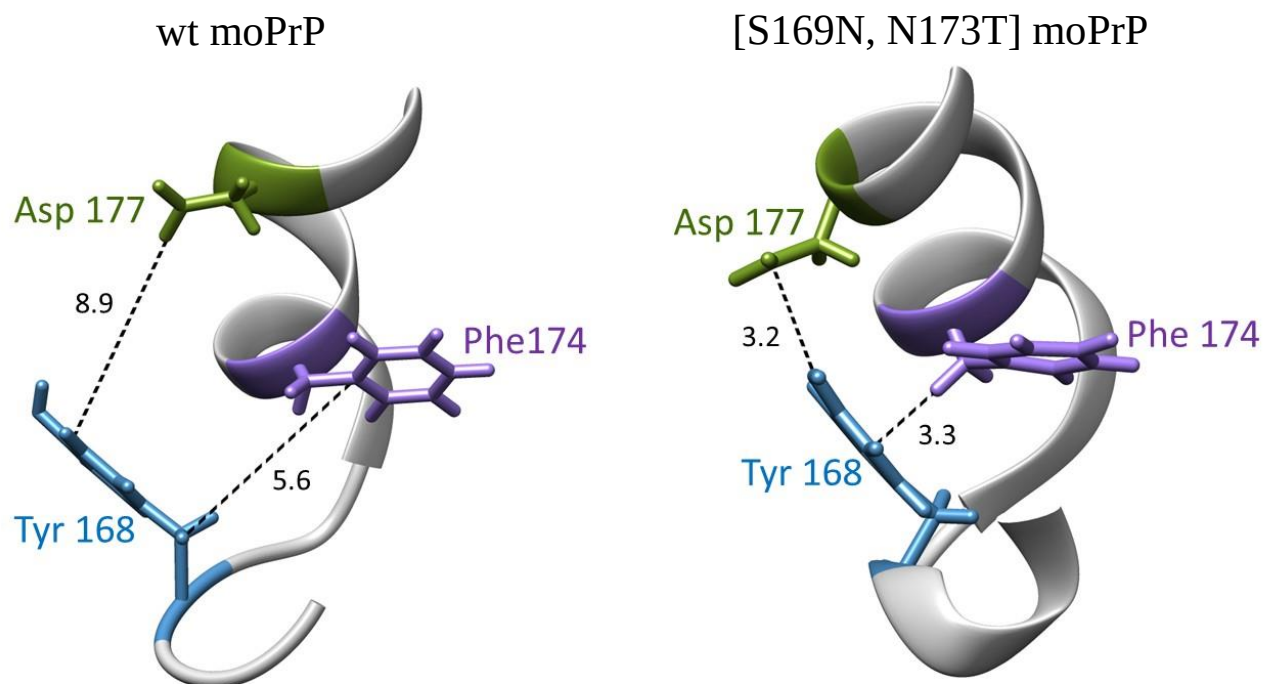


Figure 5.12. Interactions of Tyr 168 in wt moPrP and in [S169N, N173T] moPrP. In the case of wt moPrP, the side chain of Tyr168 in the $\beta 2$ - $\alpha 2$ loop is buried to the extent of 40 % and forms a weak π -stacking interaction with Phe174. In the case of the [S169N, N173T] moPrP, the side chain of Tyr168 is buried to the extent of 83 % and stabilizes the π -stacking interaction with Phe 174 as well as forms a strong hydrogen bonding interaction with Asp 177. The distances between the side chains are shown in Å. The figure was drawn using Chimera and Protein Data Bank entry [1AG2](#) (wt moPrP) and [1Y16](#) ([S169N, N173T] moPrP).

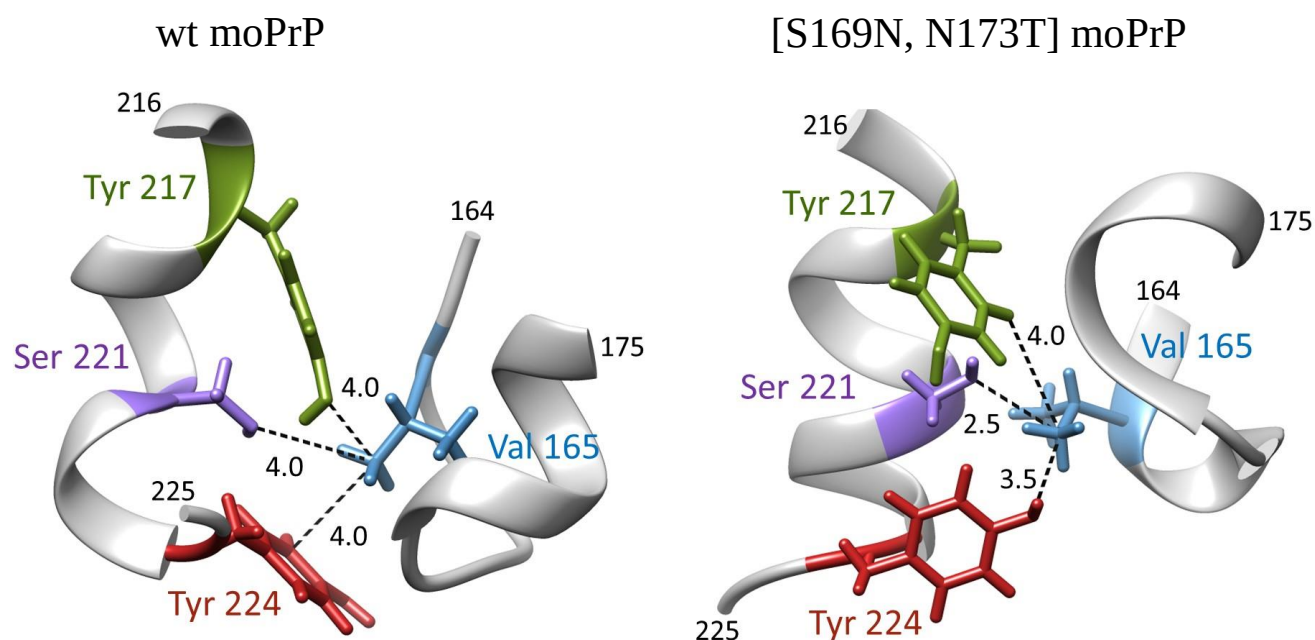


Figure 5.13. Interactions between the $\beta 2$ - $\alpha 2$ loop and the C-terminal end of $\alpha 3$. In the case of wt moPrP, the side chain of Val 165 in the $\beta 2$ - $\alpha 2$ loop (164-175) appears to form hydrophobic interactions with multiple residues (Tyr217, Ser221, and Tyr224) at the C-terminal end of $\alpha 3$ (216-225). In the case of the [S169N, N173T] moPrP, the altered conformation of the $\beta 2$ - $\alpha 2$ loop leads to a stabilization of those interactions. The distances between the side chains are shown in Å. The figure was drawn using Chimera and Protein Data Bank entry [1AG2](#) (wt moPrP) and [1Y16](#) ([S169N, N173T] moPrP).

end of $\alpha 3$ significantly reduces the formation of misfolded oligomers (Prigent and Rezaei, 2011b). In the current study, stabilization of the $\beta 2$ - $\alpha 2$ loop by making elk/deer-specific mutations at residue positions 169 and 173 appears to stabilize the aromatic and hydrophobic interactions present between the $\beta 2$ - $\alpha 2$ loop and the C-terminal end of $\alpha 3$. It thereby hinders separation of the two sub-domains. These results suggest that the conformation of the $\beta 2$ - $\alpha 2$ loop plays a critical role in modulating the separation of the $\beta 1$ - $\alpha 1$ - $\beta 2$ sub-domain from the $\alpha 2$ - $\alpha 3$ sub-domain.

It should be noted that moPrP can misfold to form various types of aggregates, including oligomers (Singh et al., 2014a), worm-like fibrils (Jain and Udgaonkar, 2008), and straight fibrils (Singh and Udgaonkar, 2013a), all characterized by β -rich structures. Previous HDX studies identified a highly protected core region within these aggregates, spanning residues 168 to 223 ($\alpha 2$ and $\alpha 3$ in the monomer) in both worm-like (Singh et al., 2012a) and straight fibrils (Singh and Udgaonkar, 2013a). Recent cryo-electron microscopy studies of amyloid fibrils formed *in vitro* by the human prion protein have also identified a core region consisting of residues 168-220 (Wang et al., 2020a), which requires the prior separation of the $\beta 1$ - $\alpha 1$ - $\beta 2$ sub-domain from the $\alpha 2$ - $\alpha 3$ sub-domain. These findings imply that the conformation of the $\beta 2$ - $\alpha 2$ loop might be crucial for the formation not only of β -rich oligomers but also of other pathological aggregates.

The $\beta 2$ - $\alpha 2$ loop conformation and correlation with the prion species-barrier

The conformation of the $\beta 2$ - $\alpha 2$ loop has been shown to have a substantial effect on the susceptibility of different species to prion infection. Transgenic mice expressing [S169N, N173T] moPrP (Tg(moPrP^{S169N, N173T})), characterized by a rigid $\beta 2$ - $\alpha 2$ loop, exhibit limited or no PrP conversion when exposed to PrP^{Sc} from cattle, sheep, and mouse strains that have a Ser at position 169, but do show conversion in the presence of PrP^{Sc} from deer and hamster, which have an Asn at position 169 (Sigurdson et al., 2010). However, when, in addition, the strictly conserved Tyr at position 168 is replaced with Gly in the $\beta 2$ - $\alpha 2$ loop, conversion to PrP^{Sc} is abolished in transgenic mice expressing this variant (Tg(moPrP^{Y168G, S169N, N173T})) (Kurt et al., 2014a; Kurt et al., 2014b). These mice show no detectable PrP^{Sc} in their brains even after extended incubation periods in the presence of PrP^{Sc} from cattle, sheep, mice, and deer (Kurt et al., 2014a; Kurt et al., 2014b). Horse PrP has the substitution D166S (Bett et al., 2012; Sigurdson et al., 2011), and both horse PrP and mouse PrP with the D166S substitution exhibit an ordered $\beta 2$ - $\alpha 2$ loop (Bett et al., 2012; Sigurdson et al., 2011). Mice expressing either wt

moPrP or D166S moPrP (Tg(moPrP^{D166S})) show no conversion when inoculated with PrP^{Sc} from species with an ordered β 2- α 2 loop, but are highly susceptible to PrP^{Sc} from species with a disordered β 2- α 2 loop. (Bett et al., 2012; Sigurdson et al., 2011). However, since both the sequence and structure of the β 2- α 2 loop are affected, it is unclear which is responsible for the altered transmission barriers in mice.

The observation that the unseeded fibrillation of the mutant variants resembles that of wtmoPrP, exhibiting lag phases in the range of 9 to 12 h (Figure S5 and Table S4), with S169N being the fastest, indicates that the rigidity of the β 2- α 2 loop did not significantly affect the lag phase of fibril formation. It should, however, be noted that the fibrillation reactions were carried out in the presence of 2 M GdnHCl, as in the absence of a denaturant, it is too slow to monitor. The similar lag phases (Table S4) and elongation rates (Table S5) observed for wt and [S169N, N173T] moPrP could be attributed to the presence of 2 M GdnHCl in the fibrillation reaction, which may have reduced the conformational differences in the β 2- α 2 loop between the two moPrP variants and the β 2- α 2 loop in [S169N, N173T] moPrP may not be as rigid as it is in its native state.

Intriguingly, both wt moPrP and [S169N, N173T] moPrP seeds were found capable of seeding both wt moPrP and [S169N, N173T] moPrP monomers to amyloid fibrils, and the addition of 10% v/v seeds was adequate to abolish the lag phase in the fibrillation process of both moPrP monomers. The rate constants of elongation, observed for the seeded fibrillation reactions of wt moPrP, were 1.6 times faster (Table S5) than those observed for the seeded fibrillation reactions of [S169N, N173T] moPrP, regardless of the seeds used. The observation that the presence of the rigid loop, in [S169N, N173T] moPrP but not in wt moPrP, makes the monomer less amenable to add on to pre-formed fibril, suggests that the conformation of this loop plays a crucial role in regulating not only oligomerisation but also fibril elongation. It also indicates that the identified PUFs are direct precursors to misfolding into both oligomers and amyloid fibrils.

Overall, the findings of the current study indicate that while the sequence of the β 2- α 2 loop is crucial in determining the prion species barrier, it is unlikely to be the sole determinant. Other factors, such as the host cellular environment and the host chaperone machinery responsible for degrading misfolded proteins, as well as post-translational modifications to PrP^C, are also likely to play key roles in influencing species barriers.

Chapter 6

**Removal of a packing imperfection in the hydrophobic core impedes
the misfolding of the prion protein**

Introduction

The cellular isoform of the mammalian prion protein (PrP^C) is a glycoprotein consisting of an unstructured N-terminal domain, and a folded C-terminal domain (CTD) containing three α -helices and two short β -strands (Bendheim et al., 1992; Riek et al., 1996). The misfolding of α -helix-rich PrP^C into β -rich aggregated forms (PrP^{Sc}) is linked to several neurodegenerative diseases (Chesebro, 2003; Prusiner, 1982). Despite significant research, the molecular mechanisms that underlie this misfolding process remain poorly understood. Uncovering the molecular mechanism is critical for advancing our understanding of prion diseases, and for developing effective anti-prion therapies.

Protein dynamics play an important role in modulating misfolding and aggregation (Ricchiuto et al., 2012). While proteins adopt a distinct folded structure through the process of folding (Dill and MacCallum, 2012), they constantly access lowly populated high-energy excited states due to thermal fluctuations (Oliveberg and Wolynes, 2005). These excited states are an integral part of their biological function but can also lead to protein misfolding and aggregation. In the case of the mouse prion protein (moPrP), it has been shown that the native (N) state is in dynamic equilibrium with multiple misfolding-prone partially unfolded states (PUFs), which act as direct precursors to misfolding and oligomer formation (Moulick et al., 2015; Pal and Udgaonkar, 2022, 2023). Earlier studies using NMR spectroscopy and pressure-induced unfolding have suggested that PUFs might be populated because of the entropic cost of packing imperfections in the hydrophobic core of the protein due to the presence of cavities (Xue et al., 2019). It is also shown that the formation of PUFs can be regulated by altering the cavity volume in the hydrophobic core of the protein (Xue et al., 2019).

NMR analysis of human PrP (huPrP) identified three cavities in its hydrophobic core, which contribute to the destabilization of the protein. Substituting methionine for valine at position 208 (V208M) eliminates two of these cavities, enhancing the thermodynamic stability of the protein (Kong et al., 2013). Transgenic mice studies suggested that V208M huPrP showed similar expression levels as well as cellular distribution patterns like wt huPrP (Kong et al., 2013). However, after inoculating these mice with brain homogenates from diseased individual, the Tg(HuPrPV208M) mice exhibited a significantly longer incubation period and lower susceptibility to prion infection compared to the wt Tg(HuPrP) mice (Kong et al., 2013), demonstrating that stabilizing the folded domain of PrP^C reduces prion propagation *in vivo*. It is therefore to examine how protein dynamics are affected when the packing integrity of the hydrophobic core is altered and, more importantly, how that impacts the formation of aggregation-prone PUFs, which act as direct precursors to misfolding.

In this study, we investigate how the cavities in mouse PrP (moPrP) influence its dynamics, ultimately affecting the formation of PUFs and the propensity to misfold and oligomerize. The removal of packing imperfections in the hydrophobic core of the prion protein, specifically the elimination of cavities around residue Val 208, is found to significantly increase the stability of the N state, and increases the energy separating between the N state from the PUFs. This results in a significant decrease of the sampling of the PUFs from the N state, leading to a suppression of misfolding. Conversely, increasing the size of the cavities destabilizes the N state, reduces the energy difference between the N state and PUFs, and leads to a 2.5-fold increase in the misfolding rate, promoting oligomer formation.

Materials and Methods

Reagents

All reagents used for experiments were of the highest purity grade available from Sigma, unless otherwise specified. Urea and guanidine hydrochloride (GdnHCl) were purchased from USB, and were of the highest purity grade.

Site-directed mutagenesis

The mutant variants of full-length moPrP were generated using the QuikChange site-directed mutagenesis kit (Stratagene). Primers containing 1-2 nucleotide changes were obtained from Sigma. The mutations in the plasmids were confirmed by DNA sequencing.

Protein expression and purification

All the moPrP variants were expressed in *Escherichia coli* BL21 (DE3) codon plus (Stratagene) cells transformed with a pET17b plasmid containing the full-length sequence (23–231) of the moPrP gene. All the moPrP variants were purified as described previously (Jain and Udgaonkar, 2008; Singh et al., 2014). The purity of each moPrP variant preparation was confirmed by mass spectrometry (Waters Synapt G2 HD mass spectrometer). Each moPrP variant showed an expected mass indicating that no chemical modification has taken place in any of the moPrP variants.

Far-UV Circular Dichroism (CD) measurements

Far-UV CD spectra were collected using a Jasco J-815 spectropolarimeter. Far-UV CD spectra were acquired using a protein concentration of 10 μ M in a 1 mm cuvette, using a scan speed of 50 nm/min, a digital integration time of 2 s, and a bandwidth of 1 nm.

Denaturant-induced equilibrium unfolding studies

Denaturant-induced and thermally-induced equilibrium unfolding transitions were determined at pH 4 in 10 mM sodium acetate buffer using 10 μ M protein. These studies were carried out in the same way as described earlier (Pal and Udgaonkar, 2022).

Oligomerization and misfolding studies at pH 4

The oligomerization and misfolding studies were carried out as described earlier (Pal and Udgaonkar, 2022). Briefly, the protein in 10 mM sodium acetate buffer (pH 4) was diluted two-fold with 2 $\times$ oligomerization buffer (10 mM sodium acetate buffer, 300 mM NaCl, pH 4), so that the protein was finally in 1 $\times$ oligomerization buffer (10 mM sodium acetate buffer, 150 mM NaCl, pH 4). The samples were then incubated at 37 $^{\circ}$ C. The final protein concentration used for all the experiments was 100 μ M.

HDX-MS measurements

The peptide map of the moPrP variants was generated as described earlier (Pal and Udgaonkar, 2022). HDX-MS measurements were carried out as described earlier (Pal and Udgaonkar, 2022).

Results

Effect of V208A/M substitution on the secondary structure and stability of moPrP

Both single-mutant variants, V208A and V208M, exhibited far-UV CD spectra are similar to that of wt moPrP (Figure 6.1a), indicating that the V208A/M mutations did not have any effect on the global secondary structure of the protein. However, urea-induced equilibrium unfolding studies revealed that these mutations had a significant impact on the stability of the N state (Figure 6.1b and Table 6.1). The V208A mutation led to a destabilization of the N state, by 0.6 kcal mol⁻¹, whereas the V208M mutation resulted in a notable increase in stability by 0.9 kcal mol⁻¹. It should be noted that studies on globular proteins indicate that the stability gain by single cavity-filling mutations can be $\sim$ 1 kcal/mol at best (Bueno et al., 2006). These findings suggest that the cavities surrounding Val at position 208 play a critical role in determining the stability of the native protein. Furthermore, these results align with previously published studies on other proteins showing that optimizing core packing through targeted mutations can significantly improve protein stability (Eriksson et al., 1992; Jackson et al., 1993; Xue et al., 2019).

Effect of V208A/M substitution on the misfolding and oligomerisation of moPrP

The prion protein is known to undergo misfolding within the endocytic pathway, particularly when it enters lysosomes and encounters the acidic environment (Borchelt et al., 1992). At pH 4, wt moPrP retains its native monomeric, helix-rich conformation but becomes prone to misfolding due to protonation of the critical residue His186 and/or D201 (Singh and Udgaonkar, 2016). However, misfolding is only initiated upon addition of salt, when a crucial network of stabilizing interactions among several charged residues—Arg155, His186, Asp201, Lys193, and Lys195—within the β 1- α 1- β 2 and α 2- α 3 subdomains is perturbed (Sengupta et al., 2017). Not surprisingly, in the absence of salt, the misfolding of wt moPrP at pH 4 occurs at an extremely slow rate ($\sim 10^{-4} \text{ h}^{-1}$) (Sengupta et al., 2017). In the present the misfolding kinetics of different moPrP variants were carried out at pH 4 in the presence of a physiological concentration (150 mM) of NaCl at 37°C. Misfolding kinetics were monitored by measurement of the changes in the CD signal at 222 nm. For both wt and V208A moPrP, the misfolding process followed monophasic kinetics that fit well to a single exponential equation. Notably, misfolding at pH 4 accompanied by oligomerization. Earlier studies have demonstrated that, for wt moPrP, the kinetics of misfolding monitored by CD and the kinetics of oligomerization measured by size-exclusion chromatography (SEC) were identical, indicating that both processes occur simultaneously (Sabareesan and Udgaonkar, 2016). Among the two mutant variants studied here, the V208A mutant variant resulted in a significantly fast misfolding rate in comparison to wt protein, with an approximately 2.5-fold increase in the misfolding rate constant (Figures 6.1c and Table 6.1). In sharp contrast, the V208M mutant variant remained in its native helical conformation, and misfolding did not initiate even by the time wt moPrP had completely misfolded into β -rich oligomeric forms (Figures 6.1c). These findings showed that eliminating cavities from the hydrophobic core of the full-length mouse prion protein significantly impedes misfolding and oligomerisation under acidic conditions in the presence of salt.

Effect of V208A/M substitution on the dynamics of moPrP

To understand the impact of the mutations on the dynamics of the protein, HDX-MS studies were carried out. Figure 6.2 represents the kinetics of deuterium incorporation into various sequence segments of the CTD of moPrP for the different mutant variants. In the case of V208A moPrP, the kinetics of deuterium incorporation were found to be increased across most sequence segments, compared to wt moPrP. However, the degree of increase varied among different segments. The most pronounced increase was observed in the middle of α 3 (205–212). Additionally, moderate increases in exchange rate constants were observed in the

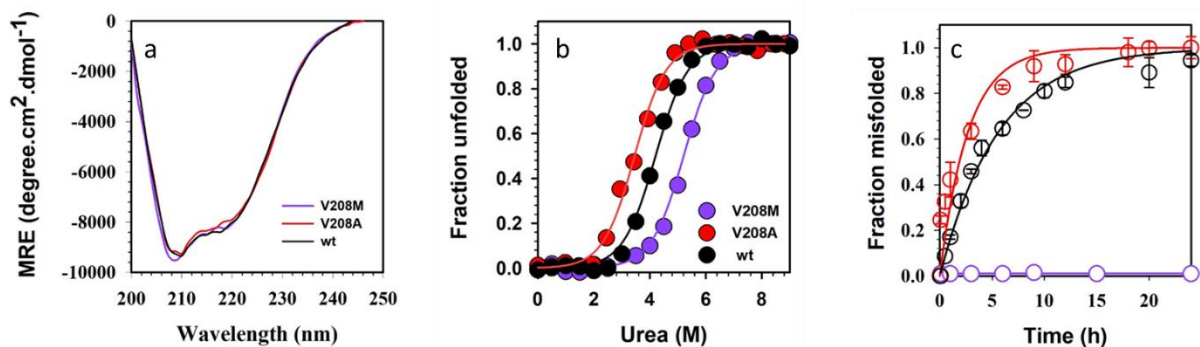


Figure 6.1. Effect of the V208A/M substitutions on secondary structure, stability, and misfolding. (a) Far-UV CD spectra of the native monomeric forms of the moPrP variants acquired at pH 4, at 25 °C. (b) Urea-induced equilibrium unfolding transitions of the moPrP variants at pH 4, at 25 °C, as monitored by measurement of the far-UV CD signal at 222 nm. The signal change was normalized to obtain the fraction unfolded. The solid line through the data points for all protein variants is a fit to an equation describing a two-state unfolding transition. (c) The kinetics of misfolding of 100 μ M protein in the presence of 150 mM NaCl at pH 4, at 37 °C, is shown. The colors of the lines represent the different moPrP variants as indicated. The error bars represent the standard deviations determined from three independent experiments.

Table 6.1. Stabilities and misfolding rate constants of the different moPrP variants

moPrP variant	ΔG_u (kcal mol⁻¹)	Misfolding rate constant (h⁻¹)
wt	4.2 ± 0.2	0.17 ± 0.03
V208A	3.7 ± 0.1	0.41 ± 0.06
V208M	5.0 ± 0.2	ND

The free energy of unfolding (ΔG_u), was obtained from urea-induced equilibrium unfolding studies at pH 4, 25 °C (Figure 6.1b). The m value was constrained to the same value (1.02 kcal mol⁻¹M⁻¹) for all the moPrP variants. The observed rate constant of misfolding was determined at 100 μ M protein concentration in the presence of 150 mM NaCl at pH 4, 37 °C (Figure 6.1c).

β 1 (96–144), the loop between β 2 and α 2 (168–173), and the C-terminal end of α 2 (182–196). In contrast, the V208M mutation resulted in a significant decrease in deuterium incorporation rate constants across all sequence segments compared to wt moPrP. The sequence segment 205–212, which exhibited substantial changes in deuterium exchange kinetics for both V208A and V208M variants, is part of the hydrophobic core and represents the most stable segment of the protein.

Figure 6.3 shows the ΔG_{op} values for different sequence segments of the mutant variants. The V208A mutation primarily reduced the ΔG_{op} values for segments within α 3 (205–212 and 210–216), as well as for β 1 (96–144), the loop between β 2 and α 2 (168–173), and the C-terminal end of α 2 (182–196). Conversely, the V208M mutation led to an overall increase in ΔG_{op} values for all sequence segments, suggesting a global stabilization of the protein. These findings imply that while the V208A mutation predominantly affects local stability near the mutation site, the V208M mutation has a global stabilizing effect, enhancing the stability of segments even distant from the mutation site.

Effect of V208A/M substitution on the stability of the PUFs

Previous HDX-MS/NMR studies identified two sparsely populated PUFs, termed as PUF1 and PUF2, which exist in dynamic equilibrium with the N state of moPrP at pH 4. The free energy required for the transition from the N state to PUF2 ($\Delta G_{op} = 3.7 \pm 0.4 \text{ kcal mol}^{-1}$) is higher than that for PUF1 ($\Delta G_{op} = 1.3 \pm 0.2 \text{ kcal mol}^{-1}$), suggesting that the sequence segments unfolded in PUF1 are also likely unfolded in PUF2 (46). Further characterization of moPrP mutant variants, in which evolutionarily conserved gatekeeper residues in the CTD were mutated to alanine, revealed that PUF2 consists of at least two iso-energetic conformations, designated as PUF2* and PUF2**. In PUF2*, the following sequence segments were found to be unfolded: 144–148, 149–153 (α 1), 154–167 (the loop between α 1 and β 2, and β 2), and 197–204 (the loop between α 2 and α 3, and the N-terminal end of α 3) (Pal & Udgaonkar, 2022). In contrast, in PUF2**, the unfolded segments included 154–167 and 217–223 (the C-terminal end of α 3) (Pal & Udgaonkar, 2023).

In the present study, HDX-MS studies revealed a significant reduction in the ΔG_{op} values for sequence segments spanning β 1 (96–144), the loop between β 2 and α 2 (168–173), and the C-terminal end of α 2 (182–196) in the case of V208A moPrP compared to wt moPrP. Notably, these regions were known to be unfolded in PUF1. On the other hand, in the case of V208M moPrP, the mutation had a pronounced effect on the stabilities (ΔG_{op}) of all sequence segments, suggesting a significant impact in PUF1, PUF2*, as well as PUF2**. This finding

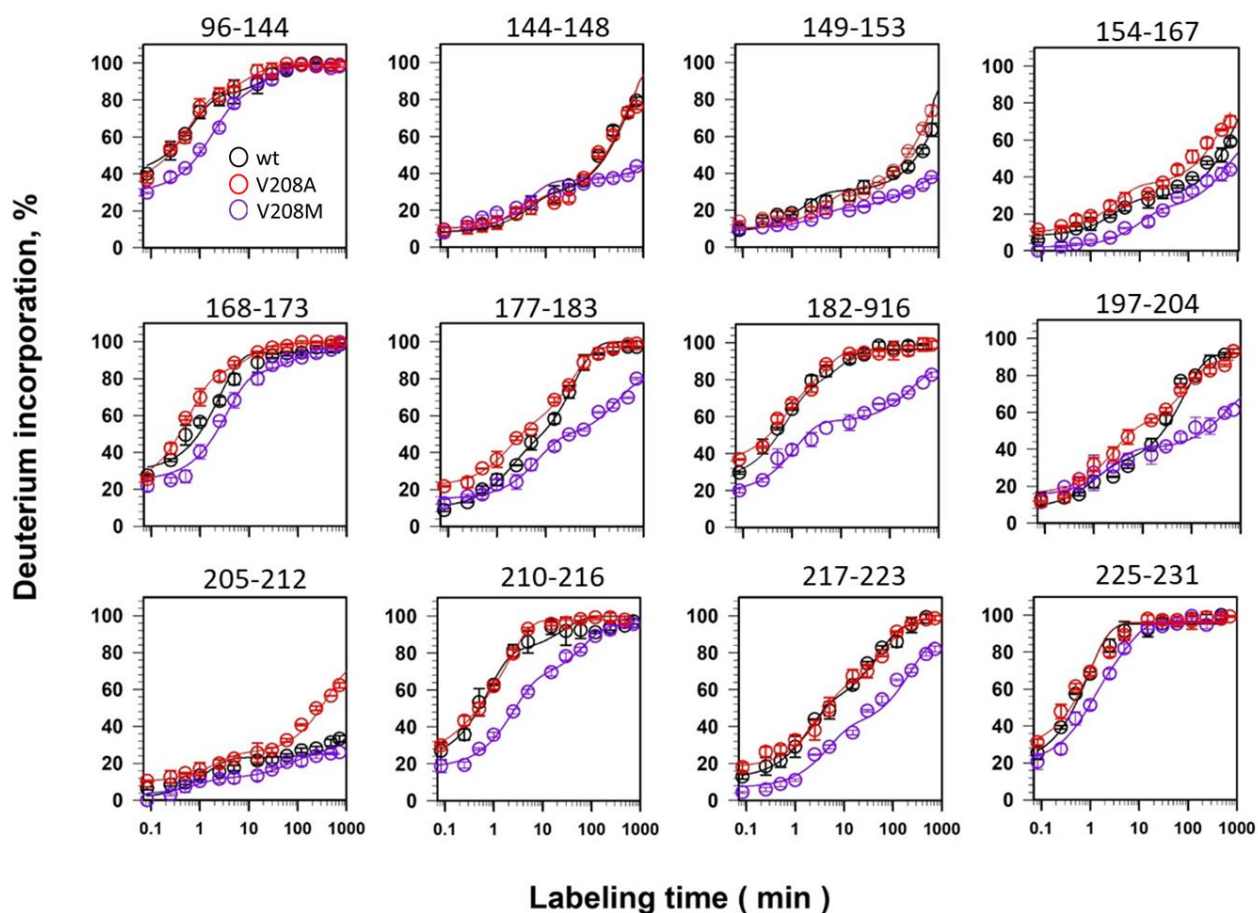


Figure 6.2. Effect of the V208A/M substitutions on the native state dynamics. The time courses of HDX into different sequence segments of the native monomeric forms at pH 4, 25 °C are shown. The differently colored circles represent the different moPrP variants, as indicated. The error bars represent the standard deviations determined from three independent experiments. The solid lines through the data represent fits to either monoexponential or biexponential equations. The data for wt moPrP is reproduced from Pal and Udgoankar, 2023.

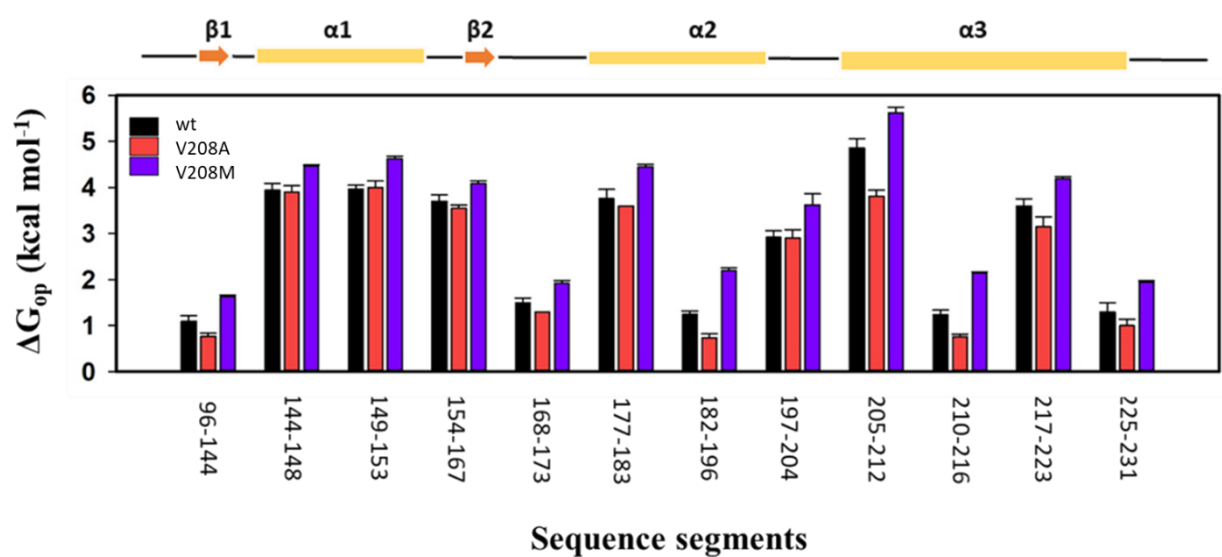


Figure 6.3. ΔG_{op} values of the different sequence segments of the wt and V208A/M mutant variants of moPrP. The secondary structural elements are shown above the sequences (rectangular boxes represent the α -helices, and arrows represent the β - strands). The differently colored bars represent the different moPrP variants, as indicated. The error bars represent the standard deviations determined from three independent experiments.

suggests that modifications in the packing of the hydrophobic core of the moPrP significantly influence the local stability of sequence segments that are known to be unfolded in the previously characterized PUFs.

Discussion

Packing of the hydrophobic core plays an important role in determining the thermodynamic stability of moPrP

The thermodynamic stability of proteins has been widely studied to understand the mechanisms that stabilize their tertiary structures and to design artificially stabilized proteins. Among the various methods developed to enhance native protein stability, a cavity-filling approach has been applied to several proteins (Eijsink et al., 1992; Ishikawa et al., 1993; Karpusas et al., 1989). Two key factors have been identified as contributing to protein stability in these cases: the seclusion of hydrophobic residues from the solvent in the U state (Chothia, 1974; Kauzmann, 1959), and the formation of tight packing within the hydrophobic core in the N state (Chothia, 1975; Richards, 1977). In the current study, V208M mutation was found to significantly increase the free energy difference between the N state and the U state, likely due to improved core packing of the hydrophobic core, that reduces cavity volume. In the case of huPrP, the same mutation is known to significantly improve packing in the core of the protein (Kong et al., 2013). In contrast, the V208A mutation, which is likely to increase the cavity size around Val 208, led to substantial destabilization of the protein. These results suggest that eliminating cavities within the hydrophobic core contributes to significant stabilization, while enlarging them disrupts lead to destabilization of the protein. These results highlight the critical role of packing of the hydrophobic core and cavity formation in determining the thermodynamic stability of the moPrP. These findings are in agreement with earlier studies on the huPrP, which identified three internal cavities that contributed to destabilization (Kong et al., 2013). Two of these cavities are located near $\alpha 2$ and $\alpha 3$, while the third is positioned at the interface of $\alpha 1$ and $\alpha 3$. Modeling and NMR studies demonstrated that replacing Val 208 with methionine in huPrP effectively removed these destabilizing cavities, leading to improved thermodynamic stability by optimizing the packing of $\alpha 1$ and $\alpha 3$.

The results of the current and previous studies, shows that cavity elimination enhances stability by increasing van der Waals interactions and optimizing hydrophobic packing. Conversely, introducing or enlarging cavities leads to structural destabilization due to reduced hydrophobic interactions and unfavorable packing forces. This is consistent with studies on T4

lysozyme, where cavity formation through Leu-to-Ala mutations resulted in a significant loss of stability (Eriksson et al., 1992).

Removing cavities from the hydrophobic core significantly decreases the populations of the PUFs

The linear correlation between the logarithm of the misfolding rate constant and the free energy difference (ΔG_{op}) between the N state and PUF1, as well as between the N state and PUF2* and PUF2**, suggests strongly that misfolding can initiate from any of these PUFs in the case of wt moPrP (Pal & Udgaonkar, 2022, 2023). Evolutionarily conserved residue mutations may destabilize only PUF2* or affect both PUF1 and PUF2**. Several disease-associated mutations have been shown to increase the population of PUF2*, accelerating misfolding (Pal and Udgaonkar, 2022; Singh and Udgaonkar, 2015). Moreover, aggregation-prone regions tend to become unfolded in the PUFs, making these intermediates highly susceptible to misfolding (Pal & Udgaonkar, 2023). Since PUF1 is more stable than PUF2* and PUF2**, it is significantly more populated at equilibrium, making it the primary precursor conformation for initiating misfolding. However, misfolding can also occur, albeit to a much less extent, from PUF2* and PUF2**.

In the current study, the V208A mutation in moPrP was found to reduce the ΔG_{op} values of the sequence segments that unfold in PUF1. The free energy of opening of the N state to PUF1 in the case of V208A moPrP was decreased to 0.78 ± 0.2 kcal mol⁻¹, suggesting that the V208A mutation significantly increased the sampling of PUF1 from the N state. This would result in increased population of PUF1, which would subsequently increase the propensity of misfolding. In contrast, the V208M mutation significantly increases the local stability (ΔG_{op}) of the sequence segments that are known to become unfolded in the PUF1, PUF2* as well as PUF2** (Figure 6.4). The free energy of opening of the N state to PUF1, PUF2*, PUF2** in the case of V208M moPrP was found to be 2.1 ± 0.3 , 4.3 ± 0.1 , 4.4 ± 0.2 kcal mol⁻¹, respectively. The reduced sampling of the PUFs from the N state, leads to a marked reduction in their equilibrium populations. The observed suppression of misfolding in V208M moPrP can be attributed to this decrease in PUF populations, and the corresponding increase in the free energy barrier to misfolding leading to oligomerisation.

Incubation of recombinant PrP with GdmCl at neutral pH results in the formation of ThT-positive amyloid structures with a fibrillar morphology (Baskakov and Bocharova, 2005; Singh and Udgaonkar, 2013). It has been shown that for both full-length (23-231) and CTD (90-231) huPrP, replacing Val208 with Met results in a significantly longer lag phase in

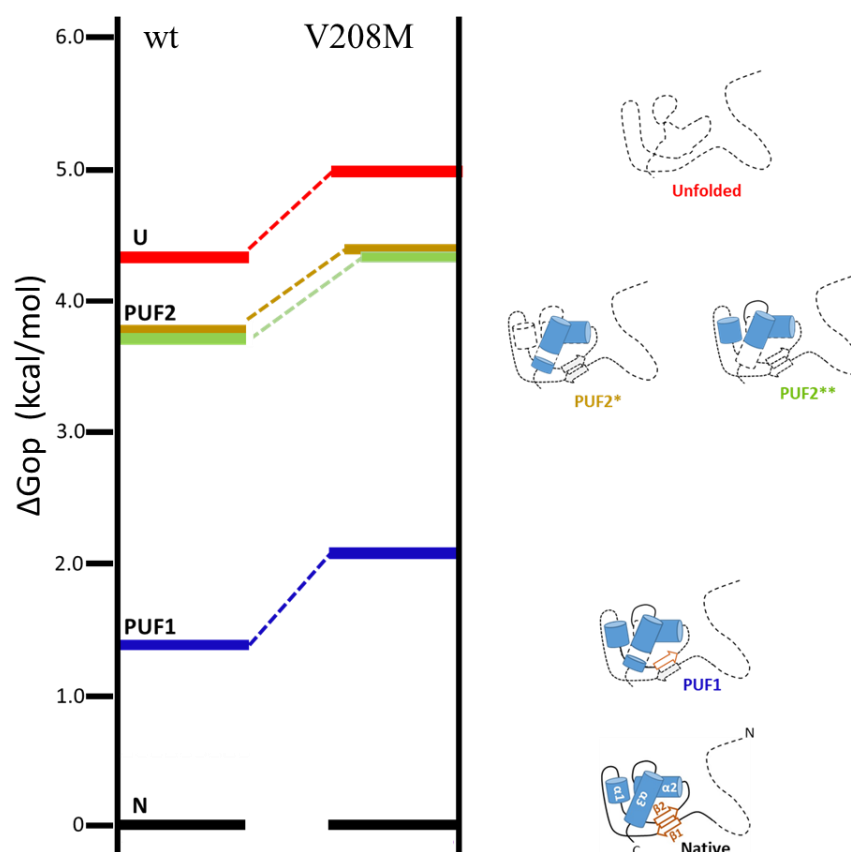


Figure 6.4. Effect of the V208M substitution on the stabilities of the PUFs. The free energy levels of the PUFs, produced from the unfolding of specific sequence segments, have been placed on a free energy ladder. PUF2 has been shown to comprise two iso-energetic sub-populations of molecules, PUF2* (gold) and PUF2** (green), which differ in structure (Pal and Udgaonkar, 2023). The structures of the PUFs are shown on the right. For each PUF, the unfolded segments are shown as dotted lines. The unstructured N-terminal domain (23-120) is shown as a dashed line. The packing of the hydrophobic core is crucial for the stability of the N state. The V208M substitution eliminates cavities and improves the packing in the hydrophobic core, resulting in the increase of the free energy levels (and decrease in the equilibrium populations) of the aggregation-prone PUF1, PUF2* and PUF2**, as indicated by differently colored broken lines. All free energies are shown relative to the same value for the free energy of the N state.

comparison to wt HuPrP (Kong et al., 2013), indicating that the V208M mutation not only slows down the formation of oligomers, but also the conversion to amyloid fibrils. These results also suggest that the PUFs are not only precursors to oligomerization but also to amyloid fibril formation.

Impact of the V208M mutation on prion dimerization

The apparent rate constant for monomer loss during oligomer formation in moPrP follows a second-order reaction, suggesting that dimerization is the rate-limiting step in the oligomerization process (Sabareesan and Udgaonkar, 2016; Sengupta et al., 2017). Similar findings were reported for human PrP misfolding in the presence of lipids, with a reaction order of 2.0 ± 0.1 (Lührs et al., 2006). However, earlier studies on Syrian hamster PrP reported a reaction order of 3 (Sokolowski et al., 2003) while AFM-based studies on human PrP misfolding identified β -rich oligomers composed of 3–4 monomers as the first stable species (Cho et al., 2011). Prion dimers have also been observed in brain samples from individuals with inherited prion diseases (Gao et al., 2019) and in murine neuroblastoma cells expressing hamster PrP (Priola et al., 1995). Furthermore, prion assemblies extracted from transgenic mouse brains expressing prion proteins from various species were found to dissociate into dimers, supporting the hypothesis that oligomers form through dimer assembly (Bohl et al., 2023). A mutant variant of moPrP (3A moPrP) that adopts a molten-globule-like conformation, spontaneously misfold into dimers and the dimers transform very rapidly into oligomers upon the addition of salt (Pal and Udgaonkar, 2024). This suggested the idea that dimers serve as the building blocks of oligomers.

HDX-MS studies had shown that the loss of native structure during the formation of the PUFs promotes dimer formation. Additionally, the structure of the β -rich dimer closely resembles that of the oligomer, except for residues 205–212 in the middle of $\alpha 3$. In the dimer, this region become unfolded but adopts a stable structured β -rich conformation in the oligomer, indicating this region may form dimer-dimer interface in the oligomers (Pal and Udgaonkar, 2024). Hence, to form the interface this region need to first undergo unfolding, followed by misfolding into β -rich conformation as dimers assembled to form oligomer. The present study demonstrates that the V208M mutation significantly increases the local stability of this segment, suggesting it would require significantly higher energy to form stable oligomer. These findings suggest that the V208M mutation suppresses oligomerization through two potential mechanisms: first, by reducing the population of aggregation-prone PUFs, thereby lowering the propensity of dimer formation, and second, by inhibiting oligomerization through the

destabilization of the key dimer interface, preventing further dimer accretion. This also suggests that blocking dimerization—whether through mutations such as V208M or targeted interventions—could be an effective strategy for preventing prion aggregation.

Targeting protein stability as an alternative therapeutic avenue for TSEs

Despite numerous proposed immunotherapeutic and chemotherapeutic approaches, no effective treatment currently exists for transmissible spongiform encephalopathies (TSEs) or Prion diseases. A past approach has focused on downregulating PrP^C expression using small interfering RNA (siRNA) (Pfeifer et al., 2006). However, this method poses potential risks due to the possible roles of PrP^C in cell signaling, neuroprotection, metal ion homeostasis and other physiological functions (Westergaard et al., 2007). The results of the present study show that cavity-filling mutations significantly stabilise the native protein, increase the energy barriers that separate the stable native state from the misfolded conformations and impede misfolding and oligomerisation (Figure 6.5). This suggests a promising strategy for pharmacological intervention in prion diseases could be stabilizing the native conformation of PrP^C without altering its expression levels, a possibility also discussed in an earlier study (Kong et al. 2013). This approach could be achieved through the screening or rational design of small molecules that thermodynamically increase the stability of monomeric PrP^C, thereby reducing prion replication *in vivo*. A similar strategy has proven successful in the development of a clinically effective drug for transthyretin amyloidosis, where kinetic stabilization prevents the dissociation of the transthyretin tetramer (Bulawa et al., 2012). This suggests that targeting protein stability could provide a viable and practical therapeutic avenue for combating prion diseases.

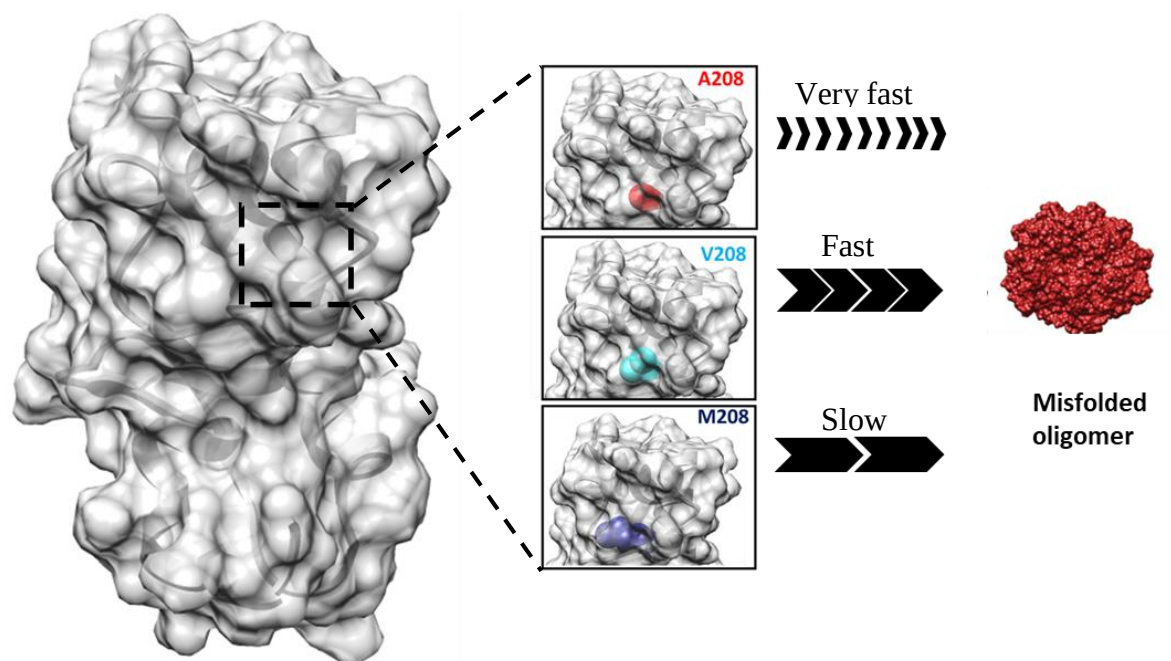


Figure 6.5. The structure of the C-terminal domain of moPrP, with the residues which are mutated in this study are highlighted. Cavity-filling mutations (Val to Met) significantly improve the stability of the N state and reduce the propensity to misfold into β -rich oligomeric forms. In contrast, shortening mutations (Val to Ala) within the hydrophobic core destabilize the N state, leading to an increased misfolding rate and accelerating oligomer formation.

Chapter 7

**Phase separation induces structure in the intrinsically disordered
region of the mammalian prion protein**

Introduction

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are fatal neurodegenerative disorders that affect humans as well as other mammals (Prusiner, 2017). Prion diseases arise spontaneously, through genetic mutations, or *via* infection, and are characterized by the conversion of the native cellular prion protein (PrP^C) into its misfolded form, PrP^{Sc} (Prusiner, 1982, 1998). PrP^C is a 208-residue glycoprotein comprised of an unstructured N-terminal region (NTR) and a structured globular C-terminal domain (CTD) with three α -helices and two short β -strands (Riek et al., 1996). PrP^C is monomeric, soluble, sensitive to protease digestion, and predominantly α -helical, whereas PrP^{Sc} is multimeric, highly insoluble, resistant to protease digestion, and rich in β -sheet structures (Aguzzi & Polymenidou, 2004; Glynn et al., 2020; Manka et al., 2022; Prusiner, 1998; Wang et al., 2020). Although prion diseases are linked to misfolded PrP, the specific misfolded conformations responsible for neurotoxicity remain unclear. Misfolding can result in fibrillar as well as non-fibrillar aggregates (Baskakov et al., 2002; Jain & Udgaonkar, 2011; Singh & Udgaonkar, 2015a).

Recently an alternative pathway to misfolding, referred to as the condensation pathway has emerged, in which proteins undergo liquid-liquid phase separation (LLPS) before forming β -rich aggregates. LLPS involves the demixing of a homogeneous liquid phase into two coexisting liquid phases of different densities, leading to the formation of liquid-like condensates with an elevated protein concentration (Michaels et al., 2023; Mukherjee et al., 2024; Ray et al., 2020). The condensates are metastable and mature into a solid-like state that facilitates amyloid aggregation (Garaizar et al., 2022; Lin et al., 2015). Condensate mediated aggregation has been observed in multiple proteins that are associated with neurodegenerative diseases, such as α -synuclein (Ray et al., 2020), tau (Wegmann et al., 2018), and FUS (Patel et al., 2015). PrP also forms biomolecular condensates, which subsequently mature into hydrogel-like structures with reduced molecular mobility (Kostylev et al., 2018). The hydrogels are thought to recruit other molecules, such as the metabotropic glutamate receptor 5 (mGluR5), which is associated with neurodegeneration (Kostylev et al., 2018). The emergence of β -rich aggregates from condensates formed by a truncated version of PrP upon maturation has been observed (Agarwal et al., 2021). However, the molecular mechanisms governing this transition remain unknown. A better understanding of these processes may provide new insights into prion disease pathology and therapeutic interventions.

The chemical environment within condensates can alter the compaction, conformation, or dynamics of changing conformations of proteins., IDPs have been found to undergo rapid conformational rearrangements and rotational diffusion, inside condensates (Farag et al., 2022). Although folded globular proteins usually retain their native structure within condensates, it has been shown that partial or complete unfolding can take place within them (Ahmed et al., 2024, Chen et al., 2024). The presence of intermolecular interactions between proteins in the condensate can also stabilize or destabilize certain conformations (Guseva et al., 2023). Moreover, at the interface of condensates, proteins can adopt more extended conformations, which may further accelerate aggregation (Linsenmeier et al., 2023). This altered conformation and stability are important considerations in understanding how proteins misfold and aggregate in condensates, as these structural changes directly impact the likelihood of aggregation.

Here the effect of the phase separation on the structure of the full-length mouse prion protein (moPrP) has been studied. MoPrP undergoes spontaneous phase separation upon addition in phosphate-buffered saline at pH = 7.4 (PBS). It is shown that while the NTR is necessary to drive phase separation, the structured CTD also plays an important role in modulating the phase behavior of this protein. PBS-induced PrP condensates initially exhibit liquid-like properties but gradually become more solid-like over 12 h. The progressive maturation into the solid-like state has been characterized by hydrogen-deuterium exchange mass spectrometry (HDX-MS) studies. The major structural transformation in the NTR occurs during condensate maturation. The major structural transformation in the NTR occurs concurrently with structural perturbation of the CTD. The study describes how LLPS can induce major conformational change in the prion protein.

Materials and Methods

Reagents

All reagents used for experiments were of the highest purity grade available from Sigma, unless otherwise specified. Urea and guanidine hydrochloride (GdnHCl) were purchased from USB, and were of the highest purity grade.

Protein expression and purification.

wt moPrP and all the mutant variants were expressed in *Escherichia coli* BL21 (DE3) codon plus (Stratagene) cells transformed with a pET17b plasmid containing either the sequence of the full-length protein (23–231) or of the CTD (121-231). The moPrP variants

were purified as described previously (Jain and Udgaonkar, 2008; Singh et al., 2014). A mutant variant with Cys at position 225 of full-length moPrP was used to label the protein using Alexa Fluor594 maleimide dye. The single Cys mutant variant of moPrP was purified as described previously (Sengupta and Udgaonkar, 2017). The purity of each moPrP variant preparation was confirmed by mass spectrometry (Waters Synapt G2 HD mass spectrometer). Each moPrP variant showed the expected mass, indicating that no chemical modification had taken place in any of the moPrP variants.

Fluorescence labeling

For labeling purified Y225C moPrP mutant variant was mixed with AlexaFluor594-C5-maleimide molar ratio dye to protein of 10:1. The reaction mixture were stirred for 1 h in the dark at 25°C. After completion of the labeling reaction, the excess free dye was removed using a desalting column. The concentration of the labeled protein was estimated using a value of ϵ_{590} of 92,000 M⁻¹cm⁻¹.

Phase separation assays

Protein in 10 mM sodium acetate buffer (pH 4) was diluted two-fold with 2X PBS (4 mM KH₂PO₄, 20 mM Na₂HPO₄, 6 mM KCl, 274 mM NaCl, pH 10.8), so that the protein was finally in 1X PBS (2 mM KH₂PO₄, 10 mM Na₂HPO₄, 3 mM KCl, 137 mM NaCl, pH 7.4). Phase separation was immediately observed upon addition of 2X PBS at 25 °C. The turbidity of the phase-separated samples was determined by taking absorbance measurements at 600 nm on Agilent Cary 300 UV-Vis spectrophotometer. Before the measurements, the solution undergoing LLPS was incubated for 5 min at 25 °C to minimize any discrepancy due to temperature fluctuation. The sample volume used for these measurements was 100 µL and raw turbidity data were plotted without background subtraction. For most of the experiments, the protein concentration was fixed at 100 µM, and salt concentration in PBS was fixed at 137 mM at pH 7.4 unless mentioned otherwise.

Confocal fluorescence microscopy

Confocal fluorescence imaging experiments were carried out at 25°C using a ZEISS LSM 710 microscope using a 63x oil-immersion objective (Numerical aperture: 1.4). To visualize condensates, 5 µL aliquots were withdrawn from the freshly prepared phase-separated samples and placed on a square coverslip. For visualizing the condensates, unlabeled protein was doped with 1% labeled protein, and the condensates were imaged using an excitation

wavelength of 590 nm. Images were processed and analyzed using ImageJ (NIH, Bethesda, USA).

Fluorescence recovery after photobleaching (FRAP) measurements

FRAP experiments on prion condensates were carried out using a ZEISS LSM 710 laser scanning confocal microscope. For image acquisitions, processing, and acquiring FRAP traces, the ZEN (2011) software was used. Unlabeled protein doped with 1 % AlexaFluor594- labeled Y225C moPrP protein was used for the FRAP experiments. Condensates of a size of $\sim 5 \mu\text{m}$ were selected. For each droplet, a region of interest (ROI) with a radius of $0.5 \mu\text{m}$ was bleached using a 590-nm laser at 80 % transmission for 5 s and post-bleach time-lapse images were collected (50 frames, 3.8 s per frame). The recovery of the bleached spots was recorded using ZEN Pro 2011(ZEISS) software provided with the instrument. The normalized FRAP curves were background corrected, plotted, and fitted using a single exponential equation to estimate the rate constant of FRAP using SigmaPlot.

HDX-MS measurements

For all HDX labeling experiments, the labeling buffer was 10 mM sodium acetate buffer ($\pm 150 \text{ mM NaCl}$) in D_2O at pH 7.4, corrected for the isotope effect.

Monomers: HDX-MS measurements of the monomeric protein were carried out as described earlier (Pal and Udgaonkar, 2022).

Condensates: Condensates were prepared by incubation of 2 mM protein with 2XPBS buffer at 25°C , so that the protein was finally in 1XPBS (2 mM KH_2PO_4 , 10 mM Na_2HPO_4 , 3 mM KCl, 137 mM NaCl, pH 7.4). Deuterium labeling was initiated by diluting 5 μl of phase separated protein into 45 μl of 1X PBS buffer prepared in D_2O . Given that the C_{sat} for LLPS under the conditions studied here is $20 \mu\text{M}$ (see results) and that HDX was conducted at a protein concentration of $100 \mu\text{M}$, over 80% of the protein undergoing HDX was that was sequestered within condensates. The FRAP results indicate that, under the conditions studied, the diffusion dynamics of the condensates (diffusion within a condensate as well as with the surrounding dilute phase) are around 10,000 times slower than the intrinsic hydrogen exchange rate of the protein. This suggests that the diffusion dynamics of the condensates have no impact on the observed deuterium exchange of the protein. At different times, a 50 μl aliquot was mixed with 50 μl ice-cold 20 mM glycine-HCl buffer (pH 2.5) to quench the labeling reaction. Online pepsin digestion was carried out by injecting the samples immediately into an immobilized pepsin cartridge (Applied Biosystems) at a flow rate of 40 $\mu\text{l}/\text{min}$ of water

(containing 0.05% formic acid) which was placed in a HDX module (Waters Corporation) coupled to a nanoAcquity UPLC. The peptides eluting from the pepsin column were collected using a trap column, washed to remove salt, and eluted over 12 min on an analytical C18 reversed-phase chromatography column using a gradient of 3 to 40% acetonitrile (containing 0.1% formic acid) at a flow rate of 40 μ l/ min. All columns were kept at 4 °C to minimize back exchange. The mass spectrometer parameters were set as follows: source temperature, 35 °C; desolvation temperature, 100 °C; capillary voltage, 3.0 kV.

Peptide masses were calculated using MassLynx from the centroid of the isotopic envelope. The shifts in the masses of the labeled peptides relative to the unlabeled peptides yielded the extent of deuterium incorporation at each time point of HDX. Since the sample was in 95% D₂O during labeling, and exposed to H₂O during quenching, control experiments were carried out to correct for back exchange. The 95% deuterated moPrP samples were processed in the same way as the labeling reaction samples. The extent of deuterium incorporation in each peptide fragment, % D, was calculated using the equation:

$$\%D = \frac{m(t) - m(0\%)}{m(95\%) - m(0\%)} \times 100$$

where $m(t)$ is the centroid mass measured at time t , $m(0\%)$ is the measured mass of the undeuterated reference peptide, and $m(95\%)$ is the measured mass of a 95% deuterated reference peptide.

To calculate the free energy of opening (ΔG_{op}) of a sequence segment, the observed rate constant of deuterium incorporation (k_{obs}) was first obtained by fitting the kinetic curve of HDX to either a single exponential or a two-exponential equation, depending upon the goodness of fit. For each sequence segment, k_{obs} was compared to the rate constant (k_{int}) that would be observed in the corresponding peptide fragment in a random coil state (Bai et al., 1993; Nguyen et al., 2018). The quantity $P_f = k_{int}/k_{obs}$ is the protection factor that slows down HDX into the sequence segment. The protection factor determined for each peptide fragment was used to calculate the local stability of the corresponding sequence segment in the protein, $\Delta G_{op} = RT \ln P_f$. For each sequence segment, ΔG_{op} is, therefore, the free energy of opening of the N state to form a HDX-competent partially unfolded form.

HDX-MS monitored kinetics of maturation of moPrP condensates

2 mM moPrP in 10 mM sodium acetate buffer at pH 4 was incubated with 2XPBS buffer at 25 °C to induce the droplet formation. At different times of maturation, 5 s or 500 s

pulses of HDX labeling were given. At each time point, a 10 μ l aliquot was withdrawn and diluted into 90 μ L of labeling buffer to initiate the deuterium labeling. After labeling, 100 μ L samples were mixed with 200 μ L of ice-cold 20 mM glycine buffer (pH 2.5) to quench the labeling reaction and dissolve the condensates. Samples were then processed and injected into the HDX module, as described above. Further processing of the sample for mass determination using a Waters Synapt G2 mass spectrometer was carried out as described above. In order to determine the rate constants of structural change in different sequence segments, the kinetic traces of structural change were fit to the following equation:

$$D(t) = D_0 + D_1(1 - e^{-k_1 t})$$

Here, D_0 is the % deuterium incorporation at 0 min, and D_1 and k_1 are the amplitudes and rate constants of structural change respectively.

Results

PBS induces liquid-liquid phase separation of full-length moPrP

To study the propensity of full-length wt moPrP to undergo liquid-liquid phase separation (LLPS), its phase behavior *in vitro* was characterized using turbidity measurements and fluorescence microscopy. The turbidity of the protein solution increased immediately after addition of PBS, suggesting phase separation (Figure 7.1b). The condensates were found to undergo fusion events (Figure 7.1e), and exhibited around 70% fluorescence recovery after photobleaching (FRAP), confirming their liquid-like dynamic nature (Figure 7.1f).

For a given set of solution conditions, the propensity of the protein to undergo phase separation can be quantified by a saturation concentration, C_{sat} , which is the threshold concentration of the protein above which it demixes into coexisting dilute and dense phases (Wang et al., 2018). Thus, when the total concentration of protein is above C_{sat} , a phase equilibrium exists between molecules in the dilute phase and molecules in the dense phase. To measure C_{sat} , the turbidity at 600 nm at different moPrP concentrations following the addition of PBS was measured. wt moPrP was found to undergo LLPS at concentrations of 20 μ M or higher (Figure 7.2). These were confirmed using confocal microscopy. No condensates were seen when the moPrP concentration was 10 μ M upon addition, full-length wt moPrP did not form any condensates upon PBS addition. At 20 μ M, a few condensates were observed, while abundant liquid condensates formed at 50 μ M and 100 μ M (Figure 7.2), corroborating the turbidity data. To determine the protein concentration within the condensates, a centrifugation method was employed (Milkovic and Mittag, 2020). The concentration in the dense phase was

found to be 2.7 mM—approximately 135 times higher than the light-phase concentration, in agreement with the C_{sat} value measured using turbidity measurements. The results therefore demonstrated that full-length wt moPrP undergoes condensation into liquid-like condensates upon PBS addition, which are protein-rich, and highly dynamic.

LLPS is driven by the intrinsically disordered domain of PrP

PrP has an NTR (23-121) juxtaposed to a highly structured CTD (123-231), containing three α -helices and two short β -strands. In order to investigate the role of the NTR (23-121) intrinsically disordered NTD, an N-terminally truncated variant, moPrP (121-231) was used. This construct did not phase separate even at high protein concentration (100 μ M) under the conditions studied here (Figure 7.3). Thus the NTR was found to have a critical role in driving phase separation of moPrP, in agreement with a previous study that have showed that the isolated N-terminal fragments, N1 (residues 23–114) and N2 (residues 23–89), underwent LLPS in physiological conditions (Kamps et al., 2021).

Role of electrostatic screening in driving LLPS of moPrP

The NTR of moPrP carries a high net positive charge (+10) at pH 7.4. This strong charge leads to interchain electrostatic repulsions, preventing the intermolecular interactions necessary for LLPS. To overcome these repulsions and enable effective interchain associations, a threshold salt concentration is required to screen the high positive charge, allowing phase separation to occur *via* homotypic multivalent interactions. To investigate the role of electrostatic screening in LLPS, turbidity experiments were carried out at different salt concentrations while varying the protein concentration. In 137 mM NaCl, phase separation occurred with 20 μ M protein. In contrast, in 100 mM NaCl the LLPS propensity of the protein was significantly reduced, and no phase separation was observed even with 100 μ M protein (Figure 7.4). These findings suggest that electrostatic interactions play a critical role in regulating phase separation of PrP. Below pH 5.6, phase separation did not occur even in 137 mM NaCl. At lower pH, the protein carries an even higher net positive charge, increasing electrostatic repulsions between positively charged residues and 137 mM NaCl is not sufficient to screen all charges. Hence, electrostatic screening of charges is critical in modulating LLPS.

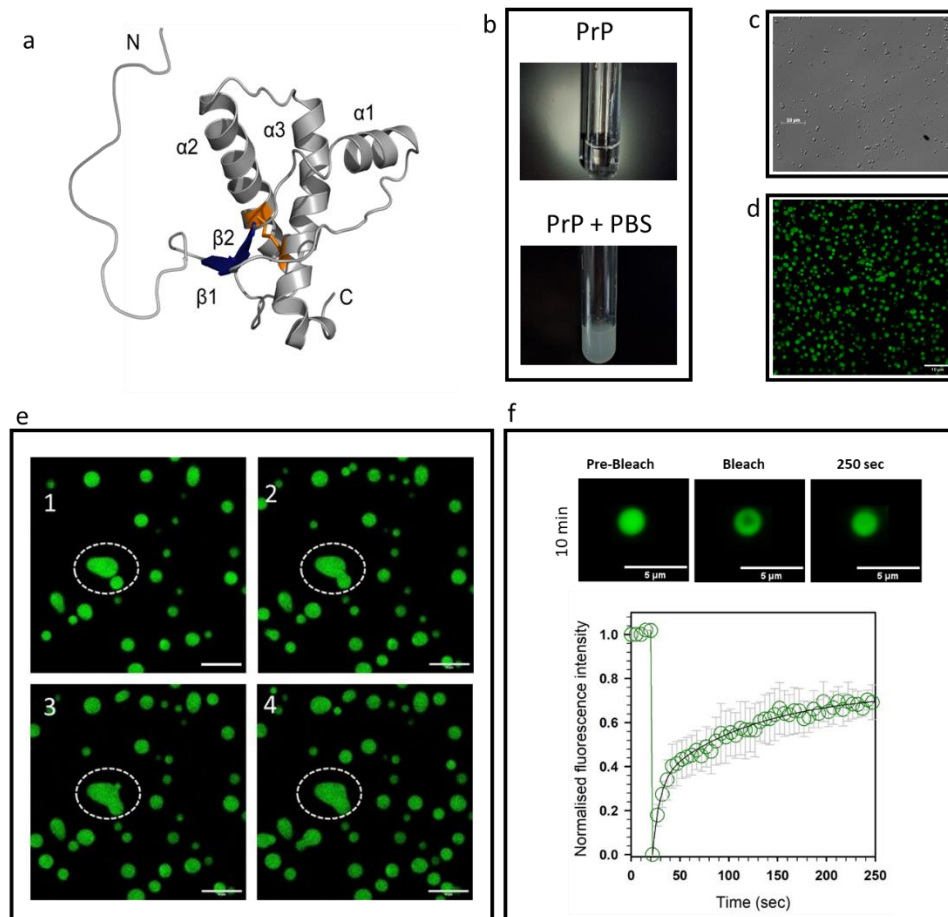


Figure 7.1. Phase separation of full-length mouse prion protein (moPrP) (a) NMR structure of moPrP. The N-terminal region, spanning residues 23-120, is unstructured and the C-terminal domain, spanning residues 121-231, is structured. The figure was drawn using Chimera and Protein Data Bank entry [1AG2](#). (b) Spontaneous LLPS of full-length moPrP (100 μ M, pH 7.4, 25 $^{\circ}$ C) from a homogeneous phase into a demixed dilute and denser phases, upon addition of PBS. Liquid condensates were observed by (c) DIC confocal microscopy and (d) fluorescence microscopy. The scale bar represents 10 μ m. (e) Droplet fusion events (scale bar 10 μ m) and (f) Kinetics of FRAP of multiple condensates ($n=5$) at pH 7.4, incubated in PBS for 10 min at 25 $^{\circ}$ C.

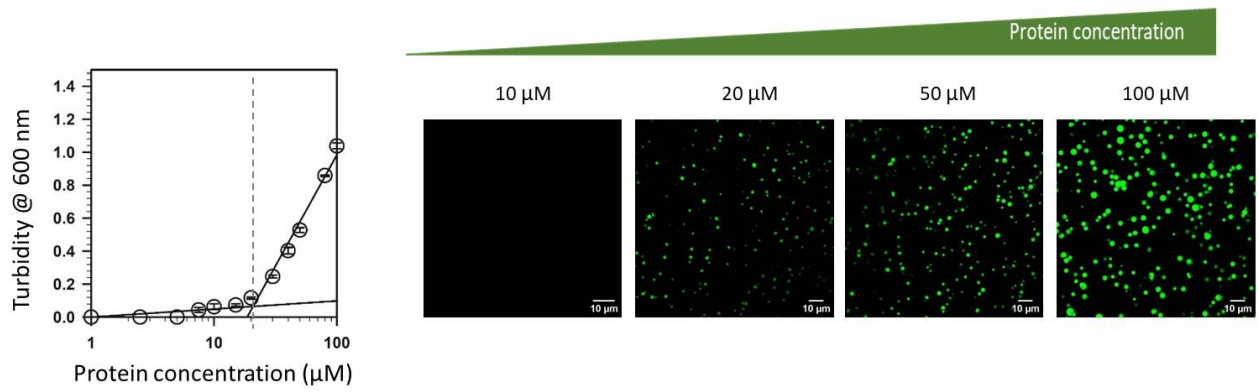


Figure 7.2. Equilibrium phase boundary of the LLPS of full-length moPrP. (a) The dependence of turbidity changes upon incubation in PBS for 10 min at 25 °C on protein concentration. The turbidity of PrP condensates was measured at 600 nm and 25 °C. The dashed line indicates the protein concentration (C_{sat}), above which LLPS was observed. The error bars represent the standard deviations determined from three independent experiments. (b) The dependence of droplet formation on protein concentration was observed by confocal fluorescence microscopy. The scale bar represents 10 μM.

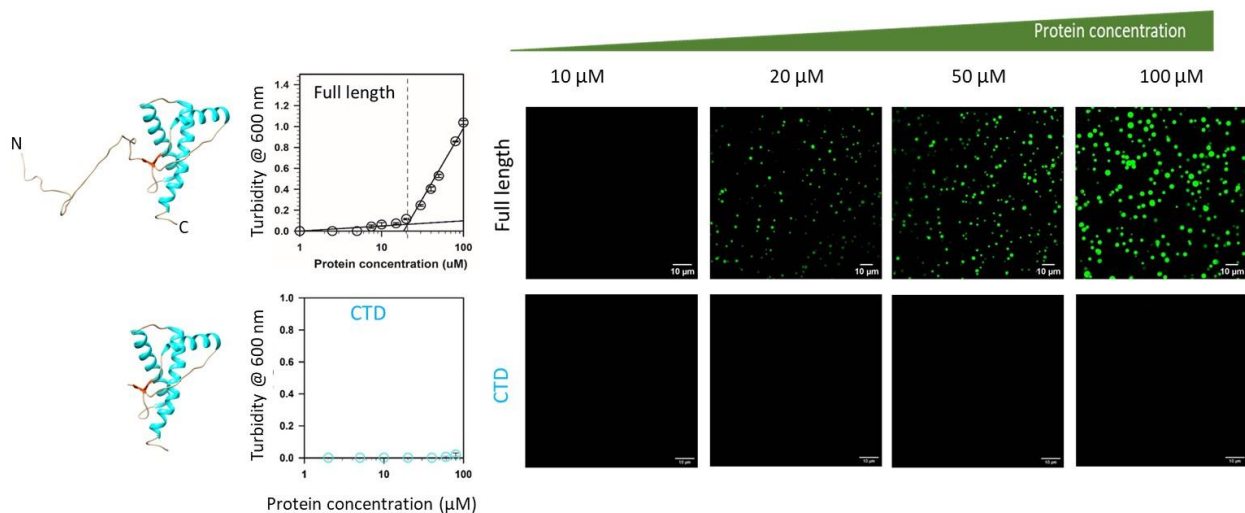


Figure 7.3. Comparison of the phase separation properties of full-length moPrP and moPrP (121-231). Turbidity measurements and confocal microscopy imaging (scale bar represents 10 μM) were used. Turbidity was measured at 600 nm, upon incubation in PBS for 10 min at 25 °C. The dashed line indicates the protein concentration above which LLPS was observed (C_{sat}). The error bars represent the standard deviations determined from three independent experiments.

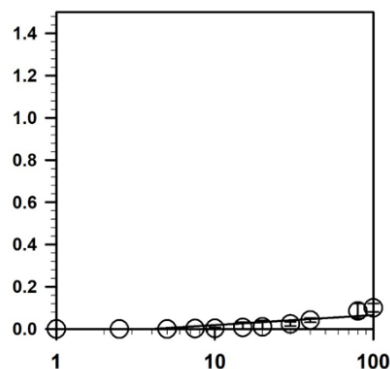


Figure 7.4. Dependence of the ability of full-length moPrP to undergo LLPS on salt concentration. The turbidity of the PrP condensates was measured at 600 nm, incubated in PBS containing 100 mM NaCl for 10 min at 25 °C. The black dashed line indicates the protein concentration above which LLPS occurs (C_{sat}). The error bars represent the standard deviations determined from three independent experiments.

moPrP condensates undergo liquid-like to solid-like transition

While biomolecular condensates are characterized by liquid-like properties, studies employing techniques such as FRAP (Taylor et al., 2019), coalescence assays (Garaizar et al., 2022), and microrheology (Luo et al., 2022) have demonstrated that these condensates can mature into gel-like or solid-like states over time. As condensates age, they exhibit reduced fusion tendencies, prolonged or absent fluorescence recovery post-photobleaching, and significantly slower molecular diffusion, indicating restricted molecular movement within the condensate. This liquid-to-solid phase transition is often linked to the formation of gel-like or arrested states in several proteins (Kato et al., 2012; Kostylev et al., 2018; Xiang et al., 2015b). FRAP is commonly used to assess this transition by measuring protein diffusion within individual condensates.

FRAP was utilized to investigate the maturation of homotypic condensates formed by full-length moPrP in PBS. Freshly formed condensates, observed at 10 min of LLPS, displayed approximately 70% FRAP recovery within 150 s, indicating dynamic molecular exchange typical of liquid-like behavior. However, condensates incubated for 30 min at 25°C showed a marked decrease in recovery, with only 35% intensity regained, suggesting the onset of reduced molecular mobility. Strikingly, condensates incubated for 4 h at 25°C exhibited almost FRAP during the observation period, indicating that the condensates had transitioned to a solid-like state with arrested molecular dynamics (Figure 7.5). These findings highlight the time-dependent maturation of PrP condensates from a dynamic, liquid-like phase to a more rigid, solid-like state. The observed liquid-like to solid-like phase transition suggests that over time, the weak multivalent interactions of PrP molecules within the condensates become stronger and more stable upon maturation, leading to restricted molecular movement and the formation of solid-like structures.

Condensate maturation involves conformational change of the protein

To determine potential changes in the structures of the protein, which accompanies the transition from liquid-like to solid-like states in condensates, native-state HDX-MS on both liquid-like (10 min) and solid-like (12 h) condensates were compared. HDX labeling pulses [5 s (weak pulse) and 500 s (strong pulse)] were applied at different stages of maturation. HDX can differentiate between stable, structured regions, which are protected against exchange, and dynamic, unstructured regions, which are unprotected. Stable segments incorporate less deuterium, whereas less stable regions incorporate more. Since deuterium-labeled segments have a higher mass than unlabeled ones, these segments are easily identified by mass

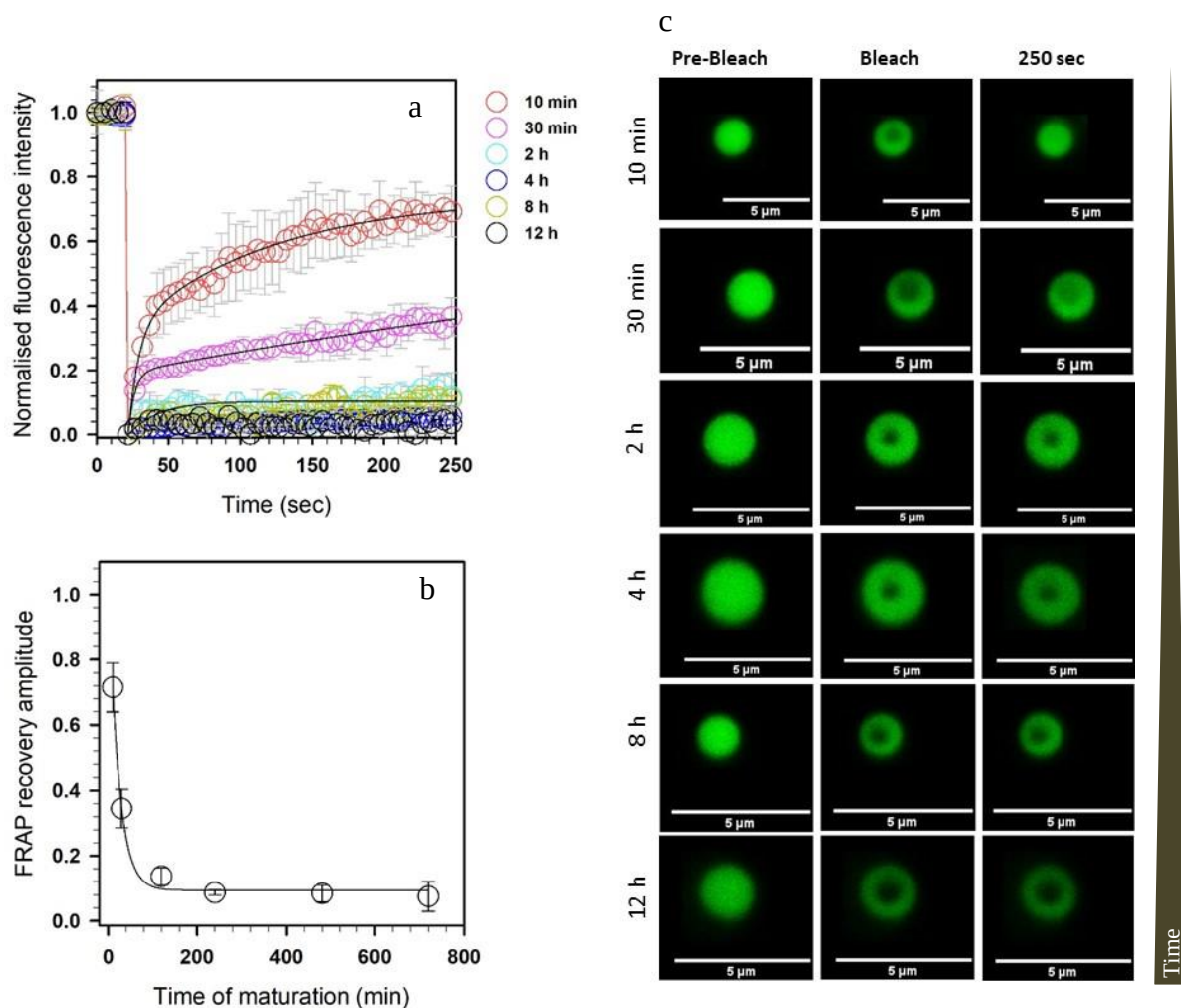


Figure 7.5. Characterization of moPrP droplet aging using FRAP. (a) Normalized FRAP recovery curves for different time points of maturation of droplets ($n=5$). The continuous lines through the data point are fits to an exponential equation. The error bars represent the standard deviations around the mean. (b) Normalized FRAP recovery amplitude for different times of maturation of droplets ($n=5$). The continuous lines through the data point are fits to an exponential equation. The error bars represent the standard deviations around the mean. (c) Time series images of droplet FRAP over maturation time, observed by fluorescence confocal microscopy. The images on the left indicate the droplet images before bleaching, the images in the middle indicate the initial photobleaching at $t=0$ s, and the images on the right show the same droplet after 250 sec. The scale bar represents 5 μ m.

spectrometry (MS) after peptic fragmentation following the exchange. It was observed, that the NTR, which remained unstructured immediately after condensate formation—as evident by its low protection factor—became significantly more stable and protected against HDX in the mature (12 h) condensates. In contrast, most segments of the CTD showed no change in their protection factors in the mature condensates compared to in the liquid-like form. However, the sequence regions 168-173 ($\beta 2$ - $\alpha 2$ loop), 182-196 (C-terminal end of $\alpha 2$), 210-216, 217-223, and 225-231 (C-terminal end of $\alpha 3$), exhibited increased P_f values and ΔG_{op} values in the 12 h condensates. Interestingly, the sequence segment 133-143 ($\beta 2$ and the loop between $\beta 2$ and $\alpha 2$) showed a decreased P_f values and ΔG_{op} values in the solid-like condensates compared to the liquid-like ones. The remaining segments of the CTD did not show any significant change in HDX protection.

To further understand the sequence of structural changes in different segments of the protein inside condensates during maturation and to identify the onset of this process pulse labeling kinetic HDX-MS experiments were carried out. The 5 s pulse labeling HDX-MS experiments revealed that the entire NTR, (residues 23–131) gained significant protection against exchange during the aging of the liquid-like condensates (Figure 7.6). Interestingly, parts of the CTD, including the sequence segments 162–175 ($\beta 2$ - $\alpha 2$ loop), 182–196 (C-terminal end of $\alpha 2$), and 217–223 and 225–231 (C-terminal end of $\alpha 3$) also become more stable during maturation (Figure 7.6). Other regions within the CTD remained unchanged in their stability. However, the sequence segment 133–143 ($\beta 1$ and $\beta 1$ - $\alpha 1$ loop), which connects the NTR to the structured CTD, lost protection during maturation, becoming significantly less stable in the matured condensates (Figure 7.6). When a longer 500-s labeling pulse was applied, even the regions that became protected during maturation also were get labeled (Figure 7.6), as observed from the P_f observed for the segments.

The rate constants of structural changes of individual sequence segments were also determined (Table 7.1). The gain in structure in NTR occurred with the same rate constant as the stabilization of the C-terminal end of $\alpha 3$. In contrast, the sequence segment 133–143 lost structure with a rate constant comparable to the rate constant at which segments 162–175 ($\beta 2$ - $\alpha 2$ loop) and 182–196 (C-terminal end of $\alpha 2$) gained structure. Interestingly, the NTR and the C-terminal end of $\alpha 3$ became structured slower than the rate at which the 133–143 segment lost its structure.

Destabilisation of CTD of PrP induces phase separation

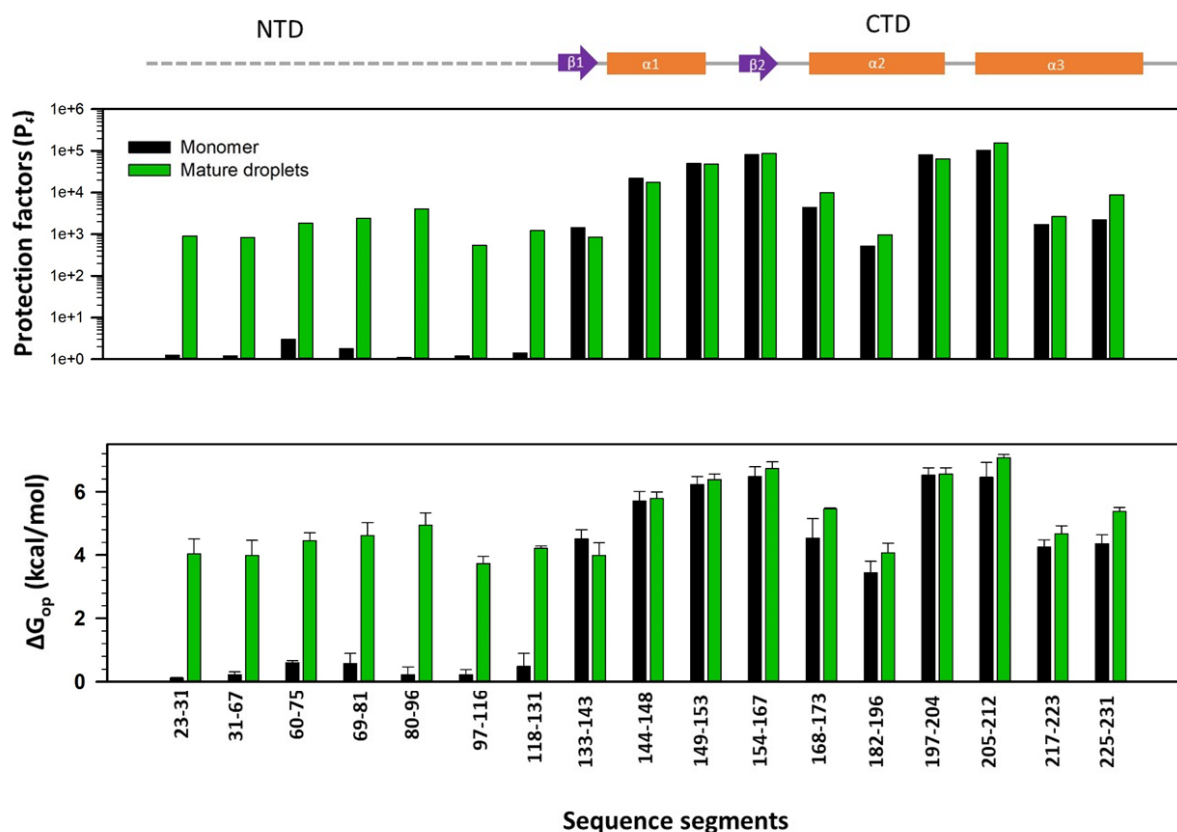


Figure 7.6. Protection factors (P_i) and ΔG_{op} values of the different sequence segments of moPrP monomers and moPrP in mature droplets. The secondary structural elements are shown above the sequences. The NTR is shown as a dashed line, and the CTD is shown as solid line. For sequence stretch 23-131 of the monomers, the extent of deuterium incorporation (% D) upon labeling of the monomers by a 30 s HDX pulse at pH 4, 25 °C (Sabareesan, and Udgaonkar, 2016) was used to provide an approximate estimate of k_{obs} , using the following equation: $\%D = 1 - e^{(-30)(k_{obs})}$. The error bars represent the standard deviations from three independent experiments.

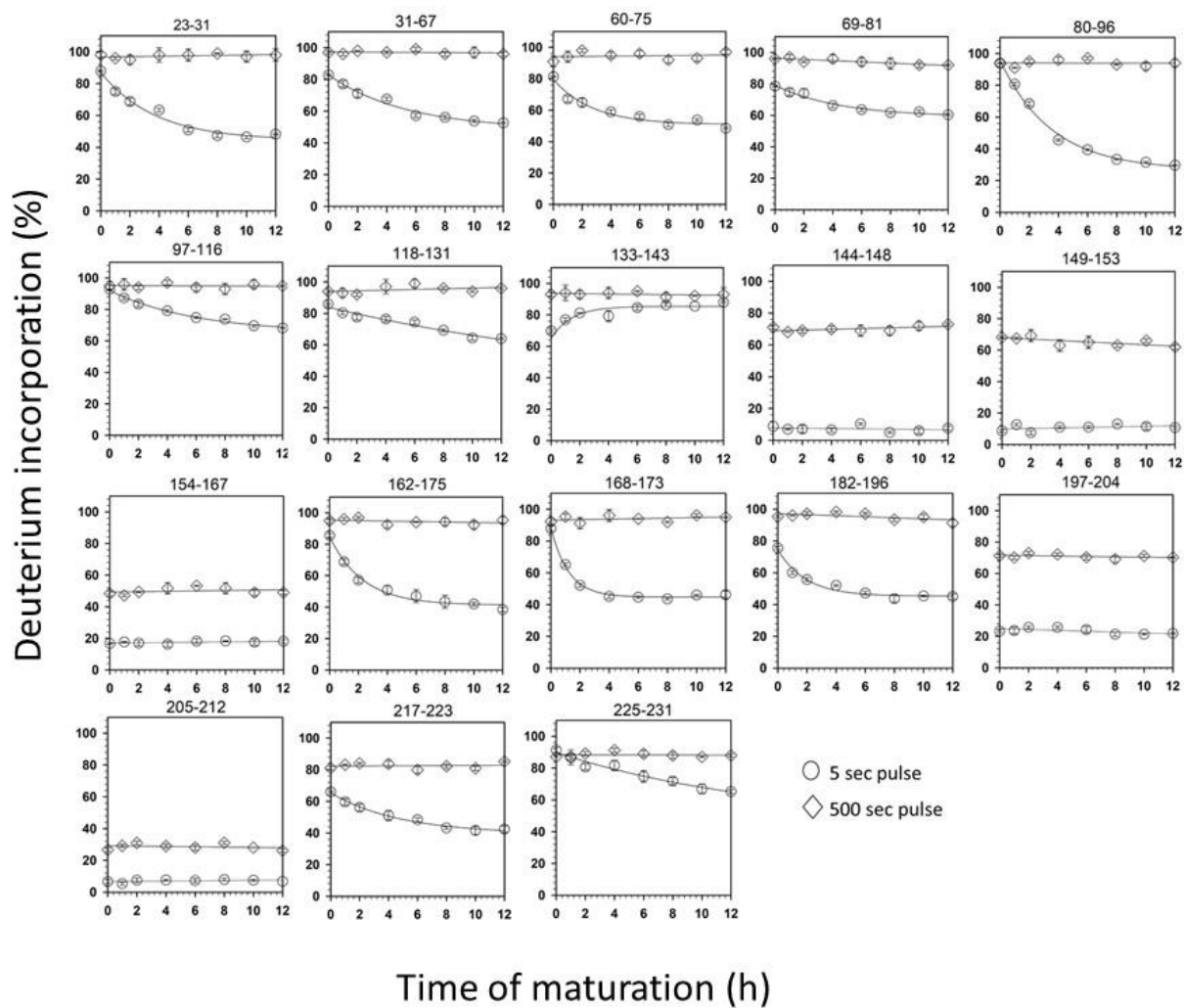


Figure 7.7. Kinetics of pulsed HDX into different sequence segments of moPrP during the aging of droplets. The time courses of deuterium incorporation into different sequence segments upon the application of 5 s and 500 s HDX-labeling pulses at different times of aging of droplets in PBS at 25 °C, are shown. The error bars represent the standard deviations determined from three independent experiments.

Table 7.1: Observed rate constants of structural change in different sequence segments of moPrP during maturation of droplets

Sequence segment	$k_{\text{obs}} \text{ (h}^{-1}\text{)}$
23-31	0.26 ± 0.06
31-67	0.18 ± 0.04
60-75	0.34 ± 0.05
69-81	0.22 ± 0.08
80-96	0.28 ± 0.04
97-116	0.17 ± 0.06
118-131 ($\beta 1$)	0.44 ± 0.12
133-143 ($\beta 1$-$\alpha 1$ loop)	0.70 ± 0.14
162-175 ($\beta 2$-$\alpha 2$ loop)	0.52 ± 0.03
168-173 ($\beta 2$-$\alpha 2$ loop)	0.73 ± 0.06
182-196 ($\alpha 2$)	0.61 ± 0.11
217-223 ($\alpha 3$)	0.21 ± 0.09
225-231 ($\alpha 3$)	0.11 ± 0.03

Formation and maturation of full-length moPrP droplets was carried out in PBS at 25 °C. The apparent rate constants of the structural change of different sequence segments were obtained from the data shown in Figure 7.7. The secondary structural elements are shown in brackets. Sequence segments 144-148 ($\alpha 1$), 149-153 ($\alpha 1$), 154-167 ($\beta 2$), 197-204 ($\alpha 2$ - $\alpha 3$ loop) and 205-212 ($\alpha 3$) do not show structural change. The errors represent the standard deviations determined from three independent experiments.

To understand the role of the CTD of moPrP, a set of mutant variants of moPrP in which evolutionarily conserved residues had mutated, were used. These mutated residues had been shown to be crucial for maintaining the energy barrier between folded and unfolded conformations (Pal and Udgaonkar, 2022, 2023). These conserved residues, referred to as gatekeeper residues, and stabilized the folded conformation of the protein. Mutating these residues destabilizes the CTD, reduces the energy difference between folded and unfolded conformations, and increases the equilibrium population of unfolded conformations. The propensity for LLPS of the mutant variants was checked using turbidity assays and it was found they underwent LLPS at significantly lower protein concentration (Figure 7.9 and 7.10). These findings suggested that the LLPS propensity of moPrP could be modulated by destabilizing the folded globular domain.

Discussion

Interactions that drive LLPS

Proteins that are intrinsically disordered (IDPs) or contain large intrinsically disordered regions (IDRs) are more likely to undergo LLPS (Borchers et al., 2021). While the disordered nature of IDRs drives LLPS, sequence composition also plays a significant role (Saar et al., 2021). Several interactions, including electrostatic, cation- π , π - π , π /sp², and hydrophobic interactions, contribute to LLPS (Das et al., 2020; Saar et al., 2021). IDR-containing RNA-binding proteins, such as those enriched in Gly, Ser, Tyr, and Gln, often undergo LLPS (Chong et al., 2018; Lin et al., 2015). Aromatic residues, particularly Tyr and Trp, are crucial in LLPS by facilitating interactions within and between IDRs (Martin et al., 2020). Cation- π interactions between arginine and phenylalanine are essential for LLPS in the RNA helicase Ddx4 (Nott et al., 2015). Mutations in tyrosine residues significantly reduce the phase-separating propensity of proteins like BugZ and Nephin (Jiang et al., 2015; Pak et al., 2016), and impair hydrogel formation by FUS (Kato et al., 2012). The presence of Ser residues and their phosphorylation can regulate interactions, preventing IDRs from entering condensates, as seen in RNA polymerase II (Kwon et al., 2013).

The "stickers-and-spacers" model helps to explain how the distribution of aromatic residues in IDRs controls phase transition behavior (Ruff et al., 2021). In the case of moPrP, the NTR contains several tryptophan and tyrosine residues, which are regularly spaced by polar amino acids like Gly and Pro. This arrangement fits the stickers-and-spacers model, with the aromatic residues acting as stickers and the polar residues as spacers. The regular spacing of

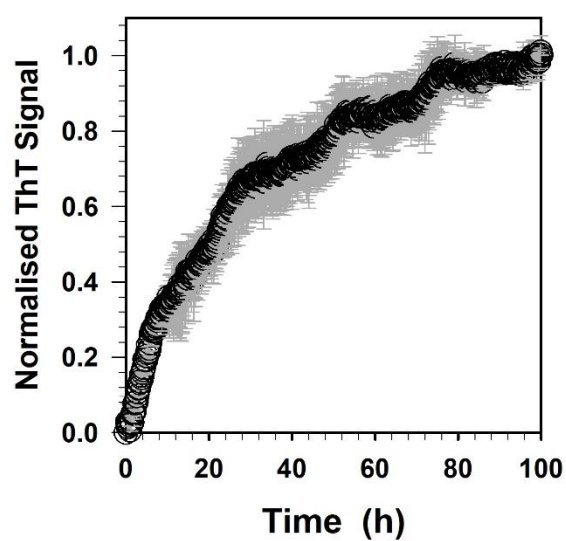


Figure 7.8. Change in ThT fluorescence of PrP condensates formed by 100 μM moPrP upon incubation in PBS containing 100 μM ThT at 25 $^{\circ}\text{C}$. The error bars represent the standard deviations determined from two independent experiments.

aromatic residues allows for efficient intermolecular interactions, facilitating phase separation. Moreover, one-third of the NTR of PrP is composed of Gly residues which plays an important role in LLPS by increasing the fluidity of the condensates (Xu et al., 2024). Studies with other disordered regions, such as those in FUS, one of the well-studied LLPS-forming proteins, have demonstrated that mutations in glycine residues can significantly reduce the condensate fusion rate (Wang et al., 2018)

Formation of structure in the NTR in the condensates

Native state HDX-MS experiments suggest that the NTR became structured upon maturation of the condensates. Pulse-labeling HDX-MS experiments showed that the gain in structure in NTR occurred with the same rate constant as the stabilization of the C-terminal end of $\alpha 3$. Therefore, it seems that formation of structure in the NTR in the condensates is likely due to the interaction between the NTR and the C-terminal end of $\alpha 3$.

Modulation of structure in the CTD in the condensates

Low sequence complexity and the ability of IDPs to adopt multiple conformations enable them to form diverse intermolecular interactions that drive their entropically unfavorable LLPS. In contrast, globular folded proteins, with their compact structures and limited flexibility, do not readily undergo LLPS. This is supported by findings that the CTD reduces LLPS propensity, as the N-terminally truncated variant of moPrP did not undergo phase separation even at high concentrations. However, mutations in the CTD, such as His177 and Asn159 substitutions, can increase the saturation concentration for LLPS, inhibiting phase separation in human PrP (Li et al., 2023).

In many globular proteins, LLPS arises from concentration-dependent interactions among unfolded protein conformations (Balchin et al., 2020; Clark, 2004; Hartl, 2016; Vendruscolo and Dobson, 2005; Ryno et al., 2013; Solomon et al., 2012; Song, 2018). In the case of SOD1, NMR studies have shown a shift in the folding-unfolding equilibrium within condensates (Ahmed et al., 2024). In the condensed phase, the folded population decreases from ~67% in the dilute phase to ~27%, indicating destabilization of the protein inside the condensed phase (Ahmed et al., 2024). The presence of CAPRIN1 significantly enhances the LLPS propensity of SOD1 by preferentially interacting with the unfolded ensemble, pushing the equilibrium toward the unfolded state without altering the folded structure (Ahmed et al., 2024). Destabilized mutant variants of Barnase, with reduced structural stability and increased population of unfolded molecules, have been shown to have a higher propensity for LLPS

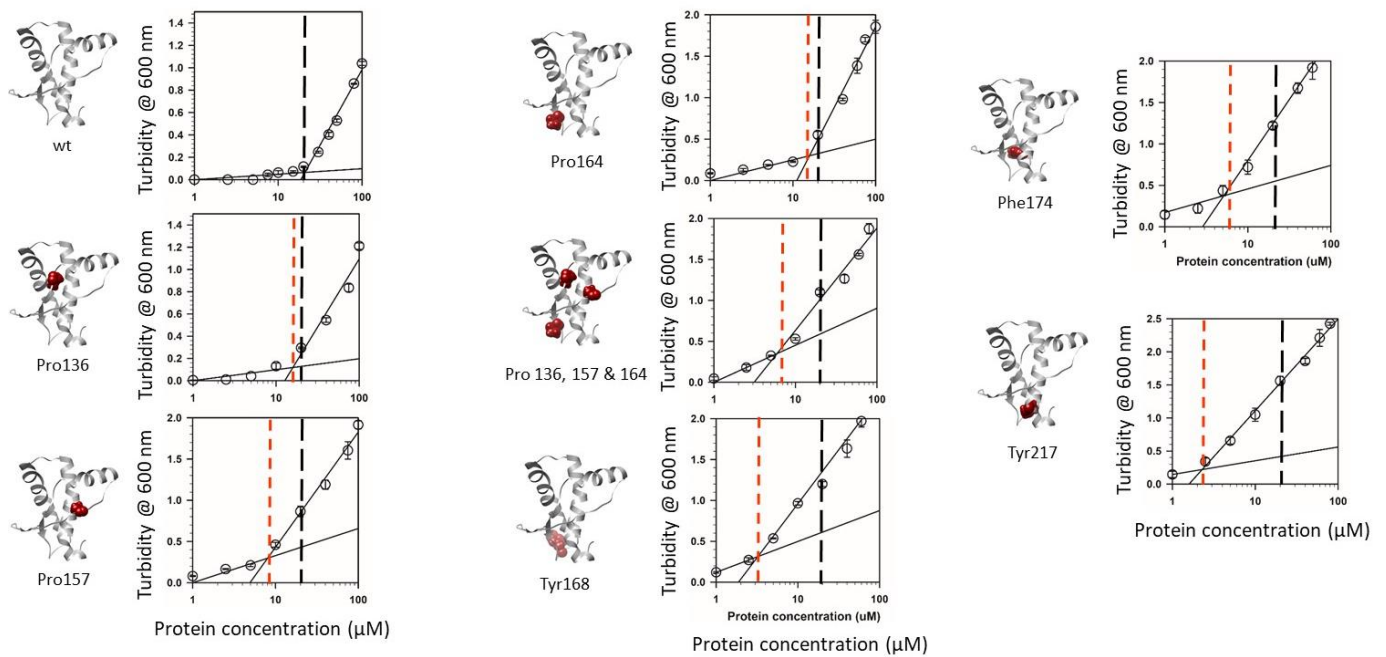


Figure 7.9. Destabilized moPrP mutant variants strongly increase the propensity of full-length moPrP, shifting the equilibrium phase boundary to a lower protein concentration. The mutated residues are shown on the left side of the plots. The turbidity of the PrP condensates was measured after 10 min at 600 nm in PBS at 25 °C. The black and red dashed line indicates the protein concentration (C_{sat}) above which LLPS has occurred for wt moPrP and the mutant variants, respectively. The error bars represent the standard deviations determined from three independent experiments.

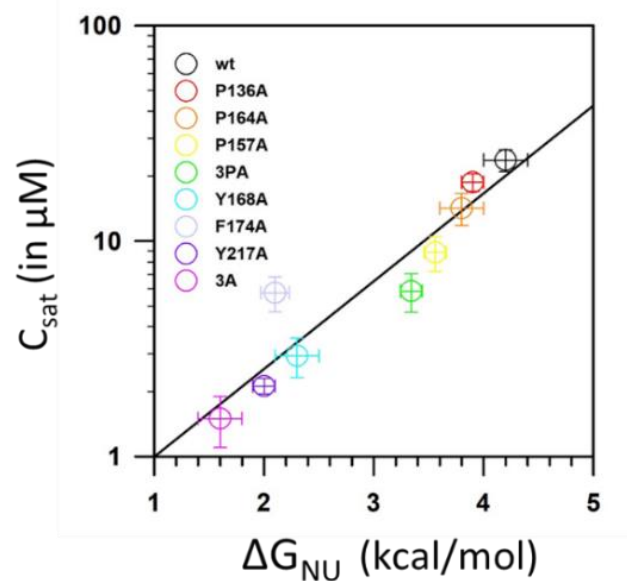


Figure 7.10. Dependence of the the observed C_{sat} values on global stability of the moPrP mutant variants. C_{sat} values were determined from the turbidity measurements at 600 nm after 10 min in PBS at 25 °C. Plot of the observed C_{sat} values of the destabilized moPrP mutant variants determined by the turbidity at 600 nm upon incubation at pH 7.4, 25 °C in PBS buffer for 10 min *versus* the decrease in global stability (ΔG_{NU}) of the destabilized moPrP mutant variants with respect to wt moPrP. The error bars represent the standard deviations determined from three independent experiments.

(Ruff et al., 2022). However, the increase in the population of equilibrium unfolded molecules for the mutant variants in the current study was not significant. For wt moPrP, this value was less than 1%, while it rose to 9% in the most destabilized mutant variant, which was capable of undergoing LLPS even at concentrations as low as 2 μ M. Therefore, it seems unlikely that there is a bias toward unfolded states in the case of full length moPrP. Moreover, disruption of the hydrophobic core of the folded part of the protein would be necessary for complete unfolding. Native state HDX data indicated that the region (sequence segment 205-212) forming the hydrophobic core remained as protected in the condensate as in the dilute phase (Figure 7.7), suggesting the core was intact.

Mutations in the folded domain of moPrP may also promote LLPS by altering the protein conformation in such a way that phase separation is favored. For instance, studies on tau protein revealed that it adopts an expanded conformation within condensates, as indicated by changes in single-molecule FRET efficiency under LLPS conditions compared to the solution (Wen et al., 2021). To explore this in moPrP, the dynamics of different protein segments using native-state HDX-MS in both the light and dense phases was compared. It was seen that the folded domain shows similar dynamics in the dense phase and the light phase, suggesting the folded domain of moPrP retains its conformation in condensates, with no major conformational changes occurring immediately after the induction of LLPS. However, in an earlier study of LLPS of moPrP using NMR spectroscopy, Thr residues in α 2 and α 3 helices exhibited shifts characteristic of α -helical to random coil transitions (Kostylev et al., 2018).

Role of the 133-143 sequence segment

The fast rate constant of structural change in sequence segment 133-143 during maturation could be reporting on the unfolding of this segment as an early event of post-LLPS conformational change. The observed conformational changes in sequence segments 162–175 and 182–196 are likely consequences of the structural alterations in the 133–143 segment, given the similarity in their rate constants for structural changes. Notably, this study also points out that the loop between β 1 and α 1 (residues 139–141), which is part of the 133–143 segment that undergoes unfolding during droplet maturation, is highly prone to unfolding (Kachel et al., 2006). Hence it appears that the loss of structure in the 133–143 segment facilitates interactions between the NTR and the C-terminal end of α 3 inside the condensates, driving the structure formation of the NTR during maturation. Since this loop acts as the linker between the NTD and the CTD, it must undergo structural rearrangement to facilitate their interaction. It should be noted, its structural rearrangement might exposes an amyloidogenic determinant maturation

of condensates (residues 137–140) (Kundu et al., 2003) that is otherwise not exposed in the full-length PrP structure. Residues within or near this region have been shown to influence the abnormal formation of PrP in both cell culture and animal models (Goldmann et al., 1996; Priola and Chesebro, 1995).

Interactions between NTR and CTD

HDX-MS experiments reveal that within PrP condensates, the intrinsically disordered NTR interacts with the structured CTD, and the interaction strengthens as the condensates mature. This process induces structural organization in the entire NTR as well as in specific CTD segments. Multiple studies have suggested transient interaction between the unstructured NTR and the CTD, specifically with $\alpha 2$ (residues 187–193) and $\alpha 3$ (residues 217–226) (McDonald et al., 2019; Schilling et al., 2020; Thakur et al., 2011; Wu et al., 2017). Disrupting the interaction between NTR and CTD have been shown to induce toxicity in cultured neurons and brain tissue (Wu et al., 2017). Since the NTR acts as a toxic effector (Wu et al., 2017) and its toxic effect is regulated by the CTD, the maintenance of the interaction between this two domain is essential for neuroprotection. The results suggest that neuroprotective self-regulation of PrP might be facilitated by condensate formation, which stabilizes the protective interaction between its toxic NTR and regulatory CTD.

Mature PrP condensates do not transform into amyloid like fibrils

While the maturation of condensates is a common phenomenon observed across all phase-separating proteins (Lu and Weitz, 2013), it has been shown that aggregation-prone proteins mature at a much faster rate (Lin et al., 2015). Intuitively, hyper-concentrated condensates are suitable for the formation of fibrillar aggregates, which follows the nucleated polymerization mechanism (Babinchak and Surewicz, 2020). The increased local concentration is expected to decrease the lag phase. Indeed, growing evidence suggests that amyloid fibrils could develop from matured condensates of many aggregation-prone proteins such as TDP-43 (Babinchak et al., 2019), FUS (Patel et al., 2015), tau (Wegmann et al., 2018), α -synuclein (Ray et al., 2020) associated with neurodegenerative disorders. In the case of full-length PrP, the formation of Th-T, positive β -rich fibrils has been emerged from matured condensates in the presence of crowding agents (Kostylev et al., 2018) and kosmotropic salt (Tange et al., 2021). A recent study also suggest that pathogenic mutant variant of PrP, E200K, accelerates the maturation of condensates (Liu et al., 2024). However, these aggregates lack the autocatalytic activity observed in the amyloid aggregates formed through the canonical

non-LLPS conditions. It was important to determine whether the mature condensates could convert into amyloid-like aggregates. The aged condensates did not show any significant increases in during the initial 12h of maturation indicating that amyloid like structures did not form during this period. However, after prolonged incubation in PBS for 100 h a slight increase in ThT fluorescence was observed (Figure 7.8), suggesting a slow onset of amyloid-like aggregate formation. The results contrasts with a previous study that reported a significant increase in ThT fluorescence in condensates of full-length PrP in PBS. The fact that full-length moPrP condensates remained mostly ThT-negative even after extended incubation suggests that the CTD might plays an inhibitory role in amyloid formation *via* condensation.

Condensate formation and aggregation

In vitro studies have shown that Cu^{2+} also promotes PrP condensation, increasing its structural compactness and stability, with PrP: Cu^{2+} condensates found to suppress aggregation (do Amaral et al., 2023). Cu^{2+} binding induces the NTR to fold around the CTD, interacting specifically with $\alpha 2$ and $\alpha 3$, which brings the $\beta 1$ - $\alpha 1$ - $\beta 2$ and $\beta 2$ - $\beta 3$ subdomains of the CTD into closer proximity (Schilling et al., 2020), enhancing overall protein stability. By facilitating these conformational changes, Cu^{2+} prevents PrP from adopting aggregation-prone structures inside condensates, acting as a safeguard against amyloid formation. Additionally, acidic pH prevents LLPS of PrP (Kostylev et al., 2018), while lowering pH is known to induce misfolding and aggregation of PrP (DeMarco and Daggett, 2007; Hornemann and Glockshuber, 1998; Singh and Udgaonkar, 2016). Moreover, experiments with recombinant PrP variants, such as PrPY145Stop (Agarwal et al., 2021), PrP90–231 (Matos et al., 2020), and PrP23-231 treated with kosmotropic salts ($\text{Na}_2\text{S}_2\text{O}_3$) (Tange et al., 2021), suggest that hydrophobically driven condensates transition from a liquid-like to a solid-like amyloid state, while electrostatically driven LLPS (do Amaral et al., 2023) may be more functionally relevant. The results suggest that LLPS and aggregation might occur independently, similar to mechanisms observed in tau (Lin et al., 2020) and α -synuclein (Lipiński et al., 2022).

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