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Review

Prion protein oligomer and its neurotoxicity

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The prion diseases, also known as transmissible spongiform encephalopathies, are fatal neurodegenerative disorders. According to the 'protein only' hypothesis, the key molecular event in the pathogenesis of prion disease is the conformational conversion of the host-derived cellular prion protein (PrP^{C}) into a misfolded form (scrapie PrP, PrP^{Sc}). Increasing evidence has shown that the most infectious factor is the smaller subfibrillar oligomers formed by prion proteins. Both the prion oligomer and PrP^{Sc} are rich in β-sheet structure and resistant to the proteolysis of proteinase K. The prion oligomer is soluble in physiologic environments whereas PrP^{Sc} is insoluble. Various prion oligomers are formed in different conditions. Prion oligomers exhibited more neurotoxicity both *in vitro* and *in vivo* than the fibrillar forms of PrP^{Sc}, implying that prion oligomers could be potential drug targets for attacking prion diseases. In this article, we describe recent experimental evidence regarding prion oligomers, with a special focus on prion oligomer formation and its neurotoxicity.

Keywords prion; oligomer; neurotoxicity; transmissible spongiform encephalopathies

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Introduction

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are a group of fatal neurodegenerative disorders that can infect both animals and humans, including Creutzfeldt–Jakob disease, kuru and fatal familial insomnia in humans, bovine spongiform encephalopathy in cattle, scrapie in sheep, and chronic wasting disease in deer and elk [1–6]. The key molecular event in the pathogenesis of prion diseases is the conformational conversion of the host-derived PrP^{C} (soluble cellular prion protein) into a misfolded form PrP^{Sc} (insoluble scrapie PrP) [1]. Until now, the pathologic mechanism of the prion disease is still unclear. According to literature reports, misfolded protein aggregates are involved in no fewer than 20 human diseases, collectively called protein misfolding disorders (PMDs). They include highly prevalent neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's diseases [7–9]. Although the proteins in PMDs are evolutionarily or structurally unrelated, the structural characteristics of the misfolded forms are highly similar and they share the same features including an increase of the β-sheet structure, oligomerization, and formation of fibrillar amyloid-like polymers [10–12]. Eventually, the protein aggregates become insoluble, resistant to proteolysis, and induce nerve cell death [13,14].

Usually, PrP^{Sc} is considered to be the culprit in prion diseases due to its morphological and biochemical properties [15–19]. Despite the supports of many researches, the amyloid hypothesis has been widely challenged [20]. A contradiction is that there is no evident correlation between amyloid deposits and state of the illness, but the synapse loss is obviously related to cognitive impairment [21,22]. The experimental evidence that small derivatives resulting from fibrillar PrP^{Sc} showed significant toxicity in amyloid deposits, suggested that there was another factor initiating the disorder [23,24]. The subsequent researches also indicated that monomeric PrP^{C} could be transformed into various forms of oligomers that showed neurotoxicity *in vivo* and *in vitro* [25,26]. Some work confirmed that prion oligomers were the transmissible X form [25,27]. However, interestingly, PrP^{Sc} showed little toxicity and was considered to be a defensive product in the defense system [28]. The crossing between misfolded proteins in AD and those in prion diseases has initiated a lot of controversies [29,30]. Further studies should be performed to exploit the potential relationship between AD and TSEs.

Although much progress has been made in the studies of prion oligomers, many controversies about the biochemistry

and biophysics of prion oligomers remain to be elucidated. In this review, we summarized the recent progress in the studies of the prion oligomer and its neurotoxicity.

Structures of Prion Proteins

PrP^{C} is soluble and sensitive to the proteolysis of proteinase K, whereas PrP^{Sc} is an insoluble and proteinase-resistant aggregate. Both circular dichroism (CD) and Fourier transform infrared (FTIR) spectra show that PrP^{C} adopts an α-helix-rich conformation whereas PrP^{Sc} possesses a β-sheet-rich conformation [31]. PrP^{C} is a conserved glycoprotein and mainly expressed in the central nervous system [32]. It has a single polypeptide chain of about 210 amino acid residues. The N terminus (residues 23–120) is flexible, with a highly conserved octa-repeating sequence PHGGGWGQ, whereas the C terminus (residues 121–231) is a globular structured domain encompassing three α-helices and two short antiparallel β-strands (**Fig. 1**) [33].

As PrP^{Sc} is insoluble, it is difficult to determine its three-dimensional structure by solution nuclear magnetic resonance (NMR) or X-ray crystallography techniques. Thereby the ternary structure of PrP^{Sc} is poorly understood up to now. Several structural models have been proposed to demonstrate the structural characteristics of PrP^{Sc} based on the studies of electron microscopy and atomic force microscopy [34–36], such as the β-helix model [37], the spiral model [38] and the parallel in-register β-sheet model [39,40]. All the structural models indicate that the ternary structures of PrP^{Sc} are more compact and compressed tightly with repetitive β-sheet-rich units (**Fig. 2**). Determination of the three-dimensional structure of the prion oligomer is really a huge challenge to scientists. Although the ternary structures of prion oligomers have not been exactly interpreted, the atomic structures of some analogue have been provided by X-ray crystallography [27]. The oligomer formed by the αβ crystalline protein, possesses the typical character of oligomers: β-sheet-rich structure, neurotoxicity, and recognition by an oligomer-specific antibody, illustrating a cylindering structure with six antiparallel β-strands (**Fig. 3**). Furthermore, the three-dimensional structures of some prion protein fragments have also been determined, which could be transformed into amyloid fibrils [41]. The small peptide of human PrP (residues 106–126), which is partially resistant to proteinase K, presents a high β-sheet-enriched structure, and forms amyloid fibrils *in vitro* [42,43]. The

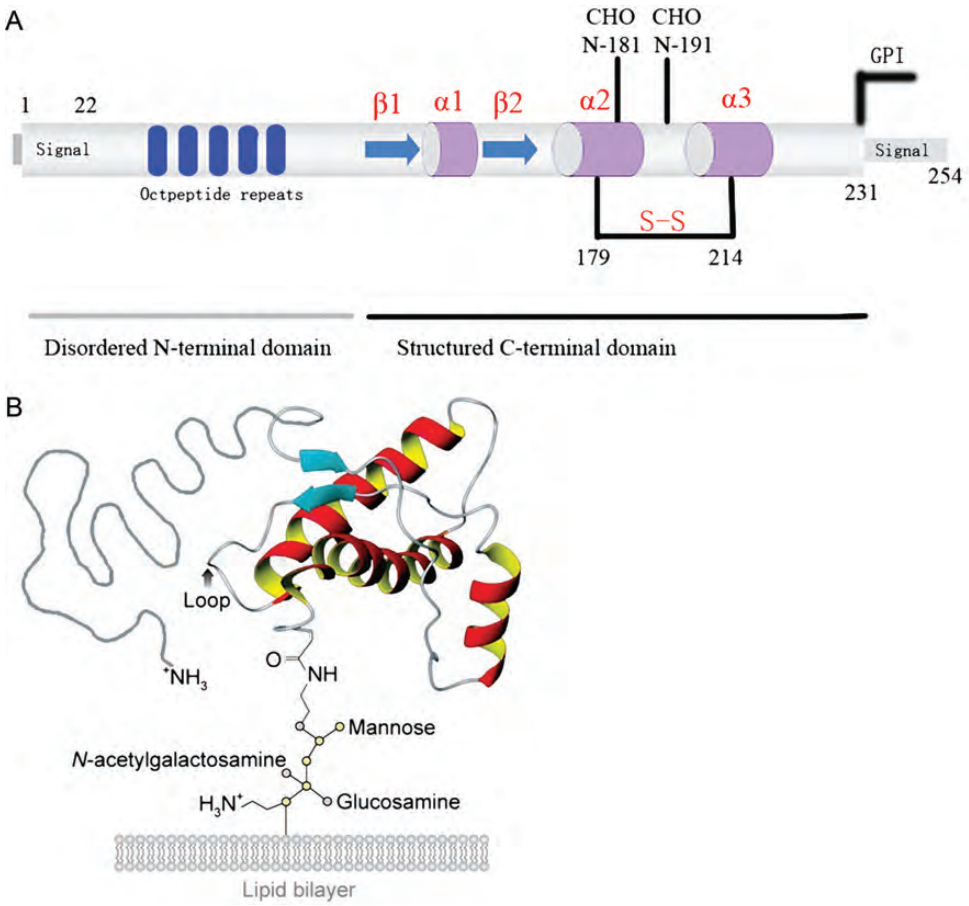

Figure 1 Structural features and biochemical properties of the cellular prion protein (A) Scheme of the primary structure of PrP^{C}. (B) Tertiary structure of PrP^{C} attached to the lipid bilayer with the GPI anchor. Figure panels are redrawn from a previous publication [4].

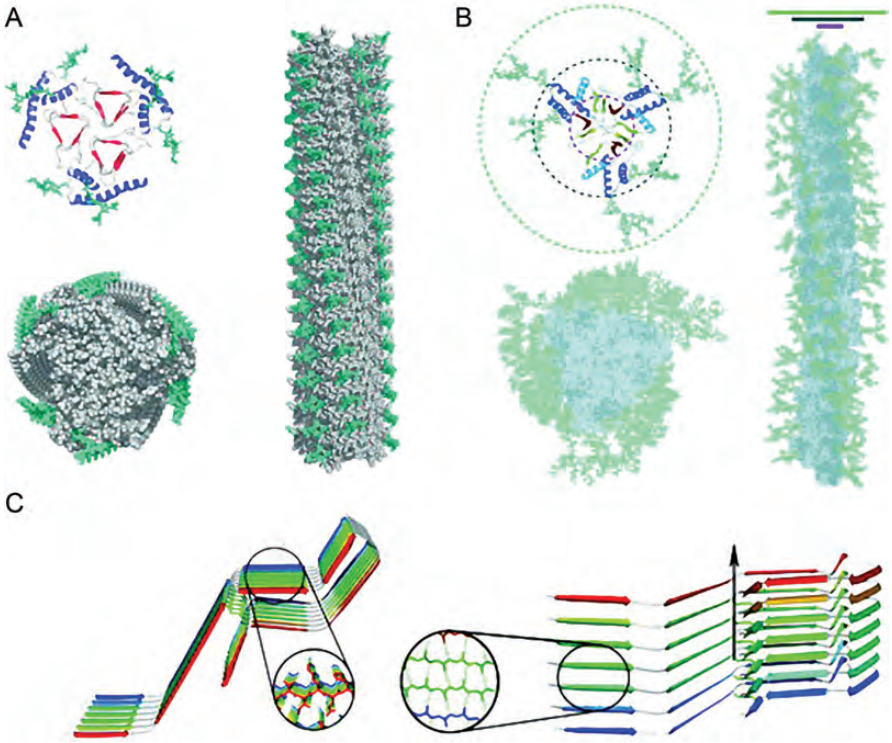

Figure 2 Structural models of PrP^{Sc} (A) The β-helix model. *Left top panel*: The residues (89–175) are transformed into the β-helical fold (red), whereas α-helix 2, 3 remain the native conformation (blue). The trimer assembles by the β-helical fold. *Bottom left panel*: Space-filling model of the trimer, the protein surface is shown in gray and sugars are shown in cyan. *Right panel*: The trimers are stacked into an assembly of a protofibril. (B) The spiral model. *Left top panel*: PrP^{Sc}-like trimer with circumferences: β-sheet core (magenta), all protein atoms (gray), and the diglycosylated protofibril (cyan). *Bottom left panel*: Space-filling model of the trimer, the protein surface is shown in gray and sugars are shown in cyan. *Right panel*: The trimers were stacked into an assembly of a protofibril. Bars at the top indicate diameters of the 35 Å extended β-core (magenta), 65 Å protein diameters (gray), and a 110 Å diglycosylated protofibril (cyan). (C) Parallel in-register β-sheet model. The arrow indicates the long axis of the fibril. Figure panels are redrawn from previous publications [37,38,40,104].

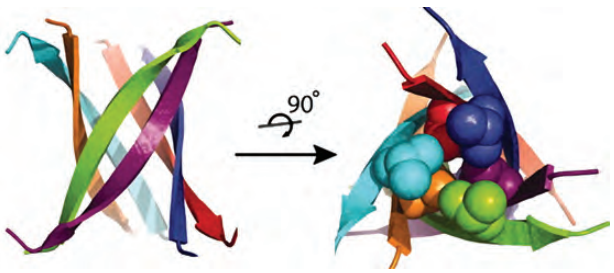

Figure 3 Crystal structure of the protein αβ crystallin oligomer Ribbon representation of the oligomeric αβ crystallin crystal structure. Pairs of strands form antiparallel dimers, which assemble around a threefold axis down the barrel axis of the oligomer. The height of the oligomer is 22 Å. The inner dimension of the oligomer, around the waist from C_α to C_α, is 12 Å, and at the splayed ends the diameter is 22 Å. Figure panels are redrawn from a previous publication [27].

secondary and quaternary structures of the fibrils have been defined with solid-state NMR spectroscopy [44]. These results indicated that the PrP peptides form in-register parallel β sheets, stack in an antiparallel manner within the mature fibril.

Although PrP^C is a conserved protein and the ternary structure is almost the same among species, there are a few mammalian species that appear to be resistant to TSEs due to the unique structural characteristic [45]. Rabbit is one of the TSEs-resistant species. However, it was recently reported that the species barrier in rabbits could be overcome by using the protein misfolding cyclic technique [46,47]. These studies suggested that it is not reasonable to attribute species-specific prion disease resistance based purely on the absence of natural cases and incomplete *in vivo* challenges. The concept of species resistance to prion disease should be re-evaluated using the new powerful tools available in modern prion laboratories, whether any other species could be at risk [47].

Recently, our laboratory has determined the solution structures of the recombinant rabbit prion protein $RaPrP^C$(91–228) and its S173N and I214V variants, and also detected the backbone dynamics of their structured C-terminal domain (121–228) using multi-dimensional NMR techniques [48,49]. Moreover, the wild-type RaPrPC protein shows a much higher structural stability compared with its S173N and I214V mutants, which was confirmed by molecular dynamics simulation [50]. Significantly, $RaPrP^C$ possesses a unique electrostatic charge distribution,

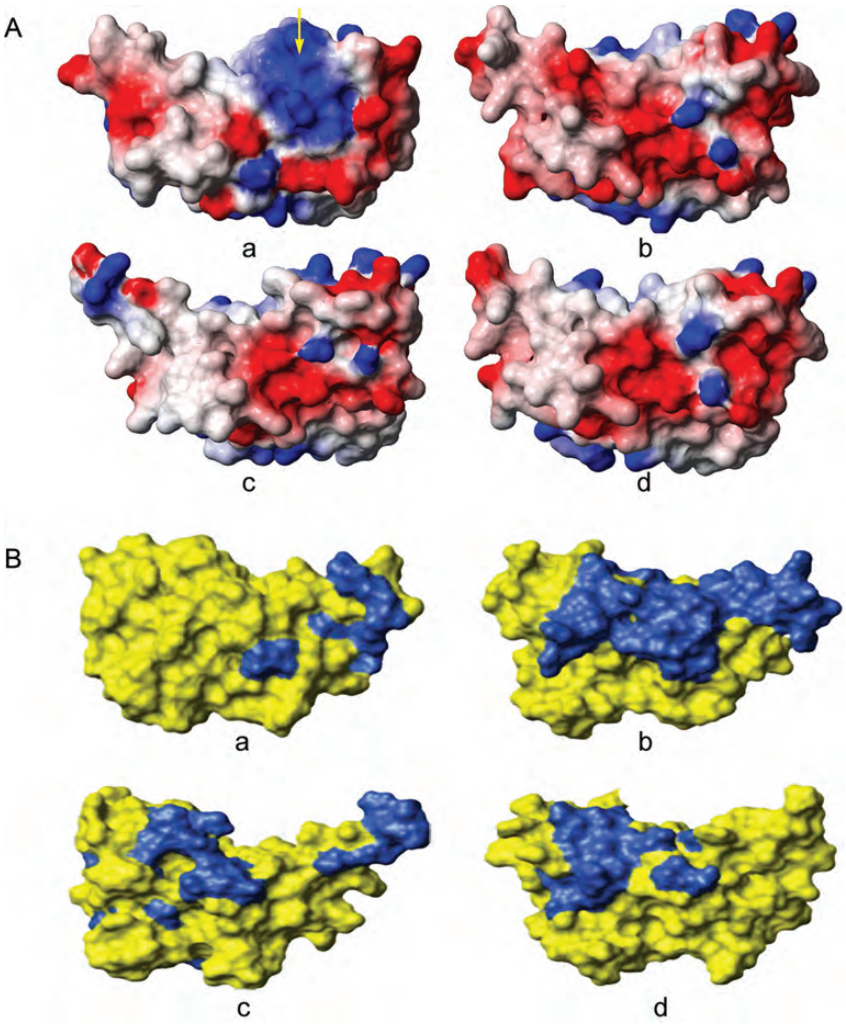

Figure 4 Distributions of electrostatic potential of PrP^C and the predicted DNA-binding sites (A) distributions of electrostatic potential of PrP^C, a: rabbit PrP^C (PDB code 2FJ3); b: human PrP^C (PDB code 1QM3); c: mouse PrP^C (PDB code 1XYX); d: bovine PrP^C (PDB code 1DWZ). Three-dimensional structures of these PrP^Cs were all determined in sodium acetate buffer at pH 4.5. Blue, positive charge; red, negative charge. (B) Molecular surface graphs evidencing the differences in the predicted DNA-binding sites (colored blue). The tab is the same as in (A). DNA-binding predictions were performed using DISPLAR. All surface graphs were generated using MolMol. Figure panels are redrawn from a previous publication [49].

carrying a continuous area of positive charge on the protein surface, which is distinguished from any other PrP^C (**Fig. 4**). Previous studies suggested that the conformational transformation of PrP^C into PrP^{Sc} was accompanied by a molecular chaperone 'Protein X', and a specific nucleic acid could bind to PrP^C and induce conformational change [51–56]. The unique distribution of electrostatic potential appearing on the $RaPrP^C$ surface is dramatically different from those on other PrP^C surfaces, which potentially makes significant contribution to protect $RaPrP^C$ from the conformational conversion. The predicted DNA-binding sites among PrP^C molecules from distinct mammalian species suggest that $RaPrP^C$ possesses a relatively small putative DNA-contacting surface [57]. The continuous positive charge on the $RaPrP^C$ surface might be a key factor that makes rabbits be resistant to TSEs.

The recent studies of ternary structures of PrP proteins lay the crucial basis for further addressing their biological functions, and may give the clue to elucidate the pathology of TSEs.

Formation of Prion Oligomers

The discovery of prion can date back to 1982, when the Nobel Prize winner Stanley B. Prusiner described prion proteins as novel proteinaceous infectious agents causing scrapie [58]. The 'protein-only' hypothesis was a challenge to the common sense at that time. In 1985, the first evidence was reported that PrP was assembled into filaments within the brain to form amyloid plaques in extracellular spaces of scrapie-infected hamsters [59]. The filaments were composed of PrP27–30 molecules, as determined

by immunoelectron microscopy using affinity-purified antibodies.

Many studies were conducted to clarify the formation of prion oligomers in different conditions with various truncated fragments. Generally, there are three recombined proteins: the full length PrP (residues 23–231), the truncated PrP (residues 91–231) that was supposed to be the core section after the proteolysis of PrP^{Sc} with proteinase K, and the truncated PrP (residues 121–231) that was confirmed to be the smallest well-structured domain. In 1997, Swietnicki *et al*. [60] discovered that conformational properties and the folding pathway of human PrP^{C} (residues 90–231) are strongly pH-dependent. In acidic solutions (pH 3.6–5.0), the urea-induced unfolding transition processes of PrP^{C} could be fitted to a three-state unfolding model by measuring the ellipticity at 222 nm as a function of denaturant concentration. However, in neutral solutions the urea-induced unfolding transition processes were fitted to a two-state unfolding model. Therefore, PrP^{C} was supposed to be transformed into an intermediate in acidic conditions. Afterwards, PrP^{C} was confirmed to form a soluble β-sheet-rich oligomer in a condition of an acidic mildly denaturing (4 M urea or 1 M guanidine hydrochloride) with a high salt concentration [61]. However, the necessity of salts has not been interpreted in details. There was a clue that the abnormal effect of salts was likely caused by the ion-induced destabilization of salt bridges (Asp^{144}–Arg^{148} and/or Asp^{147}–Arg^{151}) in the extremely hydrophilic helix 1 rather than the interaction of ions with the octa-repeating in the flexible N-terminal region of PrP^{C} [62]. The prion oligomer adopts a β-sheet-rich form as verified by CD spectrum, and shows an increased resistance to proteinase K digestion compared with monomeric PrP^{C}. Some work conducted on the reduced form of recombinant PrP^{C}(23–231), revealed four β-sheet-rich isoforms as judged by their distinct retention time in reverse-phase chromatography [63,64]. In contrast, another work demonstrated that both PrP^{C} and PrP^{Sc} maintain a disulfide bridge [65]. The three-dimensional structure of PrP^{C} illustrates that the disulfide bridge exactly exists in the stable state of PrP^{C} [48,49,66].

In 2002, Baskakov *et al*. [67] studied the mouse and Syrian hamster PrPs and found that the recombinant PrPs (recPrPs) were able to form two structurally distinct non-native isoforms: the β-oligomer form and the amyloid isoform. At acidic pH, recPrPs were converted into β-oligomers; while at neutral or slightly acidic pH, recPrPs were converted into the amyloid isoform. Analysis of both dynamic light scattering and electrospray ionization tandem-mass spectrometry indicated that the radius of the β-oligomer was 6.5 nm with an average molecular mass of 300 kDa. Although it was suggested that the β-oligomer was not the precursor of the amyloid isoform and its construction was different from the amyloid isoform, more direct evidence was needed to elucidate the relevance between the β-oligomer and the amyloid fibril. In addition, an obvious difference between the oligomer and the amyloid fibril is the ability to bind Thioflavin T [68,69]. The experimental results indicated that the surface of the oligomer had more hydrophilic residues than that of the amyloid fibril. Many studies have been performed to explore the relation between the prion oligomer and PrP^{Sc} [70,71].

Whether the conformational conversion of monomeric PrP^{C} into the β-sheet-rich oligomer is reversible is another question that remains enigmatic. Rezaei *et al*. [72] pointed out that the oligomerization pathway of the full-length recombinant ovine PrP is irreversible. They used heat-induced unfolding process instead of the mildly denaturing condition to assess the conformational conversion. Interestingly, two distinct oligomers (12 mer and 36 mer) were formed from monomeric PrP^{C} in the heat induced-unfolding process. The two oligomers possessed different secondary and quaternary structures, as confirmed by the different binding sites of the corresponding antibody. Moreover, results from the analysis of pressure-jump ^{1}H-NMR spectroscopy suggested that the monomer–oligomer transition was reversible [73]. In general, the prion oligomers could be formed either in the heat-induced unfolding process or under an acidic mildly denaturing condition (4 M urea or 1 M guanidine hydrochloride), together with the high salt concentration. These formed oligomers are stable and the transition is irreversible.

Recently, more and more studies were conducted to assess the difference among various protein oligomers and understand the diversities of the oligomers [74–77]. One of the latest 'coups de theatre' in the amyloid history is the observation that PrP^{C} was a high-affinity cell-surface receptor for soluble Aβ oligomers on neurons and is a mediator of Aβ oligomers-induced synaptic dysfunction [78]. This hypothesis, however, has been challenged by several researches [78–80], and has become a highly controversial issue that is still far from being settled [81]. Now it is widely believed that the protein oligomers are various and different from each other, and one specific oligomer could be transformed into the corresponding amyloid fibril in the lasting denaturing condition [28,82,83]. Determination of the core structures of the oligomers is the key to explore the pathologic mechanism of conformational transition [76,77].

PrP Oligomerization Proceeds *via* a Molten Globule Intermediate

In the slight acidic or neutral solution, PrP undergoes two-state change (a normally native-form to denatured-form) on the unfolding and refolding way in the degeneration experiments. However, in the acidic buffer PrP may experience an 'intermediate' state on the unfolding and

refolding way [64,65,84]. As the transient intermediate was hardly observed, the fluorescence titration experiment was the early primary method to detect the kinetics of folding and unfolding reactions for the recPrP [85]. The intermediate showed an increased absorption value compared with the native state of PrP^C, and a decreased absorption value compared with the oligomers in the particular condition [60,86,87]. It has been confirmed that the intermediate exactly exists in the acidic solution with mild denaturant, providing an important clue to explain the pathologic way of the prion diseases. Nevertheless, the intermediate is hard to be captured and its structural property remains unclear.

Early studies of the recPrP suggested that the conformation conversion of monomeric PrP^C into β-sheet-rich oligomers might go through a compatible intermediate with a preformed β-sheet subunit structure [60,87]. On the contrary, far-UV CD results demonstrated that the intermediate adopted an almost intact α-helical organization, termed as a molten globule state [70]. The near-UV CD results, which provided a qualitative measure of the tertiary structure of the protein, illustrated distinct difference between the native PrP^C and the molten-PrP^C. These near-UV CD spectra display signal loss for the molten-PrP^C, whereas the native PrP^C is clearly highly organized [70]. The two-dimensional NMR spectra also exhibit signal loss of high field methyl protons for the molten-PrP^C when compared with native PrP^C, implying a loss of the tertiary structure [70]. These data clearly identified an intermediate state (a molten globule state), which was partially unfolded, monomeric, and on the pathway to form the β-oligomer (**Fig. 5**).

Studies of molten globule states were performed to understand the intermediate states of folding and unfolding of proteins [86]. Multiparametric analyses of equilibrium folding and unfolding of several globular proteins allowed the classification of the intermediates between the native and unfolded states [88–95]. The general properties of these intermediates include a pronounced secondary structure, a high compactness without a rigid packing inside a molecule, and substantially increased fluctuations of side chains and larger parts domain. Owing to these particular structural properties, this class of intermediate states has been defined as the 'molten globule' state.

Several studies have explored the biological significances of the molten globule intermediate in the process of conformation transition [96,97]. The molten globule state provides a clue that the intermediate of the oligomer or PrP^{Sc} is a thermodynamically stable intermediate [86], which might account for the fact that in refolding and unfolding experiments, heat-induced oligomers were more stable than the urea-induced oligomers [98]. Actually, there was an energy barrier in the first step of the pathway of PrP^C converting into PrP^{Sc}. Although the CD measurement of the molten globule state showed the α-helix-rich secondary structure,

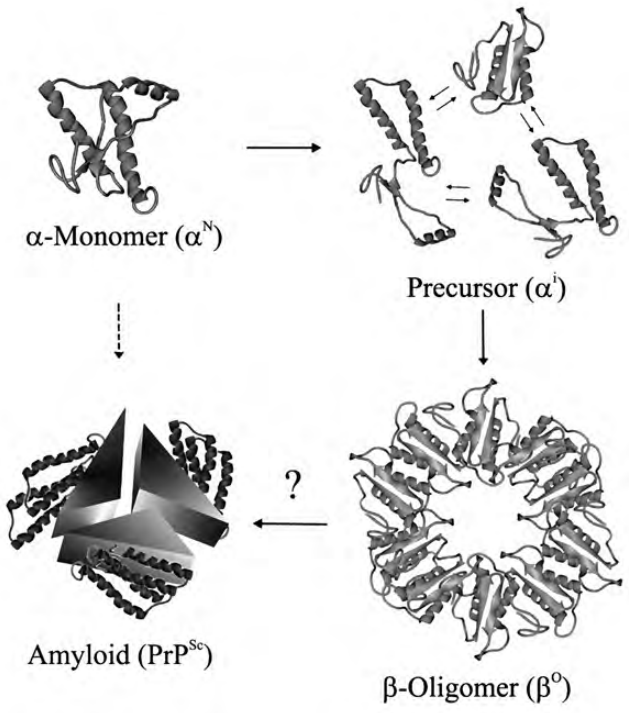

Figure 5 Schematic representation of the folding pathways of recombinant human prion protein *in vitro* PrP^C is first transformed into the molten globular state, which is the precursor of the oligomer. Then the oligomer is converted into PrP^{Sc}. The schematic diagram is inferred but not confirmed. Figure panels are redrawn from a previous publication [70].

β-sheet transition was also detected with the more sensitive β-sheet probe ANS that could bind to the intermediate. Gerber *et al*. [70] reported that 2D NMR spectra recorded on the intermediate and oligomer were similar. As the oligomer is too large to be directly observed, it could be expected that understanding the construction of the molten globule intermediate would be helpful to elucidate the structural features of the β-sheet misfolded isoform.

Neurotoxicity of Oligomers *in vitro* and *in vivo*

Recent studies indicated that the formation of large amyloid fibrils might be a protective process: the neurotoxic subfibrillar oligomers grow into relatively innocuous fibrils [99,100]. Silveira *et al*. [25] first reported that the non-fibrillar particles, with masses equivalent to 14–28 PrP molecules, were the most efficient initiators of prion diseases. Their results showed that the infective and converting active particles were ~17–27 nm (300–600 kDa). Rather than expressing the recPrP in bacterial systems, they purified PrP^{Sc} from scrapie-infected (263K strain) hamster brain and treated with proteinase K to produce a product of >90% purity [101]. To break down the large PrP^{Sc} aggregates into a range of smaller particles for evaluating the activity, they

treated PrP^{Sc} with various detergents and sonication. Their work demonstrated that sodium N-undecyl sulfate (SUS) treatment was better than sodium dodecyl sulfate treatment by comparing the levels of converting activity. The method of flow field-flow fractionation was used to fractionate the SUS-treated oligomers according to the size. The dot-blot-based solid-phase conversion assays were used to access the converting activity of different fractionation. Their results illustrated that the 14–28 mer oligomers had the most converting activity. In contrast, it was demonstrated that the oligomers formed by recPrPs possessed the almost identical neurotoxicity *in vitro* and *in vivo* [26]. These results showed that β-sheet-rich oligomers rather than α-helix-rich monomers or amyloid fibrils were toxic to cortical neurons in culture [102].

Recently, Kudo *et al.* [103] found PrP^{C} is involved in the oligomeric amyloid-β-induced neuronal cell death. They showed that $Prnp^{-/-}$ mice were resistant to the neurotoxic effect of the Aβ oligomer *in vivo* and *in vitro*. The anti-PrP^{C} could prevent the Aβ oligomer-induced neurotoxicity. These results demonstrated that PrP^{C} mediated the process of Aβ oligomer-induced neuronal cell death (**Fig. 6**). Moreover, the antibody 6D11 binding to PrP^{C}(93–109), could prevent neuronal cell death induced by the Aβ oligomer. Contrarily, another antibody 6H4 binding to PrP^{C}(144–152), failed to block the process. These results indicated that the residues 93–109 might be responsible for binding the Aβ oligomer and inducing the neurotoxicity in cells. Lauren *et al.* [56] showed that antibody binding this region could prevent the interaction of PrP^{C} with the Aβ oligomer and stop the Aβ oligomer-induced synaptic dysfunction. These observations strongly support the hypothesis that PrP contributes to neurotoxic signaling induced by Aβ oligomers, and mediates neuronal cell death.

Conclusion

The prion oligomer adopts a soluble, neurotoxic, β-sheet-rich form. More and more evidence demonstrated that the oligomers are the most infectious prion protein particles. Although there is no controversy on the neurotoxicity of the oligomers, the exact pathologic mechanism remains unclear. Furthermore, the pathogenetic mechanisms of prion diseases and other PMDs such as AD and PD are vague. The latest 'coups de theatre' in prion diseases is the observation that PrP^{C} is a high-affinity cell-surface receptor for soluble Aβ oligomers on neurons, and is also a mediator of Aβ oligomers-induced synaptic dysfunction. This discovery would promote the study regarding the biological function of PrP^{C} *in vivo*.

Since amyloid fibrils were supposed to be the pathogen in prion diseases, scientists have been considering amyloid fibrils as the drug target [104]. Dozens of lead compounds have been developed with the aim to stop the assembling of amyloid proteins in the brain. However, 10 years has past with little advance in the healing of the class of misfolding diseases. Almost all the drugs in clinical use were announced failure in the curative effect to AD, PD, or prion diseases. The toxic assays show that the most pathogenic factors in prion diseases are prion oligomers. The same phenomenon occurred in the researches of AD, and the results showed that the soluble Aβ oligomer is the crucial factor responsible for neuronal synapses. All the evidence indicated that protein oligomers possess the most significant neurotoxicity and would be the potential drug targets.

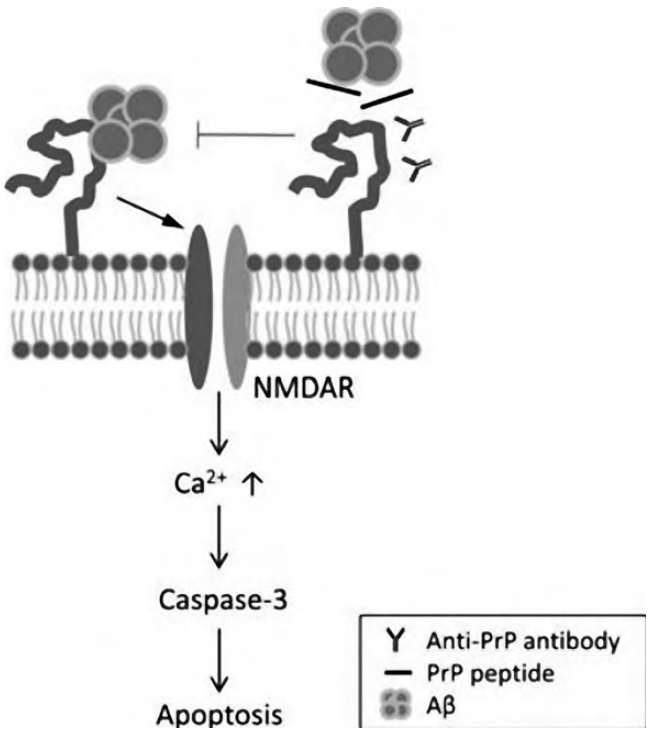

Figure 6 Hypothetical model of PrPC/Aβ oligomer induced neurotoxicity The Aβ oligomer binding to PrP^{C} at cytomembrane activates the metabolic pathway of caspase-3-dependent neuronal cell death. Treatment with anti-PrP^{C} antibody or competitive PrP^{C} peptides prevents the activation of pathway, suggesting that PrP^{C} is essential for the Aβ oligomer induced neurotoxicity. Figure panels are redrawn from a previous publication [103].

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