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**Short Communication**

# An Engineered $PrP^{sc}$-like Molecule from the Chimera of Mammalian Prion Protein and Yeast Ure2p Prion-inducing Domain

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**Abstract** Production of the pathogenic prion isoform $PrP^{sc}$-like molecules is thought to be useful for understanding the mysterious mechanism of conformational conversion process of prion diseases and proving the "protein-only" hypothesis. In this report, an engineered $PrP^{sc}$-like conformation was produced from a chimera of mammalian bovine prion protein (bPrP) and yeast Ure2p prion-inducing domain (UPrD). Compared with the normal form of bPrP, the engineered recombinant protein, termed bPrP-UPrD, spontaneously aggregated into ordered fibrils under physiological condition, displaying amyloid-like characteristics, such as fibrillar morphology, birefringence upon binding to Congo red and increased fluorescence intensity with Thioflavine T. Limited resistance to protease K digestion and CD spectroscopy experiments suggested that the structure of bPrP-UPrD had been changed, and adopted a new, high content β-sheet conformation during the fibrils formation. Moreover, bPrP-UPrD amyloid fibrils could recruit more soluble forms into the aggregates. Therefore, the engineered molecules could mimic significant behaviors of $PrP^{sc}$ and will be helpful for further understanding the mechanism of conformational conversion process.

**Key words** yeast Ure2p prion-inducing domain; $PrP^{sc}$-like molecule; conformational conversion

Prions are infectious agents causing progressive neurodegenerative disorders, such as scrapie, mad cow disease and Creutz-Jakob disease [1,2]. All of these disorders are characterized by cerebral accumulation of an abnormal protein, designated $PrP^{sc}$, which has a strong tendency to aggregate into insoluble amyloid fibrils. The covalent structure of $PrP^{sc}$ is most likely identical with its normal cellular form of $PrP^{c}$, but their conformations and physical properties are completely different. $PrP^{c}$ exists as α-helix rich structure, which is highly sensitive to protease K digestion, whereas $PrP^{sc}$ forms insoluble aggregates with ordered β-sheet rich structure and often shows amyloid characteristics with increased resistance to protease K digestion [3]. According to the "protein-only" hypothesis, $PrP^{sc}$ can act as seeds to mediate the formation of more $PrP^{sc}$ from host $PrP^{c}$.

A well-known model of exploring the mechanism of prion polymerization is yeast prion Ure2p or Sup35p. Both of them contain the short Gln/Asn-rich domain that plays a critical role in the conformational transition, termed prion-inducing domain (PrD). Ure2p- and Sup35p-PrD are both modular and transferable [4]. The green fluorescent protein (GFP) and rat glucocorticoid protein, when fused to the Sup35p-PrD, were found to exist in different heritable functional states in yeast and could be switched by experimental conditions [5,6]. The Ure2p-PrD (UPrD) could induce self-propagating amyloid formation of Ure2p *in vitro* [7].

Keen interests have been shown in producing $PrP^{sc}$-like molecules *in vitro* to better know the conformational conversion process of prion disease which remains unclear until now. Recombinant prion protein could be transformed into β-sheet oligomers from an α-helix conformation under acidic conditions (pH 3.6–5) in the presence of low concentrations of detergents [8]. Some factors, such as copper [9], nucleic acids [10] and

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$PrP^{c}$-related peptides [11], can also induce the conformational change and polymerization.

In the present study, a $PrP^{sc}$-like conformation was obtained from the chimera of bovine prion protein and yeast Ure2p prion-inducing domain. The engineered molecule had acquired all the significant characteristics of $PrP^{sc}$, such as amyloid fibril structure, increased resistance to protease digestion, and self-induced aggregation, thus providing another approach to study the "mysterious" conformational transition process of prion diseases.

## Materials and Methods

### Plasmids construction

The gene fragment corresponding to the putative mature bovine $PrP^{c}$ 25–242 residues (bPrP) was amplified with primers: 5′-GGCATATGAAGAAGCGACCAAAACCTG-3′ (upstream); 5′-GGGAATTCACTTGCCCCTCGTTGGTA-3′ (downstream).

The above PCR product was cloned between restriction sites *Nde*I and *Eco*RI of the vector pET30a(+) (Novagen), termed pETbPrP. The gene fragment corresponding to the prion-inducing domain Ure2p 1–65 residue was amplified from the genomic DNA extracted from the phenotype [URE3] of *Saccharomyces cerevisiae* (strain 3687) with primers: 5′-GGGAATTCAATAACAACGGCAACC-3′ (upstream); 5′-GGCTCGAGTTAGCGGCCGCTGTTATTG-3′ (downstream).

The PCR product was cloned between restriction sites *Eco*RI and *Xho*I of the recombinant vector pETbPrP, termed pETbPrP-UPrD. The right in-frame fusion around the *Eco*RI site was confirmed by DNA sequencing.

### Protein preparation and amyloid fibrils formation

The fusion protein bPrP-UPrD was over-expressed in *E. coli* as inclusion bodies. Its purification and refolding were performed as described [12] using nickel-nitrilotriacetic acid (NTA) agarose (Qiagen) chromatography. Similar procedures were used to obtain recombinant protein bPrP. For amyloid fibrils formation, the renatured bPrP-UPrD (>1 g/L) in 20 mM NaAc, 150 mM NaCl, pH 5.5 was incubated at 37 ℃ with constant rotation (80 r/min) overnight.

### Electron microscopy and staining assay

Samples (5 μl) of bPrP-UPrD amyloid fibrils were absorbed to glow discharged carbon-coated copper grids. They were washed twice with deionized water, negatively stained with 2% uranyl acetate for 30 s, air-dried after removing excess liquid, and were visualized under a Hitachi H600A electron microscope. For Congo red (Sigma) staining, samples were prepared as described [13] and observed on a Zeiss photomicroscope equipped with cross polars. Thioflavine T (ThT, Molecular Probes) binding was recorded on a Shimadzu RF-540 spectrofluorimeter at 450 nm. The emission intensity at maximum (482 nm) was monitored as described previously [14]. Recombinant bPrP aggregates from denaturalization were used as the control in these experiments.

### Protease K resistance assay

Experiments were performed as described [13]. Briefly, about 10 μg of bPrP-UPrD amyloid fibrils were suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 0.8 M urea. Protease K (Sigma) was added to 1 μg/ml and incubated at 37 ℃. The equal quantities of bPrP-UPrD amyloid fibrils were denatured by 8 M urea and diluted 10 fold by TE buffer. The aggregates from dilution were used as the control. At interval time, aliquots were removed and boiled for 10 min. Digestion products were analyzed by 16% SDS-PAGE.

### Circular dichroism (CD) spectroscopy

Far-UV CD spectra were recorded at 25 ℃ on a Jasco J-715 spectropolarimeter with a quartz well of 0.2-cm length. Amyloid fibrils samples were briefly sonicated before measurement.

### SDS-PAGE analysis of the interaction between bPrP-UPrD amyloid fibrils and their soluble forms

Freshly prepared bPrP-UPrD or bPrP with the same protein concentration (0.5 g/L) was added with variant volumes of suspended bPrP-UPrD amyloid fibrils (1 g/L each) and incubated at room temperature with constant rotation (80 r/min) overnight. After centrifugation at 20,000 *g* for 30 min, 10 μl of each supernatant was subjected to SDS-PAGE analysis.

## Results

### bPrP-UPrD spontaneously formed amyloid fibrils *in vitro* under physiological conditions while bPrP remained stable

The aggregation of bPrP-UPrD in 20 mM NaAc, 150 mM NaCl, pH 5.5 was in a concentration-dependent manner. After soluble bPrP-UPrD (>1 g/L) was incubated at room temperature overnight, the insoluble protein aggregates were seen. When examined by electron

microscopy, the negatively stained aggregates appeared as fibrils of 10–20 nm in diameter, and 100–300 nm in length. Some fibrils twisted around each other [Fig. 1(A)]. The diameter of bPrP-UPrD fibrils was comparable to that of fibrils formed by the infectious prion isoform $PrP^{sc}$ [15]. The observed bPrP aggregates showed no regular structures [Fig. 1(B)]. The amyloid specific dyes Congo red and ThT were further used to identify bPrP-UPrD amyloid fibrils. Fig. 2(A) showed the optical microscopy image of bPrP-UPrD aggregates stained with Congo red under cross-polarized light. The character of red-yellow birefringence is corresponding to β-sheet rich regions in amyloid fibrils. The stained bPrP aggregates have no special optical properties [Fig. 2(B)]. When amyloid fibrils were added into the ThT solution, the fluorescence emission intensity at 482 nm increased about 16 fold compared with that of bPrP aggregates (Fig. 3). On the contrary, renatured bPrP remained stable under the same conditions without any precipitation. It could not produce obvious changes on the fluorescence spectra of ThT (Fig. 3).

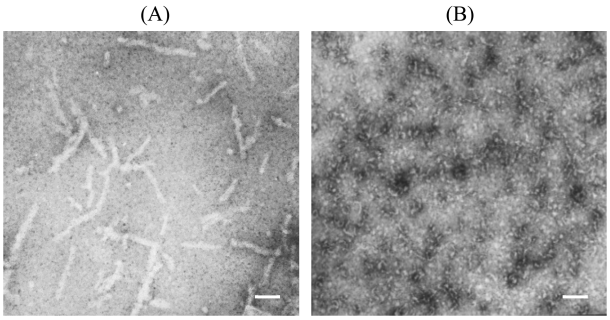


**Fig. 1 Electron micrograph of bPrP-UPrD amyloid fibrils**

Electron micrographs of negatively stained bPrP-UPrD amyloid fibrils (A) showed fibrous shapes whereas bPrP aggregates (B) had no regular structures. Scale bar=100 nm.

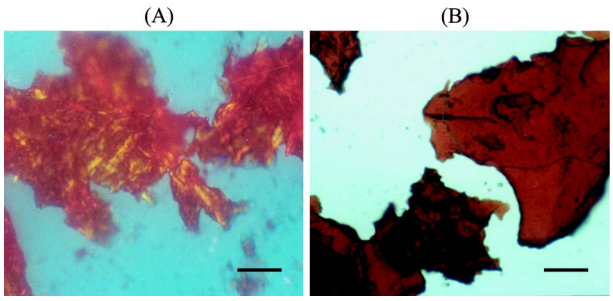


**Fig. 2 Optical microcopy image of bPrP-UPrD amyloid fibrils stained with Congo red under cross-polarized light**

bPrP-UPrD amyloid fibrils (A) stained with Congo red showed red-yellow birefringence, whereas bPrP aggregates (B) had no optical characters. Scale bar=25 μm.

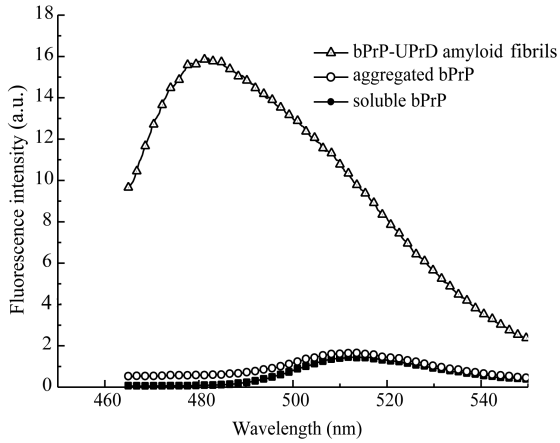


**Fig. 3 Fluorescence spectra of ThT in the presence of bPrP-UPrD amyloid fibrils**

The emission spectrum of ThT in the presence of bPrP-UPrD amyloid fibrils showed increased fluorescence intensity at 482 nm whereas the spectrum in the presence recombinant bPrP or their aggregates

## Limited proteolysis of bPrP-UPrD amyloid fibrils

To test whether the *in vitro* generated amyloid fibrils had increased protease resistance, we subjected the following two samples to protease K digestion. (1) Fibrils were precipitated from an aged bPrP-UPrD solution and resuspended in TE buffer containing 0.8 M urea. (2) The same quantities of fibrils were denatured in 8 M urea and then diluted 10 fold by TE buffer. The amorphous aggregation from the dilution process served as the control. The fusion protein of about 33 kD was detected for amyloid fibril forms [Fig. 4(A)] even after 30 min of protease K digestion. The corresponding protein for amorphous forms [Fig. 4(B)] was completely digested in the first 10 min. A set of fragments of about 26 kD in the digestion profile of fibril forms was more resistant to proteolysis compared with that of amorphous forms. This difference further illustrated the fact that amyloid fibrils possess ordered structures compared with those of amorphous forms.

## Spectroscopic analysis of soluble and aggregated bPrP-UPrD proteins

Fig. 5 showed the CD spectra of soluble and aggregated forms of bPrP-UPrD proteins. The spectrum of soluble bPrP-UPrD displayed two minima at 208 and 222 nm, characteristic of the presence of mainly α-helical structure.

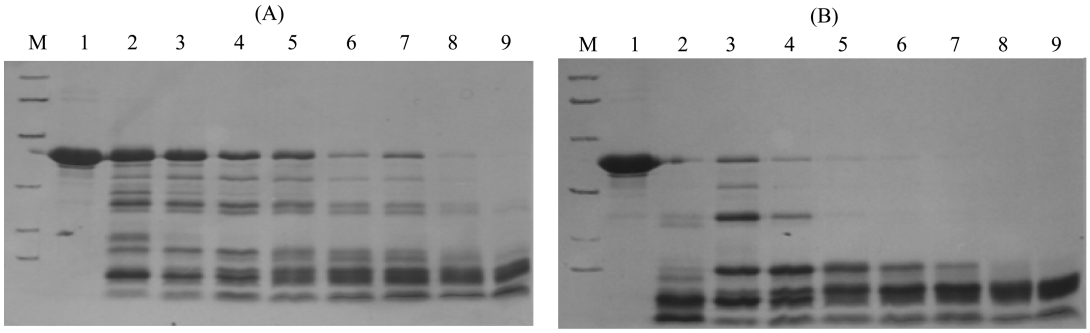


**Fig. 4 Protease K digestion of bPrP-UPrD amyloid fibrils and of amorphous aggregates**

Amyloid fibril forms (A) and amorphous forms (B) with equal protein quantities (10 μg) were subjected to protease K (1 μg/ml) digestion for variant time, then reaction was stopped by boiling and the digested products were analyzed by SDS-PAGE. Lane M shows the molecular weight markers (from top to bottom) of 14.4, 20, 31, 43, 66.2, 97 kD. The protease K digestion time (min) is indicated above each gel. M, molecular marker; 1–9, PK digestion for 0, 2, 4, 6, 10, 15, 20, 30 and 40 min respectively.

The spectrum of aggregated bPrP was radically different. The two spectra minima at 208 and 222 nm disappeared and a single minimum around 215 nm appeared.

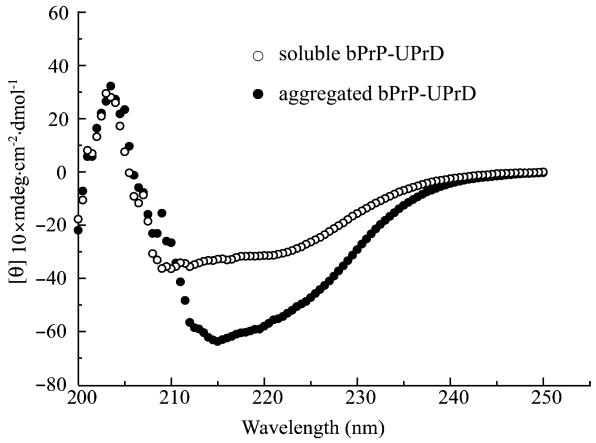


**Fig. 5 Circular dichroism of soluble and aggregated bPrP-UPrD**

The CD spectrum of freshly prepared sample (0.1 g/L) is shown in open diamonds. The CD spectrum of aged aggregated sample (0.5 g/L) is shown in filled diamonds.

### bPrP-UPrD amyloid fibrils could specifically induce aggregation of their soluble forms

To investigate the interaction between bPrP-UPrD amyloid fibrils and their soluble forms, we divided the freshly prepared bPrP-UPrD and bPrP respectively into equal aliquots and incubated with variant volumes of suspended bPrP-UPrD amyloid fibrils. Under parallel incubation conditions, the changes of protein concentrations in supernatants would reflect the recruitments of soluble forms into aggregations. Fig. 6(A) showed that soluble bPrP-UPrD concentration decreased when added more aged fibrils. However, recombinant bPrP remained soluble in spite of adding more fibrils [Fig. 6(B)].

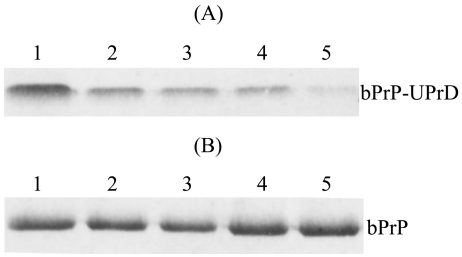


**Fig. 6 SDS-PAGE analysis of the aggregations of bPrP-UPrD and bPrP in the presence of bPrP-UPrD amyloid fibrils**

Freshly prepared bPrP-UPrD (A) and bPrP (B) with equal concentrations (0.5 g/L) in the presence of various volumes of suspended bPrP-UPrD amyloid fibrils (1g/L). 1–5, the volume of bPrP-UPrD amyloid fibrils are 0, 20, 40, 60 and 80 ul respectively.

## Discussion

Ure2p- and Sup35p-PrD retain all the prion properties, but the similar domains have not been found in mammalian prions. To enhance the propensity of mammalian prions for conformational change, we have designed a new prion-like protein by fusing UPrD to the C-terminal of bPrP. Compared with the normal recombinant bPrP, the engineered molecules spontaneously aggregated into amyloid fibrils under physiological conditions, as evidenced from electron microscopy, Congo red and ThT binding assays. Protease K limited digestion experiments showed the aggregated fusion protein had obtained increased

resistance to proteolysis. This suggested that the structure of bPrP-UPrD had been changed during the aggregation. CD experiments further revealed that it obtained a high content of ß-sheet conformation. SDS-PAGE analysis showed that bPrP-UPrD amyloid fibrils could recruit more soluble bPrP-UPrD into the aggregated deposits, but had no effect on recombinant bPrP. These findings suggested that bPrP-UPrD amyloid fibrils were not inert. They could specifically induce their soluble forms into aggregations.

The engineered $PrP^{sc}$-like conformation might shed a new light on prion research. Firstly, it can mimic mammalian $PrP^{sc}$ in two critical steps during the pathological process: spontaneous amyloid fibrils formation and self-induced aggregation. As the structural change of bPrP-UPrD occurs easily, it provides an attractive and tractable system for exploring the conformational transition mechanism of prion protein. Whether the conformation of PrP part in bPrP-UPrD amyloid fibrils is similar or equal to the infectious $PrP^{sc}$ remains further to be investigated. Secondly, the conformation of the engineered protein more prefers to change. This makes it an interesting candidate for proving the "protein-only" hypothesis.

## References


1 Prusiner SB, Scott MR, DeArmond SJ, Cohen FE. Prion protein biology. Cell, 1998, 93: 337–348

2 Prusiner SB. Molecular biology and pathogenesis of prion diseases. Trends Biochem Sci, 1996, 21: 482–487

3 Meyer RK, McKinley MP, Bowman KA, Braunfeld MB, Barry RA, Prusiner SB. Separation and properties of cellular and scrapie prion proteins. Proc Natl Acad Sci USA, 1986, 83: 2310–2314

4 Tuite MF. Yeast prions and their prion-forming domain. Cell, 2000, 100: 289–292

5 Zhou P, Derkatch IL, Liebman SW. The relationship between visible intracellular aggregates that appear after overexpression of Sup35 and the yeast prion-like elements [PSI(+)] and [PIN(+)]. Mol Microbiol, 2001, 39: 37–46

6 Li L, Lindquist S. Creating a protein-based element of inheritance. Science, 200, 288: 661–665

7 Taylor KL, Cheng N, Williams RW, Steven AC, Wickner RB. Prion domain initiation of amyloid formation *in vitro* from native Ure2p. Science, 1999, 283: 1339–1343

8 Swietnicki W, Morillas M, Chen SG, Gambetti P, Surewicz WK. Aggregation and fibrillization of the recombinant human prion protein huPrP90-231. Biochemistry, 2000, 39: 424–431

9 Qin K, Yang DS, Yang Y, Chishti MA, Meng LJ, Kretzschmar HA, Yip CM *et al*. Copper(II)-induced conformational changes and protease resistance in recombinant and cellular PrP. Effect of protein age and deamidation. J Biol Chem, 2000, 275: 19121–19131

10 Nandi PK, Leclerc E. Polymerization of murine recombinant prion protein in nucleic acid solution. Arch Virol, 1999, 144: 1751–1763

11 Nguyen J, Baldwin MA, Cohen FE, Prusiner SB. Prion protein peptides induce alpha-helix to beta-sheet conformational transitions. Biochemistry, 1995, 34: 4186–4192

12 Yin SM, Zheng Y, Tien P. On column purification and refolding of recombinant bovine prion protein: Using its octarepeat sequences as a natural affinity tag. Protein Expr Purif, 2003, 32: 104–109

13 King CY, Tittmann P, Gross H, Gebert R, Aebi M, Wuthrich K. Prion-inducing domain 2-114 of yeast Sup35 protein transforms *in vitro* into amyloid-like filaments. Proc Natl Acad Sci USA, 1997, 94: 6618–6622

14 Naiki H, Higuchi K, Hosokawa M, Takeda T. Fluorometric determination of amyloid fibrils *in vitro* using the fluorescent dye, thioflavin T1. Anal Biochem, 1989, 177: 244–249

15 Prusiner SB, McKinley MP, Bowman KA, Bolton DC, Bendheim PE, Groth DF, Glenner GG. Scrapie prions aggregate to form amyloid-like birefringent rods. Cell, 1983, 35: 349–358


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