

The yeast prion protein Ure2: insights into the mechanism of amyloid formation

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Abstract

Ure2, a regulator of nitrogen metabolism, is the protein determinant of the [URE3] prion state in *Saccharomyces cerevisiae*. Upon conversion into the prion form, Ure2 undergoes a heritable conformational change to an amyloid-like aggregated state and loses its regulatory function. A number of molecular chaperones have been found to affect the prion properties of Ure2. The studies carried out in our laboratory have been aimed at elucidating the structure of Ure2 fibrils, the mechanism of amyloid formation and the effect of chaperones on the fibril formation of Ure2.

Introduction

Certain proteins can readily convert from their soluble functional conformation into highly ordered amyloid structures. Although the formation of amyloid structure is associated with a number of neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease and the prion diseases, amyloid fibrils with positive biological functions have also been reported [1,2]. Prions are proteins that can undergo a heritable conformational change to an amyloid-like aggregated state, which is then transmitted to normal molecules of the same protein [3]. Prions have been found not only in mammals, but also in fungi [4–7]. To date, at least seven prions and the corresponding protein determinants have been identified in yeast. The first and best characterized are [PSI⁺], the prion state of the protein Sup35 which functions in translation termination, [PIN⁺], the prion state of Rnq1, a protein of unknown function, and [URE3], the prion state of Ure2, which is the subject of the present review. Recently, further yeast prions have been identified, including [SWI⁺], [MCA], [OCT⁺] and [MOT3], which are the prion states of Swi1p, MCA1p, Cyc8 and Mot3p respectively [8]. In particular, fungal prions have provided a powerful experimental model for probing and understanding the complex biological, biochemical and biophysical aspects of the prion phenomenon [9].

Overview of Ure2 structure and function

Ure2 is the protein determinant of the *Saccharomyces cerevisiae* prion [URE3], which enables yeast to grow in selective media by uptake of compounds such as ureidosuccinate. Analogous to the properties of mammalian prion proteins, Ure2 shows the ability to convey a heritable change in phenotype by undergoing a structural change at the

protein level into an aggregated form [10,11]. Ure2 functions as a nitrogen metabolism regulation factor: in the presence of a good source of nitrogen, normal soluble Ure2 interacts with the transcription factor Gln3, leading to repression of genes required to utilize poor nitrogen sources [12]. Upon conversion of Ure2 into the prion state, this function is lost and Gln3 activates transcription of nitrogen catabolic genes.

Ure2 is a 354-amino-acid homodimeric protein consisting of a poorly structured N-terminal domain and a globular C-terminal domain [13,14]. The unstructured N-terminal domain (~90 amino acids) is required for both induction of the [URE3] phenotype *in vivo* [11] and the amyloid-forming capacity *in vitro* [15–17], and is thus referred to as a PrD (prion-inducing domain). Deletion of the PrD has no detectable effect on the thermodynamic stability or folding kinetics of Ure2 under a wide range of conditions [18]. The C-terminal globular functional domain has a structure similar to the GST (glutathione transferase) superfamily [19,20] and is both necessary and sufficient for its *in vivo* nitrogen regulatory function in yeast [21]. Ure2 has been reported to confer on *S. cerevisiae* cells protection from heavy metal ion and oxidant toxicity [22], and *in vitro* studies have explored the GPx (glutathione peroxidase) and GRX (glutaredoxin) activities of Ure2 [23,24]. Conversion of the N-terminal domain into a β -sheet-rich amyloid core is not accompanied by any observable alteration in either the conformation or activity of the C-terminal domain of Ure2 [23–25]. Taken together, the data indicate that the N- and C-terminal regions of Ure2 are essentially independent from a structural point of view. In addition, the data suggest that the loss of regulatory function of Ure2 associated with prion formation is likely to be due to a steric effect rather than the loss of native structure.

Sequence determinants of Ure2 fibril formation and prion propagation

The primary structure of the N-terminal domain affects the ability of Ure2 to form fibrils *in vitro* and to act as a prion *in vivo*, as summarized in Figure 1. The natively disordered PrD,

Key words: amyloid, chaperone, prion, *Saccharomyces cerevisiae*, Ure2p, [URE3].

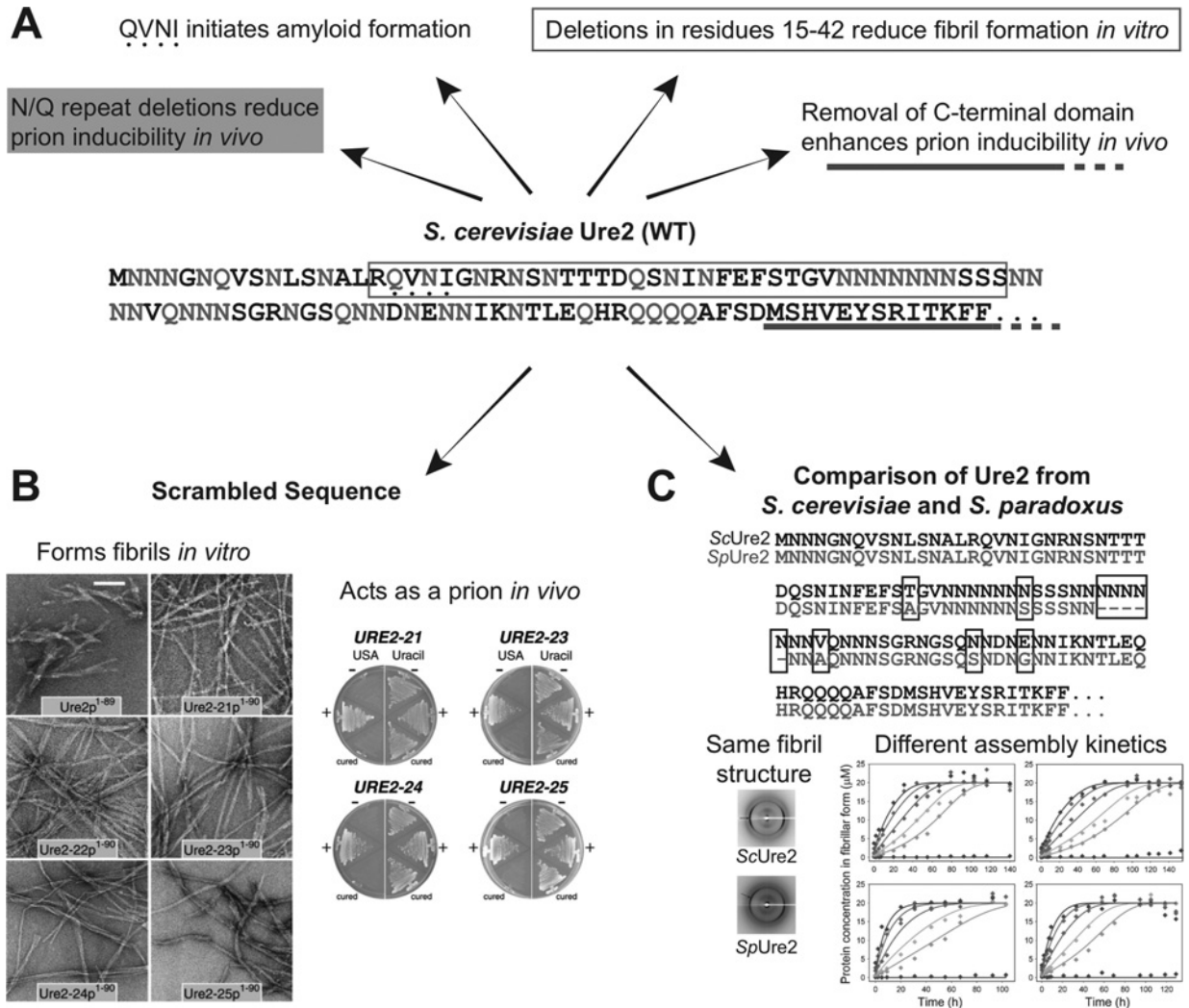
Abbreviations used: AOX1, alcohol oxidase 1; FTIR, Fourier-transform IR spectroscopy; GPx, glutathione peroxidase; GRX, glutaredoxin; GST, glutathione transferase; Hsp, heat-shock protein; PrD, prion-inducing domain.

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Figure 1 | Sequence determinants of Ure2 fibril formation

The effects of alterations to the primary structure of the N-terminal domain of *S. cerevisiae* Ure2. Portions are reprinted from [27] (American Society of Microbiology), [31] (Elsevier) and [32] (The American Society for Biochemistry and Molecular Biology) with permission. **(A)** Deletion of asparagine/glutamine residues or of residues in the region 15–42 (boxed) reduces the propensity of Ure2 to convert into an amyloid form. Deletion of C-terminal residues (underlined in grey) enhances amyloid formation. **(B)** Scrambled sequences of the PrD residues can also form fibrils *in vitro* and act as prions *in vivo*. **(C)** The *S. paradoxus* and *S. cerevisiae* homologues of Ure2 differ by a few residues in the N-terminal domain leading to a difference in the kinetics of amyloid formation, although the fibrillar structures formed are highly similar.



which is generally poorly conserved among *Saccharomyces* species, is rich in asparagine and glutamine residues, but contains a relatively conserved region, corresponding to residues 10–40 which contains normal random sequence [26]. It has been suggested that it is the amino acid composition of the PrD rather than the specific primary sequence that is responsible for the ability of Ure2 to act as a prion, in that a scrambled PrD sequence is also able to act as a prion *in vivo* and form fibrils *in vitro* [27]. Consistent with the fact that deletions of asparagine/glutamine repeats in the PrD reduce the prion inducibility *in vivo* [28], *in vitro* studies show that deletions of asparagine/glutamine repeats in the PrD inhibit amyloid formation of Ure2 [17]. Moreover,

deletion of the normal random sequence (residues 15–42) also results in reduced ability of Ure2 to form fibrils *in vitro* [17]. Interestingly, several studies have also suggested the involvement of the C-terminal region of Ure2 in its prion properties *in vivo*: deletion of residues 221–227 was shown to decrease prion induction, whereas the deletion of residues 151–158 or 348–354 increased the rate of prion induction [28]. Consistent with these results, we have found that deletion of residues 151–158 accelerates the nucleation, growth and fragmentation of amyloid-like aggregates *in vitro*. Furthermore, the aggregates formed are able to seed formation of fibrils of the wild-type protein, suggesting that the enhanced *in vivo* prion-inducing ability of the 151–158

deletion mutant is due to an increased ability to generate prion seeds [29].

We have recently compared the mechanism of fibril formation and fibril structures of two Ure2 homologues: ScUre2 and SpUre2 from *S. cerevisiae* and *Saccharomyces paradoxus* respectively [30–32]. ScUre2 and SpUre2 have identical C-terminal domains and differ by only a few residues in their PrD, but SpUre2 shows a lower prion propensity *in vivo* [33], although it readily forms amyloid fibrils *in vitro* [30]. Our results show that ScUre2 and SpUre2 have a similar cross- β core structure with the same inter-strand and inter-sheet spacings [31]. Despite their similar amyloid structures, studies of the rate constants involved in the fibril formation of ScUre2 and SpUre2 explored by a combination of kinetic theory with solution and biosensor assays have shown that SpUre2 possesses a higher elongation rate, but a lower breakage rate, which leads to the reduced prion propensity of SpUre2 [32]. In addition, the breakage rate is found to be influenced by the structure of the fibril seeds, which can be transmitted through self-propagation [32], thus suggesting that the different breakage rates observed for ScUre2 and SpUre2 *in vitro* relate to details within the cross- β structure, such as the arrangement of β -strands or the association of cross- β structures [31]. These findings illuminate the connections between the *in vitro* aggregation of proteins and the *in vivo* propagation of prions.

Insights into the structure of Ure2 fibrils

Numerous attempts to elucidate the detailed structure of Ure2 amyloid fibrils have been reported. The widely accepted model for Ure2 amyloid formation involves induction of significant conformational change in the N-terminal domain to form a parallel in-register β -sheet structure, whereas the C-terminal domain retains its native-like conformation surrounding the cross- β core [31,34–36].

Wide-angle X-ray scattering experiments have confirmed that the fibrillar forms of both ScUre2 and SpUre2 comprise a cross- β core structure. The X-ray diffraction patterns of dried and hydrated fibrils formed from both ScUre2 and SpUre2 showed anisotropic reflections at 4.8–4.9 Å and 10.3–10.5 Å, corresponding to the expected inter-strand and inter-sheet spacings respectively of a cross- β core structure [31,35].

Several lines of enquiry point towards maintenance of a functional natively folded C-terminal domain, whereas the N-terminal domain becomes incorporated into the fibril core. FTIR (Fourier-transform IR spectroscopy) analysis of ScUre2 indicates that a high degree of helical content is maintained in fibrils of ScUre2 [37]. We recently carried out FTIR analysis of ScUre2 and SpUre2 fibrils and confirmed that full-length fibrils of both proteins have a high helical content, consistent with the C-terminal domain being retained in its native conformation around the fibril core [31]. Incubation of the fibrils with proteinase K led to digestion of the C-terminal domain, but left intact the proteolytically resistant fibril cores; FTIR spectra recorded after proteinase K digestion were indicative of β -sheet-rich

secondary structure and X-ray diffraction confirmed that the cross- β core is maintained [31].

Furthermore, our systematic studies on the enzymatic activities of Ure2 in both soluble dimeric and fibrillar forms demonstrate the maintenance of a native-like dimeric conformation of the C-terminal domains in Ure2 fibrils. The C-terminal functional domain of Ure2 shows structural homology with the GST superfamily and is able to bind glutathione (GSH), but lacks typical GST activity [19–22]. We found that introduction of the point mutations A122C or N124A/V ‘restores’ the GST activity of Ure2 towards the standard substrate CDNB (1-chloro-2,4-dinitrobenzene) [25]. In addition, the GPx and GRX activities of Ure2 have also been characterized *in vitro* [23,24]. The discovery of these activities provides a reasonable explanation for the protective function of Ure2 in yeast cells from heavy metal ion and oxidant toxicity [22]. These enzymatic activities are also observed in the fibrillar aggregates of Ure2, indicating maintenance of native-like structure of the C-terminal domains within Ure2 fibrils [23–25]. Furthermore, the kinetics of the GRX activity of Ure2 in both the dimeric state and fibrils show positive co-operativity for the substrate GSH, and, in both cases, the number of essential substrate-binding sites in the catalytic unit is equal to two [24]. These results indicate that the dimer represents the minimum catalytic unit for the activity of Ure2 in both soluble and fibrillar forms, and thus the C-terminal domain retains its native-like dimeric structure within Ure2 fibrils.

Insights into the mechanism of Ure2 amyloid formation

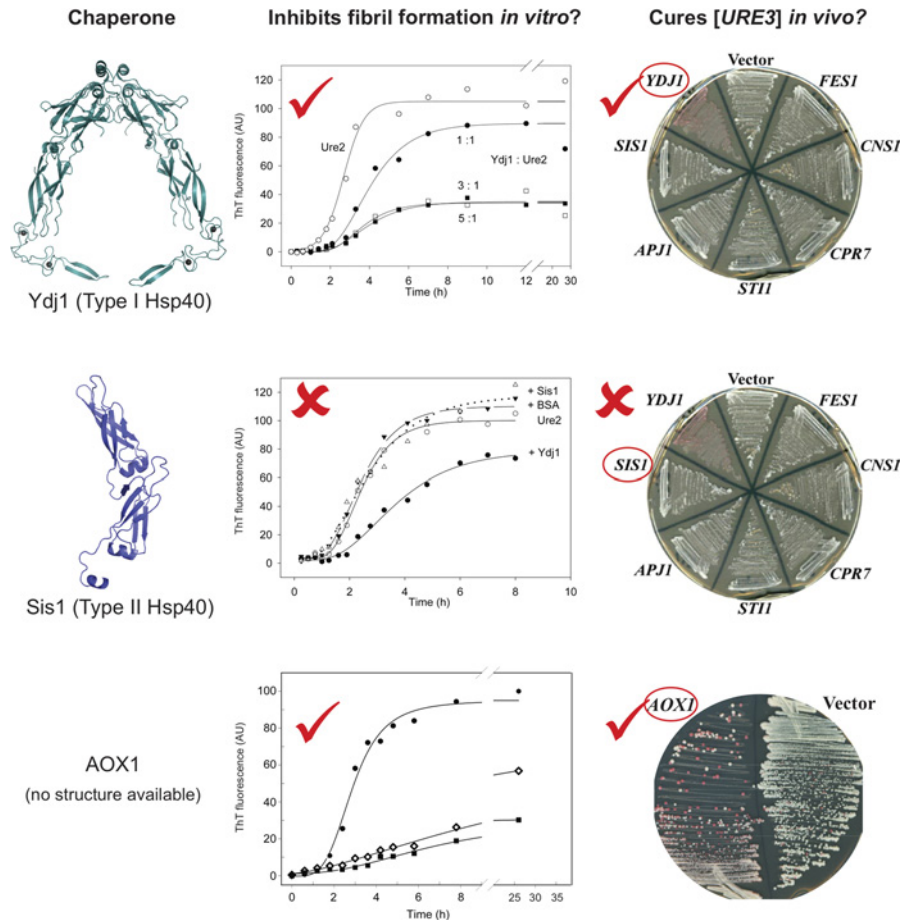
Extensive investigation in our laboratory of the molecular mechanisms underlying Ure2 amyloid formation has revealed several important principles that also inform our understanding of the mechanisms of amyloid formation in general.

First, we have demonstrated that the position of the PrD in the protein affects the ability of Ure2 to form fibrils primarily by altering its flexibility. In order to explore the effect of the PrD position on amyloid fibril formation of Ure2, two Ure2 variants were produced by swapping the position of the N- and C-terminal regions, with or without an intervening peptide linker [38]. The native structural content and stability of the variants are the same as wild-type Ure2, but the fibril-forming capabilities of the variants are different: CLN-Ure2, which contains a flexible linker between the two domains, is able to form amyloid-like fibrils, although with a longer lag time than wild-type Ure2. In contrast, the variant lacking the linker, CN-Ure2, shows limited ability to form fibrils, although very low concentrations of guanidinium chloride improve its ability to form fibrils. This suggests that the reduced ability of Ure2 to form fibrils when the positions of the N- and C-terminal domains are transposed is primarily due to effects on the flexibility of the PrD [38].

Secondly, we have identified a potential amyloid stretch that appears to act as an initiation site for fibril assembly

Figure 2 | Effects of molecular chaperones on Ure2 fibril formation

The molecular chaperone Ydj1 (structure generated from overlay of PDB codes 1NLT and 1XA0) prevents amyloid fibril formation *in vitro* and cures [URE3] *in vivo*. Ydj1 has been shown to interact with native Ure2 and the C-terminal residues 151–158 of Ure2 are thought to play a key role in this interaction. Sis1 (PDB code 1C3G) neither inhibits amyloid fibril formation *in vitro* nor cures [URE3] *in vivo*. AOX1 has also been found to inhibit Ure2 fibril formation *in vitro* and to cure [URE3] *in vivo*. Portions are reprinted from [49] (The American Society for Biochemistry and Molecular Biology) and [50] (Wiley-Blackwell) with permission.



and may be modified to control the propensity to form fibrils. Cysteine scanning and the introduction of disulfides are important approaches to obtain insight into the structure and mechanism of formation of amyloid fibrils [39]. Recently, we performed cysteine scanning within the PrD of Ure2 and identified a unique cysteine mutant (R17C) that can greatly accelerate the fibril assembly kinetics of Ure2 under oxidizing conditions [40]. The subsequent sequence segment QVNI (residues 18–21) in Ure2 appears to act as an initiation point for amyloid formation as this segment plays a critical role in the rapid assembly properties of R17C Ure2. Preceding the QVNI segment with a cysteine or a hydrophobic residue, instead of a charged residue, dramatically accelerates the rate of amyloid assembly, suggesting that the fibril-forming propensity of Ure2 is increased by the proximity of this amyloid stretch induced by either a disulfide bridge or hydrophobic interaction [40]. The wild-type octapeptide RQVNIGNR (residues 17–24) is found to form fibrils *in*

in vitro and this is the first identified short amyloid-forming peptide for Ure2. Furthermore, this octapeptide crystallizes readily and so provides a starting point towards obtaining high-resolution structural information for the amyloid core of Ure2 fibrils [41].

The effect of chaperones on the amyloid formation of Ure2

Molecular chaperones play a crucial role in the cell by safeguarding proteins from misfolding and aggregation, particularly under conditions of stress, such as heat shock or disease [42,43]. Genetic studies have demonstrated that Hsp (heat-shock protein) 104 and Hsp70 (e.g. Ssa1 and Ssa2), together with the co-chaperone Hsp40 (e.g. Ydj1 and Sis1), are involved in the propagation of yeast prions [44]. It is reported that overexpression of Ssa1 or Ydj1 is able to

cure [URE3] [45,46]. Ydj1 from *S. cerevisiae* is a molecular chaperone of the type I Hsp40 family and can bind to non-native polypeptides and pair with Hsp70 Ssa proteins to prevent aggregation and facilitate refolding of denatured proteins *in vitro* [47,48]. In contrast with Sis1 (a type II Hsp40 from *S. cerevisiae*), Ydj1 itself can act as a chaperone *in vitro*, suppressing protein aggregation by forming stable complexes with folding intermediates [47,48]. We previously examined the effect of overexpression of Hsp40 members and other Hsp70 co-factors *in vivo* and found that, of these, only Ydj1 showed a strong curing effect on [URE3] [49], as summarized in Figure 2. Surface plasmon resonance and size-exclusion chromatography experiments revealed a direct interaction between Ydj1 and the C-terminal domain of Ure2, suggesting that Ydj1 inhibits Ure2 fibril formation by binding to the native state of Ure2, thus delaying the onset of oligomerization [49]. Furthermore, the deletion of residues 151–158 in the C-terminal domain of Ure2 disrupted the inhibitory effect of Ydj1 on the fibril formation of Ure2 *in vitro* [29], suggesting residues 151–158 as a potential interaction site between Ydj1 and Ure2.

In addition, we discovered serendipitously that AOX1 (alcohol oxidase 1) from *Pichia pastoris* has an inhibitory effect on both Ure2 fibril formation *in vitro* and [URE3] prion propagation *in vivo* [50], as summarized in Figure 2. The results demonstrate that AOX1 exerts its inhibitory effect by interacting with species present during the very early stages of the fibril formation process [50].

We are currently carrying out investigations to probe the effects of other chaperones on the amyloid formation of Ure2, and to characterize the interactions between the chaperones and Ure2, in order to clarify the mechanism by which chaperones affect the folding and aggregation of Ure2.

Concluding remarks

In the present paper, we have reviewed recent progress in elucidating both the structure and mechanism of assembly of Ure2 fibrils, as well as the effect of chaperones on this process. Numerous studies have yielded results that provide valuable insights into the details of the regulation and mechanism of the fibrillation of amyloid proteins in general, which may have important implications for our understanding of diseases associated with amyloid formation and may eventually lead to the development of therapeutics to treat or prevent these conditions.

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