

Conformational change of glutathione-S-transferase by its co-expression with prion domain of yeast Ure2p*

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Abstract The Ure2 protein from *Saccharomyces cerevisiae* has a changeable structure similar to that of mammalian prion protein. Its N-terminal is the prion domain (PrD) consisting of 65 amino acids which plays a critical role in yeast prion development. In this study, PrD gene was recombined with glutathione-S-transferase (GST) gene, and a soluble GST-PrD (sGST-PrD) fusion protein was expressed in *E. coli*. sGST-PrD could spontaneously polymerize into amyloid fibrils *in vitro*, displaying typical β -sheet-type structure; it had increased resistance to proteinase K and exhibited amyloid-like optical properties. Moreover, the aggregated GST-PrD (aGST-PrD) could induce sGST-PrD to aggregate into fibrils. These results indicate that PrD could change the conformation of GST moiety in a recombinant protein with PrD to form a prion-like chimeric protein, which proves that PrD has the ability to mediate a prion-like conversion of other proteins fused with it.

Keywords: yeast prion, Ure2p, prion domain(PrD), glutathione-S-transferase(GST), conformation.

Wichner^[1] proposed that the unusual genetic behavior of two non-Mendelian determinants of yeast, [URE3] and [PSI], could be explained by prion hypothesis, and Ure2p and Sup35p are prion proteins of yeast. Ure2p is a regulatory protein for nitrogen metabolism, whose function will be lost when it aggregates into amyloid-like fibrils, which make the yeast cell exhibit a [URE3] phenotype. The first 65 amino acids of its N-terminus are the prion domain(PrD). The C-terminal domain (66 ~ 354 amino acids) contains a catalytic domain for regulation of nitrogen metabolism. The appearance and maintenance of the [URE3] phenotype requires the existence of PrD^[2-4]. Taylor et al.^[5] obtained fibrils resistant to proteinase K by a synthetic peptide comprising the first 65 residues of Ure2p, and found that these fibrils had a structure with increased β -sheet. Thual et al.^[6] expressed Ure2p in *E. coli*. And the purified Ure2p from the bacteria could be polymerized into amyloid-like fibrils, and it exhibited partial resistance to different proteases. Although Ure2p could not form amyloid-like fibrils, its regulatory function for nitrogen metabolism remained without PrD. These results show that PrD plays a very important role in the yeast prion formation.

In this study, we recombined the PrD DNA fragment of Ure2p with glutathione-S-transferase(GST) gene and expressed the fusion protein in *E. coli*. The conformational change of the purified GST-PrD fusion protein was analyzed using circular dichroism spectrum, and its resistance to proteinase K digestion and its optical property were also determined.

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1 Materials and methods

1.1 Construction of PrD expression vector

PrD gene was amplified from the genomic DNA extracted from the phenotype [URE3] of *Saccharomyces cerevisiae* (strain 3687) with the primers of 5'-GGGGATCCCATATGATGAATAACAACGGC - 3' and 5'-GGGAATTCATTAGCGGCCGCTGTATTG -3', according to the Ure2 DNA sequence^[7]. A 214bp PCR product was cloned into pGEM-T vector (Promega) and the recombinant plasmid was named pT-PrD. GST fusion expression vector pGEX-PrD was constructed by subcloning PrD gene into the *Bam*HI-*Eco*RI digested pGEX-6p-1 vector (Amersham Pharmacia).

1.2 Protein expression and purification

The expression construct of pGEX-PrD was transformed into *E. coli* BL21(DE3), grown on LB medium, and induced with 0.1 mmol/L isopropyl- β -D-galactopyranoside (IPTG). Harvested cell pellets were resuspended in phosphate buffer saline (PBS) and disrupted by sonication in 10 s bursts for 5 min, and then centrifuged at 12000 r/min for 10 min. The supernatant was purified by glutathione sepharose 4B (Amersham Pharmacia) according to the manufacturer's instruction. The purified GST-PrD protein was dialyzed against buffer I (20 mmol/L Tris-HCl, pH 7.0, 150 mmol/L NaCl, 1 mmol/L EDTA). Protein concentration was determined by using a BIO-RAD protein assay kit. PrD was cleaved from GST-PrD fusion protein by PreScission protease (Amersham Pharmacia), and purified by using a BioCAD perfusion chromatography workstation with a POROS R2 reversed-phase column (PerSeptive Biosystems Inc.). Elution buffer A was 0.1% trifluoroacetic acid (TFA) in water and elution buffer B was 0.09% TFA in acetonitrile. Flow rate was 3 mL/min. The elution was carried out with a linear gradient of 0% ~ 65% buffer B. The purified proteins were analyzed by Tricine-SDS/PAGE.

1.3 Observation by electron microscope

Samples (5 μ L) of PrD or aGST-PrD filaments were adsorbed to the 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 removal of excess liquid, and were visualized under a Hitachi H600A electron microscope.

1.4 Circular dichroism (CD) spectroscopy

The protein concentration was adjusted to 200 μ g/mL by diluting with 10 mmol/L Tris-HCl, pH 7.0, 10 mmol/L NaCl, 1 mmol/L EDTA. All samples were briefly sonicated before measurement. Far-UV CD spectra were recorded on a JASCO J715 spectropolarimeter at the wavelength from 190 to 250 nm.

1.5 Congo red staining

The protein aggregates were pelleted by centrifugation at 8000 g, washed twice with buffer I, stained with 2% Congo red. After incubation for 1 h at room temperature and centrifugation at 8000 g for 30 s, the aggregates were washed twice with water, and the pellets were resuspended. 10 μ L of

sample was placed on a slide and allowed to dry, then viewed under an Olympus BH2 polarization microscope.

1.6 Proteinase K digestion

Protein concentrations of GST, sGST-PrD, aGST-PrD and PrD were adjusted to 1 mg/mL with buffer I, then proteinase K was added to a final concentration of 20 $\mu\text{g}/\text{mL}$ and incubated at 37°C for 0, 5, 10, 15, 30, 60, 120, 240 min respectively. Proteolysis was stopped by addition of 5 mmol/L PMSF. The samples were boiled for 3 ~ 5 min, and proteins were detected by Tricine-SDS/PAGE.

1.7 Seeding assay

One percent of PrD or aGST-PrD filaments was added to sGST-PrD solution, after 24 h undisturbed incubation the aggregates were sedimented at 15000 g for 10 min, and pellets were dissolved in 20 μL

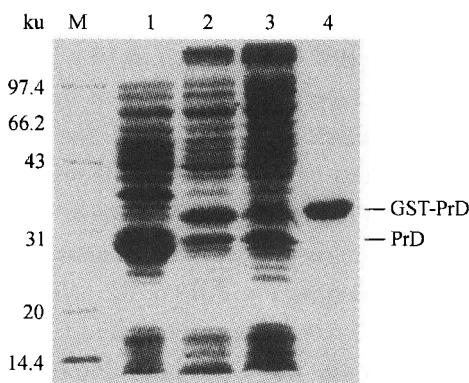


Fig. 1 Expression and purification of GST-PrD fusion protein. M, Protein molecular weight standards; 1, BL21 transformed with pGEX-6P-1; 2, BL21 transformed with pGEX-PrD; 3, supernatant after sonication; 4, GST-PrD eluted from glutathione sepharose 4B.

loading buffer, boiled for 3 ~ 5 min, and separated by Tricine/SDS-PAGE.

2 Results

2.1 Expression, purification and cleavage of GST-PrD fusion protein

DNA sequencing of the cloned fragment showed that it was the same as the reported PrD sequence by Coschigano^[7]. The plasmid pGEX-PrD was expressed successfully in *E. coli* BL21(DE3), and sGST-PrD was isolated by glutathione sepharose 4B affinity chromatography to a 90% purity (Fig. 1). After being cleaved by PreScission protease, and purified by a reversed-phase column POROS R2, PrD was obtained (Fig. 2), while

the aggregated GST-PrD was shown to resist PreScission protease digestion.

2.2 Fibrilization and aggregation of GST-PrD fusion protein

The purified sGST-PrD remained soluble at concentrations below 4 mg/mL, aggregated quickly when the concentration was above 4 mg/mL. When the concentrations below 4 mg/mL sGST-PrD was fibrilized and aggregated after being incubated at 4°C for over 24 h. Under the same conditions, PrD was more likely to aggregate than sGST-PrD. GST alone did not aggregate under any conditions.

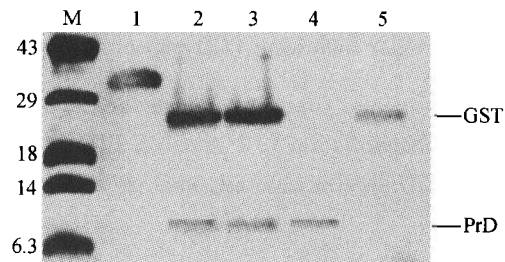


Fig. 2 Cleavage of GST-PrD fusion protein and purification of PrD. M, Protein molecular weight standards; 1, purified GST-PrD; 2, 3, products of PreScission-cleaved GST-PrD; 4, eluted PrD; 5, eluted GST.

2.3 GST-PrD aggregates under an electron microscope

Negative stain electron microscopy revealed that both PrD and GST-PrD aggregates were formed as amyloid-like fibrils. aGST-PrD filament was 10 ~ 20 nm in diameter, and 100 ~ 300 nm in length (Fig. 3(a)), and a small amount of amorphous aggregates could also be observed (data not shown). PrD filament was 6 ~ 15 nm in diameter and 100 ~ 500 nm in length (Fig. 3(b)).

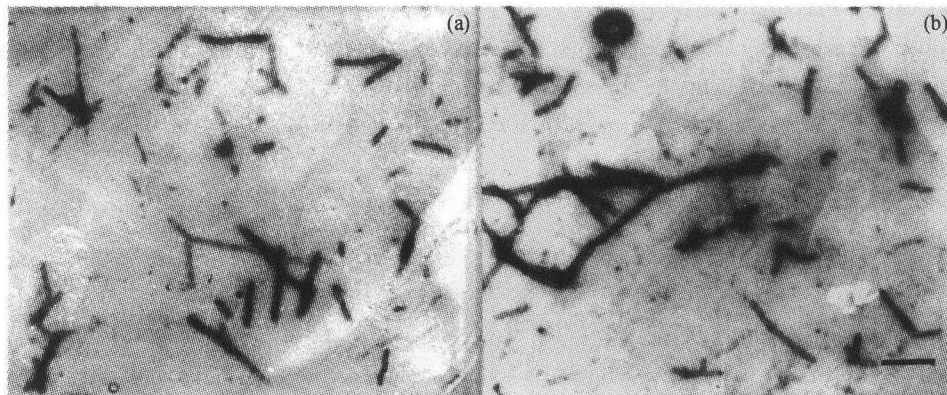


Fig. 3 Micrographs of negatively stained GST-PrD filaments. (a) Filaments formed by GST-PrD, (b) filaments formed by PrD.

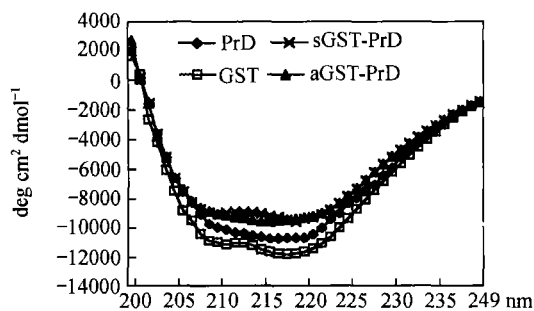


Fig. 4 CD spectra of GST-PrD.

2.4 CD spectra of GST-PrD

CD spectra of GST and sGST-PrD shown in Fig. 4 display double minima at 208 and 222 nm, with a characteristic α -helical structure. But CD spectra of PrD and aGST-PrD show that 208 nm minimum has disappeared, and the spectra changed into the shape of the spectrum of proteins rich in β -sheet structure. These results suggest that the conformation of GST-PrD is changed during aggregation, and the amount of β -sheet content is increased

as well (Table 1).

Table 1 Secondary structure content estimated from CD spectroscopy (%)

	α -helix	β -sheet	Turn	Random
PrD fibrils	16	43	14	27
GST	42	15	20	13
sGST-PrD	30	26	24	17
aGST-PrD fibrils	24	40	17	19

2.5 Birefringence of GST-PrD stained by Congo red

Numerous Congo red-stained PrD and GST-PrD aggregates were observed in a bright field under a polarized microscope. The stained aggregates exhibited strong green-yellow birefringence in the polarized light (dark field), suggesting that both PrD and GST-PrD aggregates are amyloid-like fibrils, containing ordered secondary structures (Plate II).

2.6 Proteinase K resistance

The proteinase K digestion results showed that GST was completely digested after 15-min digestion, while sGST-PrD and aGST-PrD were completely digested after 120 and 240 min, respectively; PrD was also digested after 240 min; which indicates that PrD and aGST-PrD had a strong resistance to proteinase K. Comparison of the electrophoresis patterns between the products of digested sGST-PrD and aGST-PrD shows that GST protein in aGST-PrD is more resistant to proteinase K than GST in sGST-PrD (Figure 5).

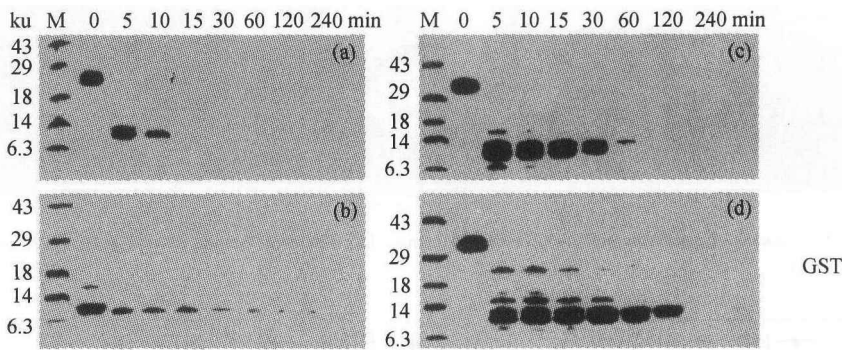


Fig. 5 Proteinase K resistance of GST-PrD aggregates. M, protein standards. (a) GST, (b) PrD filaments, (c) sGST-PrD, (d) aGST-PrD.

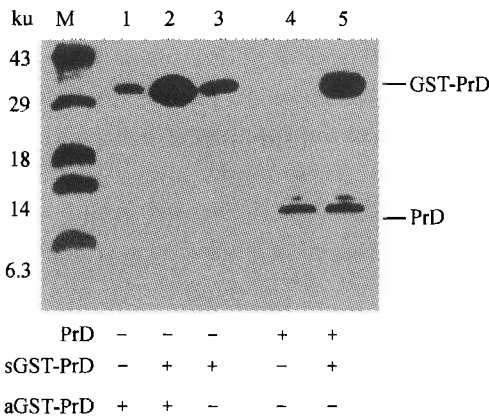


Fig. 6 SDS-PAGE pattern showing the self-aggregation of GST-PrD fusion protein.

2.7 Self-aggregation of GST-PrD fusion protein

By adding 1% aGST-PrD to freshly prepared GST-PrD solution (1 mg/mL) and incubating the mixture overnight at room temperature, a large amount of aGST-PrD was obtained, which was much more than the aggregates formed in sGST-PrD without adding aGST-PrD or PrD filaments (Figure 6).

3 Discussion

Yeast prion protein has provided an important model to study the conformational conversion of prion protein, and data about mammal prion protein could be obtained by this model. The yeast prion domain

(PrD) is necessary for the appearance and maintenance of the yeast prion phenotype as well as the propagation of the prion determinant^[1], but similar domain has not been found in animal prion protein so far. To further elucidate the function of yeast prion domain, we fused PrD gene with GST gene and

expressed soluble recombinant GST-PrD protein in *E. coli*. The purified GST-PrD protein had the structural characteristics of prion protein *in vitro*. The GST-PrD fusion protein could form amyloid-like aggregates, rich in β -sheet, and showed green-yellow birefringence upon binding of Congo red. All this suggested that GST-PrD aggregates were amyloid-like fibrils and contained ordered secondary structure. It was observed that aGST-PrD had a stronger resistance to proteinase K than sGST-PrD, so it is possible that the conformation of GST moiety in fusion protein was changed. CD spectra data indicated that β -sheet content of aGST-PrD(40%) was higher than that of sGST-PrD(26%) and GST(15%), and the amount of aGST-PrD was increased when PrD or aGST-PrD filaments were added. This suggests that PrD or aGST-PrD filaments could accelerate the aggregation of sGST-PrD, which agrees with the "seed" model of conformational conversion in mammalian prion proteins^[9].

We observed that the GST-PrD protein molecule was partially resistant to proteinase K, which was indicated by a prolonged existing time of GST in aGST-PrD digest, and the aggregates of GST-PrD was fully resistant to PreScission protease cleavage, implying that the region containing the cleavage site became inaccessible to the PreScission protease and its conformation was altered. CD spectra data of sGST-PrD and aGST-PrD showed a substantial increase in β -sheet structure in the GST moiety. All these results provided evidence that the conformation in the GST moiety of GST-PrD fusion protein was changed.

Recently Li et al.^[10] fused the prion domain of a yeast prion protein Sup35 to the rat glucocorticoid receptor. Like the known yeast prion, this chimeric protein altered the biochemical properties of yeast cells that could be inherited by progeny cell. And a new yeast prion Rnq1 has been identified^[11] based on the characteristics of the N-terminal octapeptide repeats in mammal prion and the prion domain of yeast rich in glutamine and asparagine. These new findings combined with our results further proved that PrD plays an important role in yeast prion formation and the prion domain can alter the conformation of proteins fused with it.

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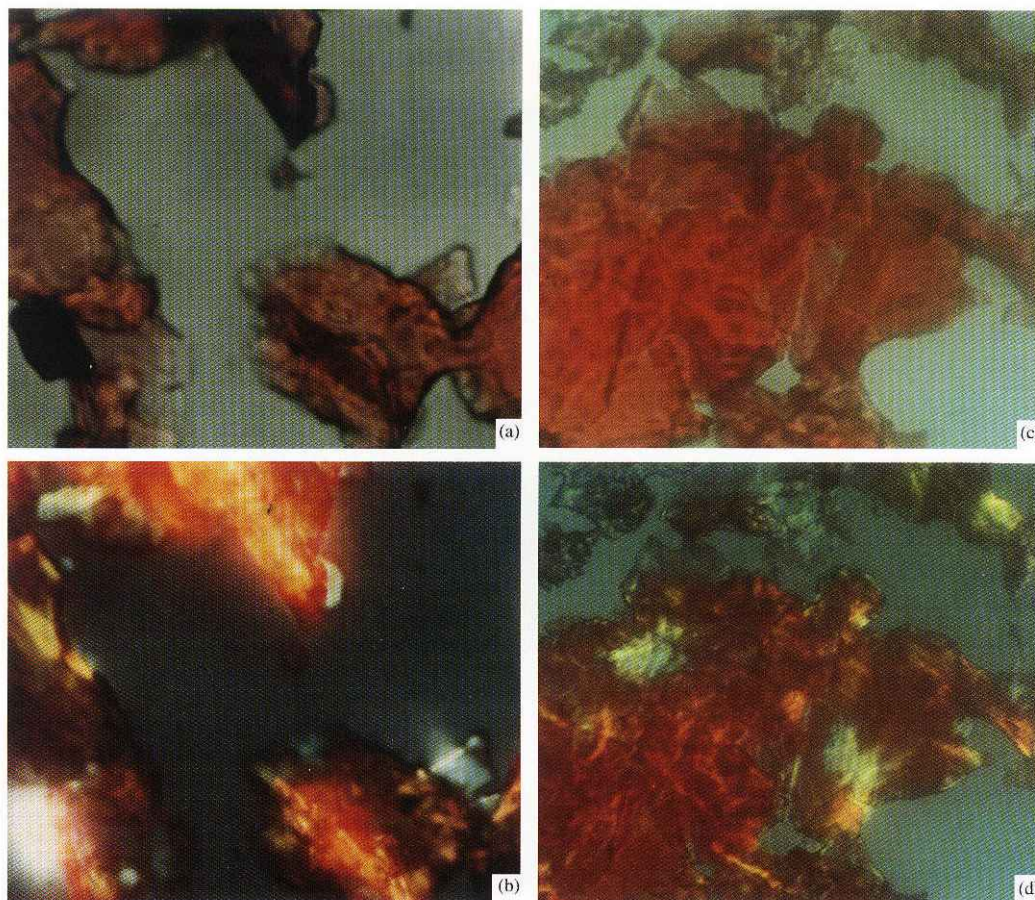


Plate II Photomicrographs of PrD and GST-PrD aggregates stained with Congo red. (a) and (c), in bright field; (b) and (d), in dark field, showing green-yellow birefringence.