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Molecular Mechanism of the Misfolding and Oligomerization of the Prion Protein: Current Understanding and Its Implications

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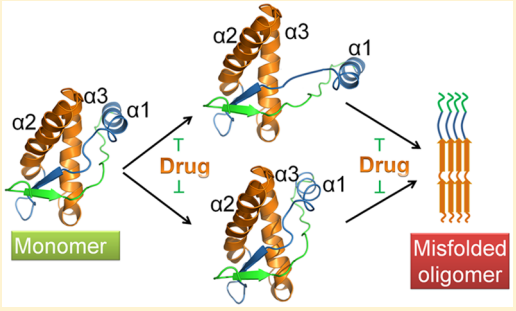
ABSTRACT: Prion diseases, also known as transmissible spongiform encephalopathies, make up a group of fatal neurodegenerative disorders linked with the misfolding and aggregation of the prion protein (PrP). Although it is not yet understood how the misfolding of PrP induces neurodegeneration, it is widely accepted that the formation of misfolded prion protein (termed PrP^{Sc}) is both the triggering event in the disease and the main component of the infectious agent responsible for disease transmission. Despite the clear involvement of PrP^{Sc} in prion diseases, the exact composition of PrP^{Sc} is not yet well-known. Recent studies show that misfolded oligomers of PrP could, however, be responsible for neurotoxicity and/or infectivity in the prion diseases. Hence, understanding the molecular mechanism of formation of the misfolded oligomers of PrP is critical for developing an understanding about the prion diseases and for developing anti-prion therapeutics. This review discusses recent advances in understanding the molecular mechanism of misfolded oligomer formation by PrP and its implications for the development of anti-prion therapeutics.



PRION PROTEIN AND PRION DISEASES

The prion protein (PrP) is a highly conserved glycoprotein, expressed ubiquitously in mammalian neurons.[1−3] The human prion gene *Prnp* encodes a 253-residue precursor protein (Figure 1a). The first 22 N-terminal signal residues are post-translationally removed during transport to the cell surface.[4] The last 23 C-terminal residues are excised after the addition of a glycosylphosphatidylinositol (GPI) anchor.[5] Thus, mature cellular prion protein, PrP^{C}, is a GPI-anchored protein present on the cell surface and consists of 208 residues (Figure 1a). PrP^{C} is variably glycosylated at two asparagine residues (Asn181 and Asn197) and exists as un-, mono-, and diglycosylated forms.[6] The structure of PrP^{C} from several mammalian species is known from NMR studies[7−9] to consist of an unstructured N-terminal domain (NTD) and a structured C-terminal domain (CTD). The NTD has four or five octapeptide repeats and has a high affinity for divalent metal ions, such as Cu^{2+}, and becomes partially structured upon interaction with Cu^{2+}.[7,10,11] The CTD consists of three α-helices, comprising residues 144−156, 174−194, and 200−228, and a two-stranded antiparallel β-sheet that flanks helix 1 (α1), spanning residues 128−131 (β1) and 161−164 (β2). Helices 2 (α2) and 3 (α3) are linked by a disulfide bond (Figure 1b).

The exact biological function of PrP^{C} is not yet known. Common strategies employed to identify possible PrP^{C} functions include the development of different transgenic (Tg) mouse lines that are knockout for the gene *Prnp* ($Prnp^{0/0}$). Despite the wide distribution of PrP^{C} in the mammalian central nervous system, $Prnp^{0/0}$ mice surprisingly failed to show any gross pathological phenotype in terms of development and behavior.[12] However, detailed evaluations revealed that $Prnp^{0/0}$ mice display mild behavioral phenotypes such as increased excitability of hippocampal neurons,[13,14] altered long-term potentiation,[15] and deficits in spatial learning and circadian rhythms.[16] PrP^{C} has also been linked to multiple physiological processes.[17,18] The functions that have been attributed to PrP^{C} include immunoregulation, signal transduction, copper binding, synaptic transmission, and induction of apoptosis or protection against apoptotic stimuli.[19] To exclude any compensatory mechanism occurring during the development of $Prnp^{0/0}$ mice, a conditional knockout model was created to explore the effects of PrP^{C} depletion on neuronal survival and function in the adult brain.[14] No evidence of histological changes or neurodegeneration was found up to 15 months postknockout. Similarly, disruption of PrP^{C} expression in cattle and goats resulted in no apparent developmental, physiological, or anatomical abnormalities.[20,21] Taken together, these studies are consistent with the idea that PrP^{C} is a pleiotropic protein with different functions but is not an essential protein.

On the other hand, the misfolding of PrP^{C} into the aggregated form, PrP^{Sc} (scrapie PrP), is linked with several fatal neurodegenerative diseases known as transmissible spongiform encephalopathies (TSEs).[22,23] TSEs can be of an infectious, sporadic, or genetic nature. While the infectious forms arise from exposure to preformed misfolded prion, sporadic forms arise *de novo* without any exposure to any preformed misfolded form or without any genetic modification.[22] In genetic or familial diseases, single-nucleotide mutations, which lead to single-amino acid residue changes in PrP, as well as insertions/deletions in the gene *Prnp*, appear to increase the likelihood of

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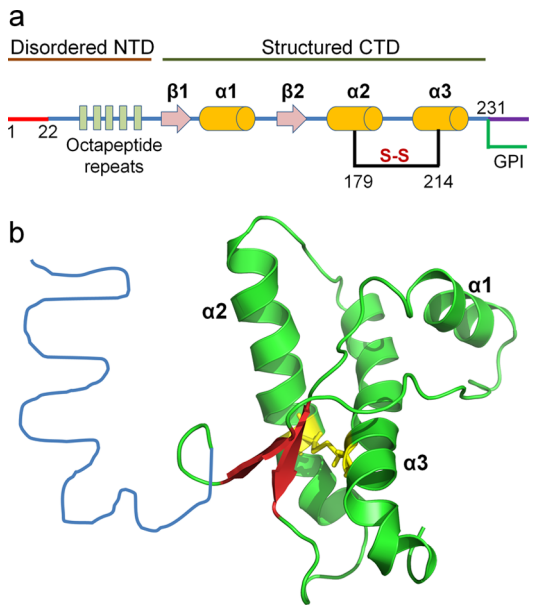

Figure 1. Structural features of the full length mammalian prion protein (PrP). (a) Scheme of primary structure of the immature cellular prion protein (PrP^C). Amino acids 1−22 (shown as a red line) represent the N-terminal signal sequence, while the last 23 amino acid residues from position 231 onward represent the GPI anchor signal (shown as a purple line). The mature full length mammalian prion protein has 208 amino acid residues and consists of two domains. The N-terminal domain (NTD) (23−120) is unstructured and contains four or five octapeptide repeats (shown as light green boxes). The C-terminal domain (CTD) (121−231) is folded into two small β-strands, which form an antiparallel β-sheet, and three α-helices. The mature protein is anchored to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor at the C-terminus. A disulfide bond links α2 to α3. (b) NMR structure of the CTD of the recombinant mouse prion protein. The disulfide bond linking α2 to α3 is colored yellow. The blue unstructured part is not present in the NMR structure and represents the NTD. The structure has been drawn from Protein Data Bank entry 1AG2, using PyMOL.

neurodegeneration.[24] TSEs are characterized by motor and cognitive impairments, extensive brain damage, and neuronal dysfunction.[22,23] After typically long incubation periods, individuals affected by TSEs deteriorate rapidly and progressively, once the clinical symptoms arise, with lethal consequences in all cases. TSEs include diseases such as Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI), Gerstmann-Straussler-Scheinker (GSS) syndrome, mad cow disease, and scrapie.[22,23] TSEs are unlikely to be caused by a loss of function of PrP^C because of its misfolding to PrP^{Sc}: mice lacking PrP^C do not show neurodegeneration.[14] TSEs are likely to be caused by a gain of function because of the formation of PrP^{Sc}.[25] Perhaps the strongest evidence in the support of PrP^{Sc} causing TSEs came from the generation of infectious material in the test tube by *in vitro* conversion and replication of PrP^C of both mammalian and recombinant origin.[26−28] There are no chemical differences between PrP^C and PrP^{Sc}, and their distinction is at the level of the structure and aggregation of the protein.[29,30]

IDENTIFICATION OF THE PATHOGENIC CONFORMATIONS OF THE PRION PROTEIN

Although it is now widely accepted that misfolded PrP is linked with prion diseases, the exact composition of the protein form(s) causing the prion diseases remains to be fully understood.[31] While the accumulation of PrP^{Sc} in the central nervous system is a characteristic feature of the prion diseases, the mechanisms of misfolded prion protein infectivity and toxicity are yet to be fully understood. Some studies reveal that the deposition of PrP amyloid fibrils in the brain is not linked with neurodegeneration.[32,33] Moreover, in several cases, amyloid deposits of PrP were not observed in the brain of animals despite the occurrence of neurodegeneration.[34,35] Similarly, several pathogenic mutations in humans do not result in any accumulation of amyloid plaques in the brain despite the occurrence of neurodegeneration.[36−38] These studies indicate that PrP amyloid fibrils may not be responsible for neurodegeneration in prion diseases.

Alternative forms of PrP, different from PrP^{Sc} in both structural and biochemical properties, have been reported to have important roles in prion-mediated neurodegeneration.[39] In some forms of the GSS syndrome, it has been shown that transmembrane forms of PrP induce neurodegeneration even when no PrP^{Sc} can be detected in the brain.[40] PrP can also exist in cytosolic[41] and secreted[42] forms. Interestingly, these forms of PrP have been shown to cause neurodegenerative features in the absence of any significant accumulation of PrP^{Sc}, both in cultured neuronal cell lines and in transgenic mouse models.[40,41,43] Apart from existing in these different forms, PrP can also exist in the form of misfolded oligomers. Several lines of evidence implicate misfolded oligomers as playing a role in prion diseases.[44]

Traditionally, PrP^{Sc} is considered to be a proteinase K (PK)-resistant form, but recent studies have indicated the involvement of infectious PK-sensitive, soluble oligomeric forms in several prion diseases.[45−48] The PK-sensitive, soluble oligomeric forms can be isolated from brain homogenates either without protease digestion[45] or upon digestion with the protease thermolysin that does not digest the PK-sensitive oligomeric forms.[47] The PK-sensitive oligomeric forms can be fractionated by sucrose gradient sedimentation and gel filtration[45,48] and have been shown to exist as a heterogeneous population.[48,49] Interestingly, different conformations of PK-sensitive, soluble oligomeric forms isolated from the brains of individuals suffering from sporadic CJD (sCJD) conformationally converted PrP^C at different rates *in vitro*.[49] Importantly, small oligomeric forms, with masses equivalent to those of 20−78 PrP molecules, were the most efficient initiators of PrP^C conversion, and the seeding efficacy of sCJD prions actually decreased with the size of the aggregates.[49] Surprisingly, the PK-sensitive, soluble oligomeric forms share basic structural features with PK-resistant PrP^{Sc}, as probed by limited proteolysis, despite the presence of differences in sensitivity toward PK digestion, and in their sizes.[48] By definition, PrP^{Sc} is protease-resistant, but the protease-sensitive forms of PrP are ambiguously termed as PK-sensitive disease-related PrP, or PrP^{Sc}, or protease-sensitive PrP^{Sc} ($sPrP^{Sc}$).[45,46,48,50] These species remain poorly defined in physical terms, and an internationally accepted, even provisional, nomenclature is lacking.

PK-resistant but soluble oligomeric forms isolated from cell cultures have also been shown to be infectious.[51] Moreover, recent studies indicate that it is the oligomeric PrP^{Sc} forms rather than the fibrillar forms that are linked to neurotoxicity.[52,53] The critical roles of PrP oligomers versus fibrils are also supported by studies of GPI-anchorless PrP Tg mice.[32] These animals either do not develop disease or develop disease after very long incubation times, despite the fact that they have large quantities of fibrillar PrP^{Sc} in their brains. Several other studies also indicate that the accumulation of insoluble, PK-resistant PrP^{Sc} is not linked with prion pathogenesis.[33,53] However, some studies show that amyloid fibrils of PrP formed *in vitro* could be cytotoxic,[55,56]

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making the role of PrP amyloid fibrils debatable in prion diseases. Irrespective of the role of amyloid fibrils in prion diseases, small oligomers appear to be infectious as well as neurotoxic. The oligomeric forms of PrP linked with disease appear to be very heterogeneous in sizes and conformations.[49,57] This heterogeneity in the sizes and conformations of oligomeric PrP^{Sc} forms makes it difficult to identify distinct neurotoxic and infective forms.[44] Importantly, oligomeric PrP^{Sc} forms of different sizes show differences in the conversion efficiency and the duration of the disease, indicating that different oligomeric forms of PrP may act as prion strains,[57] which are different conformations of PrP leading to different disease phenotypes.

Although much experimental evidence suggests that neurotoxicity in prion diseases is mediated by misfolded oligomers, how neurotoxic forms of PrP kill nerve cells remains an open question and a research priority. Misfolded oligomers have been shown to induce cell death by inducing cellular apoptosis[58] and by activating the classical complement pathway.[59] In another study, it has been shown that soluble oligomers specifically inhibit the proteolytic β subunits of the 26S proteasome, thereby inducing cell death.[60] Pore formation in lipid membranes by soluble oligomers is another proposed mechanism for the toxicity of misfolded oligomers.[61−64] Another possible mechanism is the specific PrP-mediated modulation of the *N*-methyl-D-aspartate (NMDA) receptor, which plays a crucial role in mediating a wide range of important nervous system functions;[65,66] excessive NMDA receptor activity may lead to cytotoxicity and neuronal damage.[67] These studies show that a consensus about the mechanism by which neurotoxic forms of PrP kill nerve cells remains far from clear. Because the misfolded forms of PrP are very heterogeneous, the identities of misfolded forms of PrP that lead to toxicity and of forms that cause infectivity remain to be determined. Identification and structural characterization of these forms are likely to help in understanding the molecular basis of their toxicity and infectivity.

FORMATION AND CHARACTERIZATION OF MISFOLDED OLIGOMERS *IN VITRO*

The isolation and characterization of PrP^{Sc} by using brain-derived material from diseased animals have several obstacles because of the physical properties of PrP^{Sc}.[31] The difficulty in obtaining PrP^{Sc} from diseased brain led to attempts to produce synthetic PrP^{Sc}. Such efforts included either chemically or physically altering the conformation of recombinant PrP produced in bacteria to form aggregates.[55,58,68−71] The aggregation of PrP *in vitro* is highly dependent on the environmental conditions.[69−71] PrP forms β-rich misfolded oligomers at low pH, and the propensity of misfolded oligomer formation increases with a decrease in pH.[64,72] Conversely, amyloid fibrils are formed at neutral or slightly acidic pH, and the level of amyloid fibril formation decreases with a decrease in pH.[69,71,73] This decrease in the level of amyloid fibril formation with a decrease in pH is a consequence of misfolded oligomers being off pathway to amyloid fibril formation. The formation of misfolded oligomers over amyloid fibrils with a decrease in pH is most likely caused by the difference in the molecular structures of the two forms.[69] Interestingly, aggregation of PrP has been shown to occur in the endocytic pathway,[74,75] in which lysosomes have a low internal pH. It should be noted that the stability of PrP at pH 7 is significantly higher than that at pH 4, and that at the latter pH, the native protein undergoes substantial structural fluctuations.[76] It is likely that PrP misfolds to oligomeric forms when it encounters the low pH in the endocytic pathway. Importantly, oligomers formed *in vitro* at low pH have been shown to be cytotoxic,[56,60,77,78] and sporadic prion disease susceptibility appears to correlate well with the propensity of recombinant PrP to form these oligomers.[77] The oligomers formed at low pH can disrupt lipid membranes,[62−64] pointing toward a putative mechanism of their toxicity.

Misfolded oligomers of PrP at low pH have generally been prepared in the presence of chemical denaturants.[69,77] However, oligomers also form in the absence of denaturants but in the presence of salt.[64,70,79,80] A reduction of the disulfide bond of PrP also leads to its misfolding to oligomers.[68,81] Moreover, misfolded oligomers can also be generated by high temperatures,[58,82] metal-induced oxidation,[83] and high pressures.[84] Hence, misfolded oligomers can be generated in several ways *in vitro*, and these oligomers formed under different conditions appear to be very heterogeneous in size and conformation.[85] Moreover, PrP can form different types of misfolded oligomers under identical conditions; for example, oligomers of different sizes are formed at pH 2,[86] which have been shown to have different structures (see below), as well as at pH 4 in the presence of urea.[85] Importantly, misfolded oligomers formed under different physicochemical conditions have been shown to be cytotoxic.[58,59,77,78] However, the population(s) of oligomers in a heterogeneous mixture of oligomers, which lead to cytotoxicity, remains to be determined. Determining the precise correlation between the size as well as conformation of PrP oligomers and cytotoxicity is important because oligomers of different sizes showed different PrP^{C} conversion efficiencies *in vivo* (see above). Hence, understanding the molecular mechanism of formation of the misfolded oligomers is very crucial for developing an understanding about prion disease and for being able to develop anti-prion drugs.

STRUCTURE OF MISFOLDED OLIGOMERS

Understanding the structure of misfolded oligomers is the first step in understanding the molecular mechanism of their formation. The molecular structures of PrP oligomers remain poorly described. However, in the past decade, great progress has been made in determining the structure of fibrillar PrP^{Sc}.[31] The results of low-resolution biophysical techniques as well as of computational studies were used to develop structural models for fibrillar PrP^{Sc}. The three most prevalent models for the structure of fibrillar PrP^{Sc} are the β-helix model, the spiral model, and the parallel in-register β-sheet model (Figure 2).[31] While the β-helix model was built on a low-resolution three-dimensional structure of PrP^{Sc} derived from electron crystallographic data and theoretical modeling, the spiral model was derived from a molecular dynamics (MD) simulation of PrP under amyloidogenic conditions. According to the β-helix model, α1 turns into a left-handed β-helix, whereas α2 and α3 retain an α-helical conformation, similar to those in PrP^{C} (Figure 2a).[87,88] On the other hand, the spiral model suggests that the β-core of PrP^{Sc} consists of parallel and antiparallel β-strands within the region of residues 116−164, whereas all three α-helices retain their native conformation (Figure 2b).[89]

The two models that retain helical structure in PrP^{Sc} are consistent with some of the epitope mapping studies probing conformational changes in PrP^{C},[90] but they fail to explain several biophysical properties of PrP^{Sc}. The conclusion that PK-resistant brain-derived PrP^{Sc} retains helical content appears to have been derived, in part, from the interpretation of infrared spectra and, especially, the presence of the ~1656−1660 cm^{-1} band,[30,91] but the assignment of this band is not certain. In fact, the infrared

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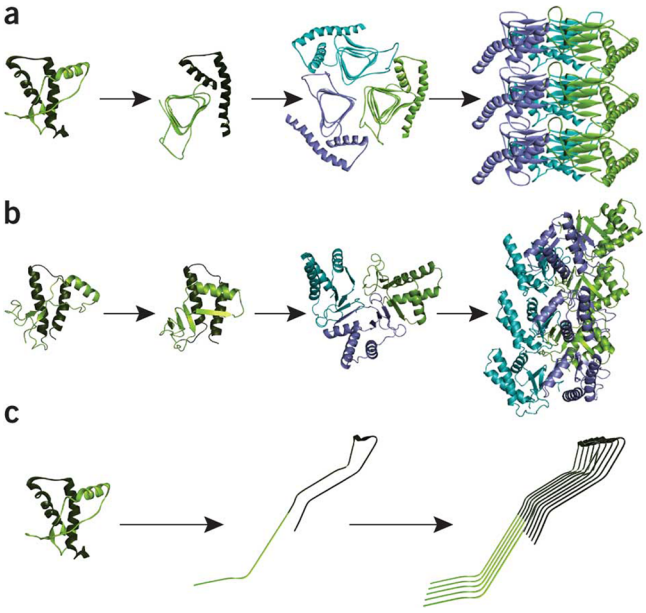

Figure 2. Alternative structural models proposed for PrP^{Sc}. (a) In the β-helical model, a major refolding of the N-terminal region of PrP into a β-helix motif from residue 90 to 177 (light green) is proposed. The C-terminal region (residues 178−230, dark green) covering α2 and α3 maintains the α-helical secondary structure organization, as in PrP^{C}. (b) The β-spiral model posits a spiraling core of extended sheets comprising short β-strands, spanning residues 116−119, 129−132, 135−140, and 160−164. In this model, all three α-helices retain their native conformation in PrP^{Sc}. (c) The parallel in-register extended β-sheet model of PrP^{Sc} proposes complete misfolding of PrP^{C} into a structure composed mainly of β-sheets. To facilitate comparison, the same color assignment for structural motifs has been used in all panels. The figure is reprinted with permission from ref 31. Copyright 2012 Nature Publishing Group.

spectrum of brain-derived PrP fibrils is identical to that of recombinant amyloid fibrils of PrP,[92] which clearly lack any helical structure (see below). Hence, it is unlikely that brain-derived PrP^{Sc} aggregates possess any helical structure. The two models also do not account for the proteolysis data: the C-terminal part of PrP^{C} is easily cleaved by proteases, while that of PrP^{Sc} has a high resistance to proteolytic degradation, indicating that the C-terminal domain must possess substantially different structures in PrP^{C} and PrP^{Sc}. Indeed, in both these models, the α-helical domains face the outside of the polymer (Figure 2); hence, they should be at least partially accessible to proteases. These two models also do not provide a rational explanation for the decrease in α-helical content that is observed during the conversion of PrP^{C} into PrP^{Sc}.[30] Importantly, more recent experimental and computational studies of different types of aggregates of PrP show conclusively that the α2−α3 region is converted into β-sheet during aggregation (see below). Clearly, the β-helix and spiral models now stand discredited.

Structure of Fibrillar PrP^{Sc}. On the other hand, the parallel in-register β-sheet model for fibrillar PrP^{Sc} has strong experimental support.[93−95] This model posits conversion of the CTD of PrP^{C} to β-sheet in PrP^{Sc} (Figure 2c). Recent experimental studies of amyloid fibrils generated *in vitro* under different environmental conditions suggest that α2 and α3 have converted into β-sheet, regardless of whether they were probed by electron paramagnetic resonance,[93] hydrogen−deuterium exchange mass spectrometry (HDX-MS),[73,96] solution nuclear magnetic resonance (NMR),[97] or solid-state NMR.[94,95] Worm-like amyloid fibrils formed at pH 2 also appear to have α2 and α3 converted into β-sheet,[63] although the stability of the core region of the wormlike amyloid fibrils seems to be low compared to the stability of the core region of the straight amyloid fibrils formed at pH 7.[73] PrP aggregates prepared in the presence of cofactors like palmitoyloleoylphosphatidylglycerol (POPG) and RNA also show an increased level of protection against HDX in the α2−α3 region.[98] In brain-derived fibrils of PrP, too, major conformational rearrangements either in the CTD[99] or in both the CTD and NTD (starting from residue ∼89 or 90)[92] appear to have taken place. Brain-derived fibrils as well as PrP^{Sc}-seeded amyloid fibrils appear, however, to have extended core regions toward the N-terminus compared to the core region of recombinant amyloid fibrils.[92,93,95,100] Importantly, the idea that α2 and α3 are intrinsically unstable and have the propensity to convert into β-sheet during conformational conversion was first suggested by computational studies[101,102] and only later corroborated by experimental evidence.

Structure of Soluble Misfolded Oligomers of PrP. Recently, structures of the misfolded oligomers of PrP formed under different conditions have been studied experimentally using several different low-resolution biophysical probes. The misfolded oligomers do not appear to retain any substantial helical structure: their infrared spectra[86] are similar to that of brain-derived fibrils as well as that of recombinant amyloid fibrils that lack any helical content (see above).[92] Oligomers formed under different conditions appear to have similar structures in terms of their core region.[63,73,103−105] Structural studies of misfolded oligomers using NMR, HDX-MS, and electron spin resonance show that the α2 region of PrP^{C} has converted into β-sheet.[63,81,105,106] Moreover, several studies indicate that the region covering α1 and β2 is unfolded in oligomers.[63,82,105] The status of the α3 region in oligomers remains unclear.[63,81,105] The α3 region of PrP^{C} is structured in oligomers and is likely to have converted into β-sheet, but that remains to be determined. Because oligomers are rich in β-sheet,[64] it is likely that the α3 region has converted to β-sheet in oligomers.[104,105] Strikingly, high-pressure NMR studies have identified a sparsely populated metastable conformation of PrP^{C}, in which α2 and α3 are preferentially disordered, that is likely to be a precursor for misfolded oligomers.[107,108] Recently, several computational studies have suggested that the monomeric precursor to oligomeric PrP^{Sc} has the α2−α3 region converted into β-sheet.[109−111] It seems very likely now that the misfolded oligomers have the α1 region unfolded and the α2−α3 region converted into β-sheet (Figure 3a).

Structural Similarity between Amyloid Fibrils and Soluble Oligomers. Misfolded oligomers and amyloid fibrils formed by recombinant PrP appear to have similar regions in their structured core (see above); however, the core region of misfolded oligomers appears to be less stable than the core region of amyloid fibrils.[63,73,96] Interestingly, a subpopulation of misfolded oligomers formed at low pH appears to be more similar to brain-derived PrP^{Sc}, in terms of their core region, than to recombinant amyloid fibrils.[63,105] Thus, it appears that the misfolded oligomers formed *in vitro* may be a better model for PrP^{Sc} than fibrils formed under other conditions. Misfolded oligomers formed under different conditions, or even under identical conditions, show conformational heterogeneity. Within the same population of oligomers, a subpopulation of oligomers has either or both of the β1 and β2 regions structured, while in the major fraction of oligomers, these regions are unstructured.[63,105] Misfolded oligomers of different sizes formed under

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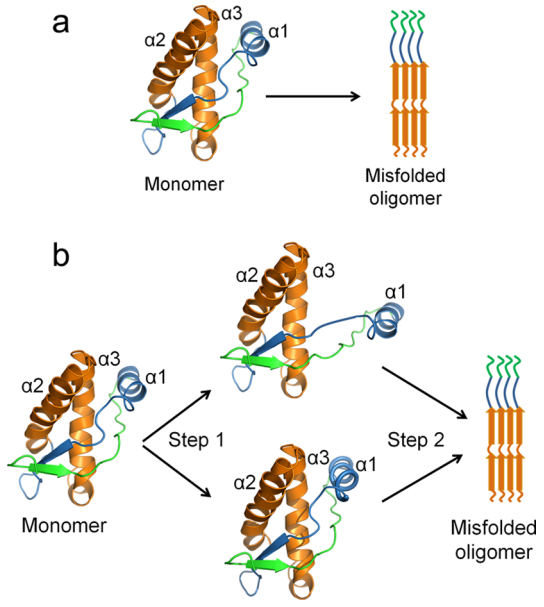

Figure 3. Model for the molecular mechanism of PrP misfolding and oligomerization. (a) Misfolded oligomers of PrP have the α1 region unfolded and the α2−α3 region misfolded into β-sheet. The high-resolution structure of misfolded oligomers remains to be determined. (b) Misfolding in native PrP may commence by the loss of structure in α1 and the loop between α1 and β2. The loss of structure in α1 could be caused by either its movement away from the α2−α3 region or its unraveling. The high intrinsic propensity of α2 to convert into β-sheet/random coil then drives the conversion of α2 and α3 into β-sheet.

identical conditions appear to have differences in the regions covering α1 and the C-terminus of α2.[63] The high-resolution structures of the misfolded oligomers of PrP formed under different conditions remain, however, to be determined. High-resolution structures of misfolded oligomers are likely to help in understanding the molecular basis of their pathogenicity.[112]

MOLECULAR MECHANISM OF MISFOLDED OLIGOMER FORMATION

Regions Critical for Misfolded Oligomer Formation. Developing an understanding of the regions in native PrP^C that are critical in misfolded oligomer formation is an important step in understanding the molecular mechanism of their formation. Different studies have pointed toward the role of different regions as being critical for PrP misfolding. Some studies have suggested that α1 is critical for misfolded aggregate formation, and that its unraveling followed by interaction with misfolded aggregates triggers the misfolding of the protein.[113] On the contrary, other studies have suggested that α1 is likely to delay conformational changes initiated somewhere else in the protein during its conformational conversion.[114] Several computational studies have showed increased structural dynamics in the α1 region during the early stages of PrP misfolding.[115,116]

The loop between β2 and α2 is another region that has been suggested to be critical for the misfolding of PrP.[117] The conformation and rigidity of this loop appear to determine prion disease transmission and susceptibility of a species. Several studies have suggested that mammals carrying a flexible β2−α2 loop can be easily infected by prions, whereas prions are poorly transmissible to animals carrying a rigid loop.[118] Importantly, horse and rabbit have so far displayed resistance to prion infections, and there are no reports of these species developing spontaneous prion diseases.[119] NMR studies showed that the structures of their PrPs are characterized by a rigid β2−α2 loop and by closer contacts between the loop and α3.[9,120] Interestingly, replacement of the β2−α2 loop residues in hamster PrP with rabbit PrP residues reduced the propensity of hamster PrP to form misfolded oligomers.[121] Conversely, replacement of the β2−α2 loop residues in rabbit PrP with hamster PrP residues increased the propensity of rabbit PrP to form misfolded oligomers. Hence, the rigidity of the β2−α2 loop appears to be important for the formation of misfolded oligomers. Nevertheless, the role of this region in prion pathogenesis is yet to be firmly established.

The C-terminus of α2 and the loop between α2 and α3 have also been shown to be a critical region linked to PrP misfolding. This region has been shown to acquire a β-sheet conformation in the domain-swapped crystal structure of a PrP dimer.[122] Moreover, several studies have shown that a reduction in pH or the addition of chemical denaturants leads to structural perturbations mostly in the C-terminus of α2.[72,123−125] Indeed, several studies point out that this region could act as a nucleation site for PrP misfolding, and hence, the initial conformational changes could begin in this region.[115,126,127] Interestingly, the C-terminal stretch of α2 (sequence stretch TVTTTT) is very unusual in its sequence composition. Several of the amino acid residues at the C-terminus of α2 have a high propensity for random coil/β-strand formation, making this region energetically frustrated.[101,102] Importantly, stabilization of the C-terminal region of α2 of PrP by replacing amino acid residues with a high propensity for β-sheet formation with the amino acid Ala, which has a high propensity for helix formation, prevents the misfolding and oligomerization of PrP,[64] highlighting the critical role of this region in PrP misfolding.

Molecular Events during Misfolded Oligomer Formation. Several studies have probed the molecular mechanism of misfolded oligomer formation by PrP. Because a reduction in pH is known to induce misfolded oligomer formation, it was important to understand the effect of pH on PrP. A reduction in pH would lead to the protonation of some critical residue(s), which could trigger the misfolding of PrP. Indeed, the relative amount of misfolded oligomers formed in the presence of salt but in the absence of chemical denaturants increases with a decrease in pH, and the misfolding/oligomerization transition is characterized by a pH midpoint (pH_m) of 4.7.[64] It is possible that the critical residue is either an acidic residue that has an abnormally high pK_a value in the native state or a histidine residue that has an abnormally low pK_a value in the oligomeric state. MD simulations have pointed to the protonation of His155 and His187 as critical steps in the pH-induced conformational conversion of human PrP.[128] The apparent pK_a values for the protonation of His155 and His187, calculated by MD simulations and NMR measurements, were estimated to be ~4.5−5.0.[124,128]

The H187R mutation, which is analogous to the protonation of His187, introduces a positive charge at residue 187 and is linked with familial prion diseases[129] indicating that the protonation of His187 might be important for the misfolding of PrP. Indeed, the H187R mutation shows an increased level of misfolding of PrP.[125,130] MD simulations suggest that the protonation of His187 disrupts the electrostatic network and other interactions between the C-terminal region of α2 and the loop between α1 and β2 involving residues Arg156, Asn159, Gln160, Glu196, and Asp202.[115,128,131,132] Because of the loss of these interactions, it is likely that the protonation of His187

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might lead to increased structural dynamics in these regions. Indeed, MD simulations of the wild type and pathogenic mutant variant H187R of human PrP show that the side chain of Arg156 moves significantly away from its original position in H187R PrP.[133] This loss of interactions of the C-terminus of α2 with other regions is likely to trigger the misfolding and oligomerization of PrP because the C-terminal region of α2 of PrP is very prone to misfolding and oligomerization.[64]

The destabilization of a protein leads to a reduction in the difference in free energy between the native state and an aggregation prone, sparsely populated N* state, from which misfolding commences. Such destabilization is therefore expected to increase the propensity of the protein to misfold.[134] A structural characterization of such a high-energy N* state, either through the use of the native-state thiol labeling[135] and hydrogen exchange[136,137] methodologies or through the use of advanced NMR[138,139] methodologies, is likely to help in understanding the molecular mechanism of misfolding of a protein. Several pathogenic mutations in PrP are known to thermodynamically destabilize the protein.[105,140,141] This reduction in the thermodynamic stability is linked with an increase in the misfolding rate of the pathogenic mutant proteins.[105,142,143] Because destabilized pathogenic mutations show increased misfolding rates, studies of the structural dynamics of the pathogenic mutant variants are likely to help in understanding the molecular mechanism of PrP misfolding. Indeed, a recent study showed that destabilizing pathogenic mutations that are present in the α2−α3 region lead to a very similar structural perturbation: α1 shows increased structural dynamics.[105] Importantly, the misfolding rate of PrP increases exponentially with the extent of destabilization of α1. Interestingly, another recent study characterized the structure of an acid-induced molten globule form of PrP: the acid-induced molten globule form has the β1−α1−β2 region unfolded, while the α2−α3 region shows marginal stability.[144] This molten globule form appears to act as a precursor to misfolded oligomer formation.[144]

Hence, the loss of α1 structure appears to be the first step during conformational conversion in misfolded oligomer formation. Strikingly, several antibodies that inhibit prion replication *in vivo*[145,146] bind α1 and might act by preventing the unfolding of α1. These results also indicate that the misfolding events captured *in vitro* might reflect the events taking place *in vivo*.

The tethering together of subdomains β1−α1−β2 and α2−α3 prevents the oligomerization of PrP,[82] indicating that separation of the subdomains might be a prerequisite for oligomer formation. Indeed, the pathogenic mutations H187R and E196K induce subdomain separation and, hence, show an increased level of misfolding.[130] Several computational studies conducted on either wild-type PrP or the pathogenic mutant variants show that α1 has a high mobility and moves away from the α2−α3 region,[116,133,147,148] an event that eventually triggers the misfolding of the protein.

Hence, misfolded oligomer formation by PrP appears to take place in at least two steps (Figure 3b). The first step involves an increase in the structural dynamics of α1. The increased structural dynamics of α1 could be caused by either its movement away from the α2−α3 region, its unraveling, or a combination of both. The details of the nature of the structural perturbation of α1 remain to be determined. Native-state hydrogen exchange NMR experiments, yet to be done, will determine whether a partially unfolded form with a structurally perturbed α1 is populated as a high-energy intermediate. FRET or high-resolution NMR experiments will provide details of the structural and dynamical changes taking place in α1. Nevertheless, the outcome of this step would be the loss of the interactions between α1 and the α2−α3 region. It is likely that any perturbation, whether a mutation or any chemical perturbation, which destabilizes the interactions between α1 and the α2−α3 region, would lead to an increased level of misfolding of PrP. Indeed, a reduction in pH has been shown to lead to a reduction in the number of tertiary contacts between α1 and α3 in the pathogenic mutant variant V210I.[149] Upon the loss of α1 structure, the high intrinsic propensity of the C-terminal sequence of α2 for a random coil/β-sheet conformation[101] would drive the misfolding of the α2−α3 subdomain[105] (Figure 3b). Interestingly, the isolated sequence segment comprising only the α2−α3 subdomain forms oligomers faster than does full length PrP,[150] suggesting that the lack of interactions with α1 in the isolated α2−α3 subdomain leads to faster oligomerization. Strikingly, misfolded oligomers of PrP formed at high temperatures,[82] by several pathogenic mutations,[105,130] or by a reduction in pH[144] appear to have been formed by very similar molecular mechanisms. It should, however, be noted that while several pathogenic mutations of PrP are known to destabilize the monomeric protein, not all pathogenic mutations destabilize the monomer.[140,151] Hence, all pathogenic mutations might not show an increased level of misfolding, and it is likely that different pathogenic mutations exert their pathogenic effects by different mechanisms.[40,152,153] Moreover, despite the apparent similarities in their core regions, misfolded oligomers formed under different conditions might have been formed by alternative pathways.

The molecular mechanism of misfolded oligomer formation (Figure 3b) by PrP is consistent with different studies that have implicated the regions that are crucial for misfolding (see above). The loss of α1 structure triggers misfolding of PrP; not surprisingly, α1 appears absent in the core of misfolded oligomers.[105] Hence, an intact α1 would prevent misfolding of PrP, providing a rationale for the anti-prion properties of antibodies that target α1 and prevent prion disease in mice.[145,146] Because the separation of β1−α1−β2 and α2−α3 subdomains triggers misfolding of PrP, the flexibility of the β2−α2 loop, which connects the two subdomains, should affect their separation. Not surprisingly, animals that have a PrP with a rigid β2−α2 loop that would reduce subdomain separation are less susceptible to prion diseases.[118] Finally, the high intrinsic propensity of the C-terminal sequence of α2 for a β-sheet/random coil conformation seems to be critical for the misfolding of PrP upon the loss of interactions between the two subdomains, as stabilization of the C-terminus of α2 even when α1 is destabilized prevents misfolding of PrP.[105]

IMPLICATIONS OF THE MOLECULAR MECHANISM OF PRP MISFOLDED OLIGOMER FORMATION FOR PRION DISEASE THERAPY

Although high-resolution structures of misfolded oligomers remain to be determined, the understanding of the molecular mechanism of misfolded oligomer formation suggests potential sites on PrP for therapeutic intervention. According to the molecular mechanism of formation of the misfolded oligomers, at least two sites on PrP^C could be used as targets for anti-prion drugs. Because the loss of α1 structure triggers misfolding, the stabilization of α1 by chemical chaperones or drug molecules should prevent misfolding. Indeed, several antibodies are known to bind to α1.[145,146,154,155] These antibodies are able to prevent

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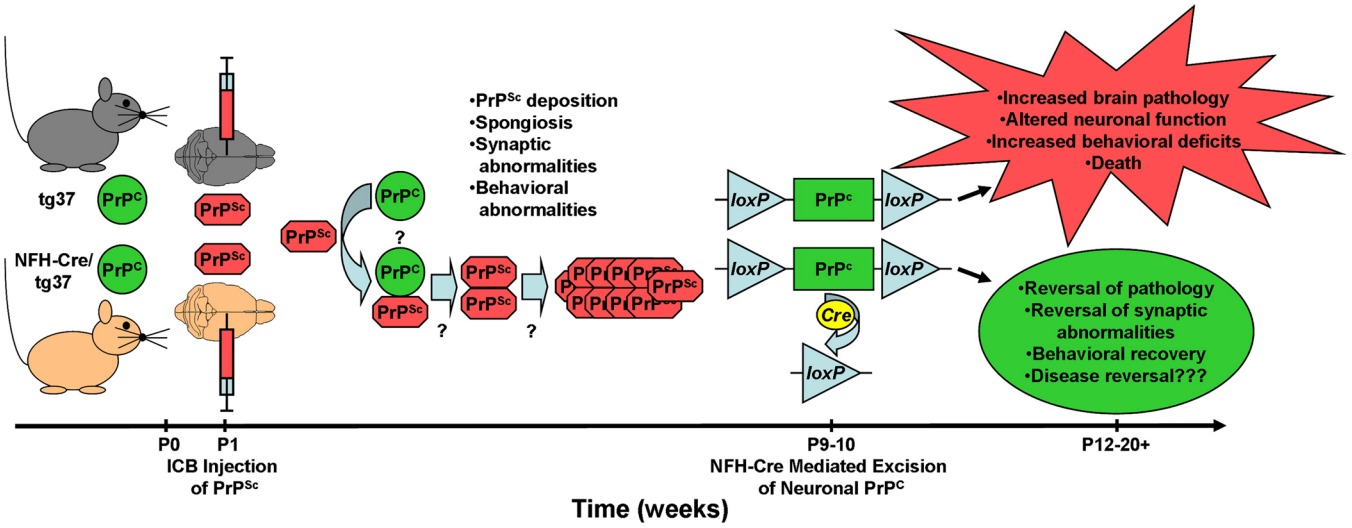

Figure 4. Postnatal excision of neuronal PrP^C reverses disease processes in prion-infected mice. Intracerebral (ICB) injection of PrP^{Sc} (Chandler/Rocky Mountain Laboratories mouse-passaged scrapie strain) in 1-week-old tg37 mice results in a stereotyped pattern of disease progression, including deposition of PrP^{Sc} and a variety of histological, synaptic, and behavioral pathologies. Cre-mediated knockout of neuronally expressed PrP^C (under the control of the murine neurofilament H control elements, NFH-Cre) at 9−10 weeks of age resulted in a halting and reversal of disease processes in these bitransgenic mice. The figure is reprinted with permission from ref 164. Copyright 2007 Elsevier.

prion disease in animals. The C-terminus of α2 of PrP is another potential site for developing anti-prion drugs, because the stabilization of the C-terminus of α2 by mutation completely inhibits misfolding of PrP.[64] Moreover, several drugs are known to bind to the C-terminus of α2 and, thereby, prevent PrP misfolding and oligomerization.[156−158]

More generally, any ligand, whether small or large, that binds to the native conformation of the protein would stabilize that state and can therefore be expected to decrease the native-state dynamics that drive misfolding. The binding energy for a ligand binding to either the native state or the misfolded monomeric intermediate would pull the equilibrium between the monomer and oligomer in the direction of the monomer. It should not be difficult to design binding partners for native PrP^C, whose high-resolution structure is well-known.[157] A high-resolution structure of the monomeric misfolded intermediate could possibly be determined by modern advanced NMR methods.[138,139] It would, however, not be advisable to design a drug that binds specifically to it, because any drug that binds to the misfolded intermediate more strongly than it does to the native conformation would pull the equilibrium between the functional native state and presumably nonfunctional misfolded intermediate in the direction of the latter. A ligand that is specific for the monomeric misfolded intermediate could, however, be used as a diagnostic agent, if it were to bind tightly enough so that it could be used at a sufficiently low concentration that would not significantly affect the concentration of functional PrP^C.

Would inhibiting misfolding of PrP *in vivo* be an effective treatment for prion diseases? The protein quality control system degrades irreversibly misfolded proteins using proteases.[159−161] Under normal physiological conditions, the rate of clearance of misfolded proteins would be higher than the rate of their formation, and hence, the misfolded proteins would not accumulate in the cell. However, under pathogenic conditions, misfolding could become higher than clearance, which would lead to the accumulation of misfolded proteins,[159−161] and to disease. Inhibition of the misfolding of a protein by drug molecules would reduce the load on the protein quality control system, and hence, the misfolded protein would eventually be degraded by proteases. Indeed, mice brain cells show clearance of PrP^{Sc} in the absence of PrP^C expression.[162] In this way, inhibiting the misfolding of a protein should be able to effectively reverse the pathogenic effects. Strikingly, the prevention of PrP misfolding in mice neurons by postnatal knockout of the *Prnp* gene using the *cre/loxP* system not only prevented disease but also resulted in the reversion of pathogenic effects (Figure 4).[14,163,164] Interestingly, mice lacking endogenous PrP do not get prion disease upon being infected with PrP^{Sc}.[165] Moreover, mice expressing a thermodynamically stabilized mutant form of PrP^C, which shows a reduced level of misfolding *in vitro*, are much more resistant to infection with PrP^{Sc}.[166] Hence, the misfolding of the endogenous PrP is a key event in PrP pathogenesis. In the absence of endogenous misfolding of PrP, PrP^{Sc} would get cleared by the protein quality control system, and hence, a reversal of pathogenic effects would be seen.

■ SUMMARY AND OUTLOOK

In the past decade, great progress has been made in understanding the role of misfolded oligomers in prion disease. Consequently, developing an understanding about the molecular mechanism of misfolded oligomer formation by PrP has become a research priority. Although several low-resolution structural studies of oligomers have been conducted, high-resolution structures of the misfolded oligomers of PrP have yet to be obtained. Obtaining high-resolution structures of PrP oligomers would be one of the most important steps toward understanding prion pathogenesis. Despite the lack of high-resolution structures of misfolded oligomers, tremendous progress has been made toward understanding the molecular mechanism of PrP misfolding. The current understanding of the molecular mechanism of misfolded oligomer formation could provide a template for developing anti-prion drugs. Because the prevention of conversion of PrP^C into PrP^{Sc} in neurons can prevent disease progression and reverse early degenerative changes, developing drugs that could inhibit conversion of PrP^C into PrP^{Sc} would be a major step toward treating prion diseases.

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ABBREVIATIONS

PrP^{C}, cellular prion protein; PrP^{Sc}, scrapie PrP; PrP, recombinant prion protein; moPrP, mouse prion protein; *Prnp*, prion protein gene; GPI, glycosylphosphatidylinositol; NTD, N-terminal domain; CTD, C-terminal domain; α1, helix 1; α2, helix 2; α3, helix 3; β1, β-strand 1; β2, β-strand 2; Tg, transgenic; TSEs, transmissible spongiform encephalopathies; CJD, Creutzfeldt-Jakob disease; FFI, fatal familial insomnia; GSS, Gerstmann-Straussler-Scheinker; PK, proteinase K; sCJD, sporadic CDJ; NMDA, *N*-methyl-D-aspartate; MD, molecular dynamics; HDX, hydrogen−deuterium exchange; MS, mass spectrometry; NMR, nuclear magnetic resonance; FRET, Förster resonance energy transfer; ICB, intracerebral.

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