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Review

The yeast prion protein Ure2: Structure, function and folding

Hui-Yong Lian [a,b], Yi Jiang [a], Hong Zhang [a,b], Gary W. Jones [c], Sarah Perrett [a,*]

[a] National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Chaoyang District, Beijing 100101, China
[b] Graduate School of the Chinese Academy of Sciences, 19 Yuquan Road, Fengtai District, Beijing 100039, China
[c] Department of Biology, National University of Ireland, Maynooth, Co. Kildare, Ireland

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Abstract

The *Saccharomyces cerevisiae* protein Ure2 functions as a regulator of nitrogen metabolism and as a glutathione-dependent peroxidase. Ure2 also has the characteristics of a prion, in that it can undergo a heritable conformational change to an aggregated state; the prion form of Ure2 loses the regulatory function, but the enzymatic function appears to be maintained. A number of factors are found to affect the prion properties of Ure2, including mutation and expression levels of molecular chaperones, and the effect of these factors on structure and stability are being investigated. The relationship between structure, function and folding for the yeast prion Ure2 are discussed.

Keywords: Prion; Amyloid; Glutathione transferase; Peroxidase; Protein Folding; Chaperone

1. Overview

The structure and function of a protein are intimately related. A large number of disease states have been attributed to protein misfolding or aggregation. Disease may arise from loss of normal function or, as suggested in the case of amyloid aggregates, by gain of toxic function. A subset of amyloid diseases arises apparently due to infection with a misfolded protein, or prion [1]. The prion diseases include Creutzfeldt–Jacob disease (CJD) in humans, and bovine spongiform encephalopathy (BSE) and scrapie in animals. The BSE epidemic in the UK [2], and subsequent appearance of a new variant of the human disease [3], has highlighted the importance of research in this area. However, despite more than a decade of intensive research, the molecular mechanism of prion propagation, and even the normal function of the mammalian prion protein, PrP, remain mysterious. While progress has been made towards showing that infectivity of the mammalian prion resides with the PrP protein alone [4,5], nevertheless, controversy continues [6].

Wickner proposed that the genetic behaviour of the *Saccharomyces cerevisiae* non-Mendelian element [*URE3*] could be explained if it were a prion of the Ure2 protein [7]. Since then much evidence has accumulated to support this proposal. To date, there are at least two other proteins in *S. cerevisiae* that also satisfy the biochemical and genetic criteria to be classed as prions [8,9], namely Sup35/[PSI^+] [10–12] and Rnq1/[RNQ/PIN^+] [13,14], and a number of other candidate prions have been identified by searching for prion domain-like sequence regions [15]. In addition, [*Het-s*] has been identified as a prion of the filamentous fungus *Podospora anserina* [16,17]. The relative simplicity and tractability of yeast and other fungal systems has allowed convincing proof of the protein-only hypothesis for Het-s [18], Sup35 [19,20] and Ure2 [21].

These various proteins are related via the prion concept; however, comparison of their structural and functional properties shows limited similarity (see Fig. 1). The Het-s protein lacks the Asn/Gln-rich region characteristic of the yeast prions identified so far, although it is similarly divided into a globular functional region and a flexible prion domain [22]. The relationship between domain structure and function for the PrP protein is more complex. The PrP protein has a flexible N-

Abbreviations: BSA, bovine serum albumin; CDNB, 1-chloro-2,4-dinitrobenzene; GdmCl, guanidine hydrochloride; GPx, glutathione peroxidase; GSH, glutathione; GSSG, oxidized glutathione; GST, glutathione transferase; CHP, cumene hydroperoxide; *t*-BH, *tert*-butyl hydroperoxide; ThT, Thioflavin T

* Corresponding author. Tel.: +86 10 6488 8496; fax: +86 10 6484 0672.
E-mail address: sarah.perrett@iname.com (S. Perrett).

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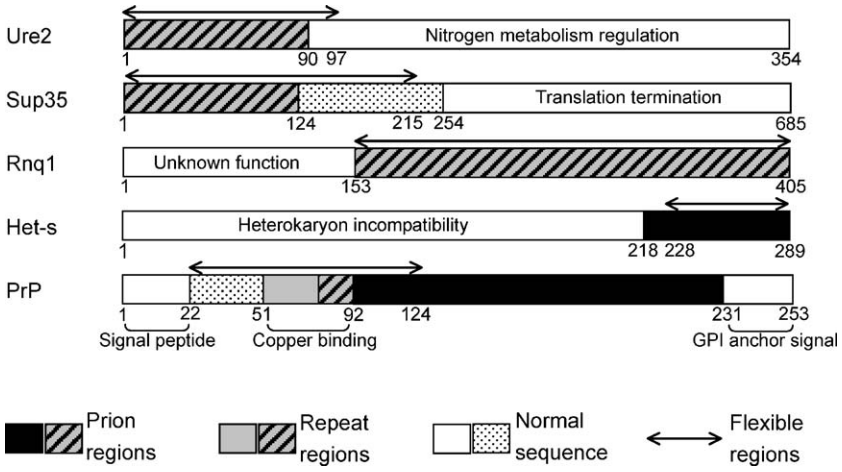

Fig. 1. Comparison of structural and functional properties of prion proteins: Ure2 [27,29,58], Sup35 [114,115], Rnq1 [13], Het-s [22] and PrP [23,24,116,117]. The functional regions are as indicated. Repetitive regions correspond to Asn/Gln-rich regions, except for PrP, in which residues 60 to 91 include four copies of an octapeptide repeat sequence Pro–His–Gly–Gly–Gly–Trp–Gly–Gln (PHGGGWGQ) [25,118].

terminal tail [23,24] containing an octapeptide repeat region involved in copper binding [25], but this flexible region only partially overlaps with the segment that is required for infectivity (see Fig. 1). As further prion proteins are identified and characterized, it may become clear whether the presence of a flexible tail in the native state is a necessary feature in order to undergo a switch to a prion structure. The fact that prions exist in yeast provides an ideal environment for detailed genetic analysis of factors affecting prion maintenance; this coupled with biochemical characterization should contribute to understanding of the molecular mechanism of amyloid formation and prion propagation.

2. Structure and function of Ure2

2.1. Introduction

Ure2 is a 354-amino acid protein consisting of a relatively flexible and protease-sensitive N-terminal region, and a globular C-terminal region [26–28]. The C-terminal region of Ure2 shows structural similarity to glutathione transferases (GSTs) and is necessary and sufficient for its regulatory function: Ure2 interacts with the transcription factor Gln3 allowing control of nitrogen catabolite repression, blocking the uptake of poor nitrogen sources in the presence of a good nitrogen source [29,30]. The N-terminal region of Ure2 is required for its prion properties in vivo [31] and to form amyloid-like filaments in vitro [32–34]. However, deletion of the N-terminal region has no detectable effect on the stability or folding of the protein in vitro [27,33,35,36]. The N-terminal approximately 90 residues of Ure2 are rich in asparagine and glutamine (see Fig. 2). Glutamine-repeats are associated with a number of neurodegenerative diseases, including Huntington's disease [37]. The ability of glutamine (or asparagine) repeats to aggregate by forming a hydrogen bonded β-sheet structure is thought to lead to disease [38–40], either by incorporation of vital cellular proteins into the aggregates [41,42], or by direct toxicity of the aggregates themselves [43]. The Ure2 system is therefore a useful model not only to investigate the prion concept, but also to understand the properties of Gln/Asn-repeat proteins and hence the molecular basis of the related diseases.

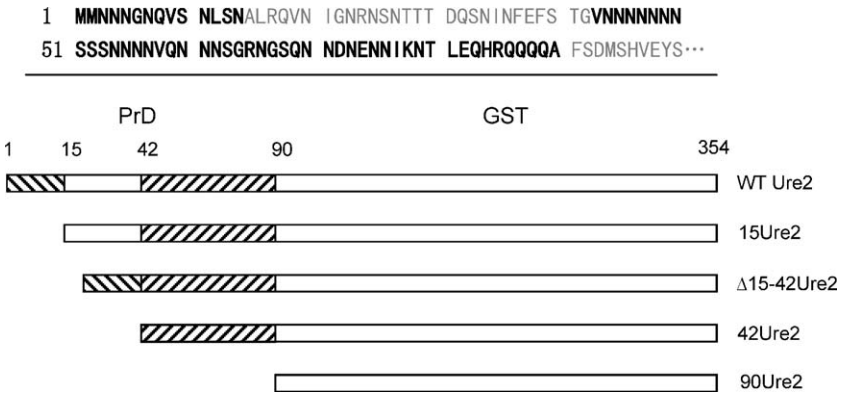

Fig. 2. Primary sequence of Ure2 and design of N-terminal deletion mutants. Repetitive regions are indicated by diagonal stripes or bold type [27]. The C-terminal region has homology to the glutathione transferase protein family [29].

2.2. Ure2 as a glutathione transferase

The glutathione transferases are a multifunctional family of enzymes, broadly distributed in nature, that play a critical role in cellular detoxification [44–49]. GSTs have the general function of conjugating glutathione (GSH) to electrophilic substances to reduce their toxicity. As a consequence, GSTs are involved in development of resistance towards drugs, insecticides and herbicides, and have a protective role against a range of diseases, including cancer. GSTs are dimeric proteins, with a relatively conserved N-terminal thioredoxin-like domain and a more variable C-domain. GST structures have been divided into a series of classes, including alpha, mu, pi, sigma and theta, on the basis of differences in structure and activity. The theta class, thought to be the progenitor of the other classes, is the most diverse [45] and further subdivisions have been suggested, such as beta for bacterial GSTs [46]. GST activity is typically tested using the 'universal' GST substrate 1-chloro-2,4-dinitrobenzene (CDNB). However, a number of GSTs identified by structural criteria have failed to show activity towards CDNB, particularly among theta class and bacterial enzymes, which are found to catalyze a diverse range of specific reactions [44]. Some GSTs have been shown to have overlapping functions with other glutathione binding enzymes, such as glutathione peroxidases (GPxs) and glutaredoxins [47–49]. These enzyme families share the GSH binding thioredoxin domain, but are otherwise structurally and mechanistically dissimilar. In particular, GPxs contain a selenocysteine, which reacts covalently with GSH, generally via a ping-pong enzyme reaction mechanism [50]. In contrast, GSTs use a conserved tyrosine, serine or cysteine residue to interact with the thiol group of GSH, thus increasing the reactivity of GSH, typically via a sequential mechanism [51–53]. In *S. cerevisiae*, the two GSTs that have been identified show activity towards CDNB, but do not possess peroxidase activity [54]. On the other hand, the two glutaredoxins are active towards both CDNB and hydroperoxides [55] and the three phospholipid hydroperoxide glutathione peroxidases show activity towards both lipid and non-lipid hydroperoxides [56]. The versatility of the glutathione binding enzymes, and their tendency to show overlapping functions, may contribute significantly to the ability of the host organism to adapt to change. Utilizing this potential, the GST fold has been used as a scaffold to design proteins with altered activity and specificity [49,57].

The Ure2 protein was identified as belonging to the theta class of glutathione transferases on the basis of sequence similarity [29,45]. Subsequent availability of crystal structures of the truncated C-domain of Ure2 in both the apo form [28,58] and with glutathione bound [59] has confirmed its classification as a theta (or beta) class GST (see Fig. 3). However, Ure2 does not show a detectable level of GST activity with typical substrates, such as CDNB [27,29,54,60]. Recent studies have found that deletion of the *URE2* gene increases the sensitivity of *S. cerevisiae* cells to heavy metals and cellular oxidants, such as hydrogen peroxide [60–62]; and a Ure2 homologue from *Aspergillus nidulans*, while lacking the nitrogen metabolite repression activity of Ure2, also contributes to heavy metal and xenobiotic resistance [63]. Peroxidase activity of Ure2 towards oxidant substrates including hydrogen peroxide as well as typical organic hydroperoxides, such as cumene hydroperoxide and *tert*-butyl hydroperoxide (*t*-BH), has been demonstrated for the purified protein in vitro, confirming the function of Ure2 as a glutathione dependent peroxidase [64].

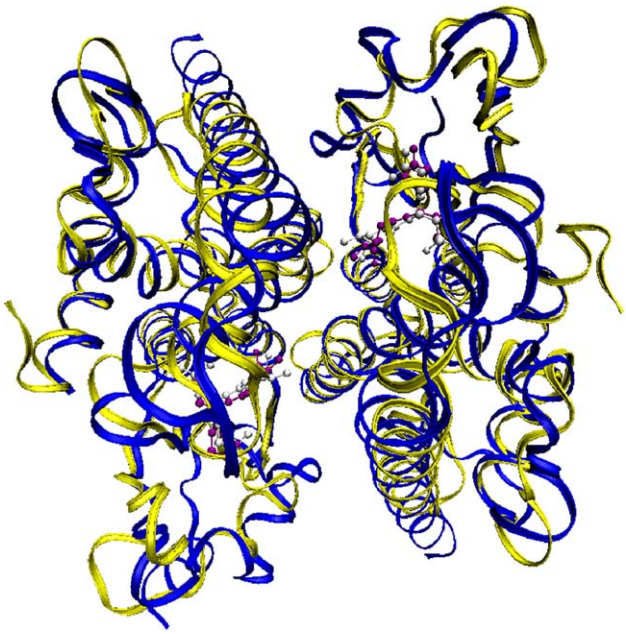

Fig. 3. Comparison of crystal structures for the C-terminal globular domain of Ure2 (PDB code 1k0d; blue) with glutathione bound (purple) and the β-class glutathione transferase for *E. coli* (PDB code 1a0f; yellow) with glutathione sulfonic acid bound (white). The figure was produced using the program VMD [119].

A number of questions remain regarding the relationship between Ure2 structure and its enzyme function. Interestingly, the residue suggested to be involved in activation of GSH on the basis of the crystal structure [59] is an Asn residue, N124; whereas in other known GSTs, the crucial catalytic residue is a Ser, Tyr, or Cys. Another interesting question regards GPx activity, which is observed in GSTs from a variety of organisms to exist as an adjunct of GST activity, while in the case of Ure2, GPx activity is present but typical GST activity is lacking. Mutagenesis studies will be required to pin-point the residues essential for substrate activation and binding. Further studies are also needed to understand the mechanistic and structural basis of the relationship between GST and GPx activity, for GST-like proteins in general and Ure2 in particular.

2.3. Relationship between enzymatic, regulatory and prion functions of Ure2

Strains of *S. cerevisiae* containing the [*URE3*] prion were found to show normal sensitivity towards hydroperoxides [60]. Further, it was found that formation of amyloid-like fibrils had negligible effect on the level of GPx activity detected for purified protein in vitro [64]. Taken together, this indicates that Ure2 GPx activity is maintained upon structural conversion to the aggregated prion form. This is in contrast to the loss of

regulatory function of Ure2 in the prion state, which is indeed the basis of detection of the [*URE3*] prion phenotype [7,65]. This suggests that the loss of regulatory function is attributable to a steric blocking mechanism, rather than to conformational changes per se. This also raises potential questions about the relative importance of the enzymatic and regulatory functions in evolutionary terms. Deletion of the *URE2* gene is non-lethal, implying that, at least in the lab environment, both enzymatic and regulatory functions are dispensable. It remains uncertain whether the [*URE3*] prion ever crops up in the wild [66] and the ability of Ure2 protein homologues to switch to the [*URE3*] prion form is conserved in some yeast species but not others [67,68]. There nevertheless remains the possibility that the additional epigenetic variability provided by the prion-switch provides an advantage in surviving changes in environment. The loss of the nitrogen regulatory function of Ure2 in strains containing the [*URE3*] prion, while GPx activity is maintained, is probably due to the relative sizes of the ligands involved in these reactions, and not any reflection of the relative importance of the respective activities. The purpose and evolutionary implications of fungal prions are discussed elsewhere [8,9,66,67,68].

3. Factors affecting [*URE3*] prion formation

3.1. Mutation

A number of mutations in the *URE2* gene have been shown to affect the prion behaviour (see Figs. 4 and 5). The N-terminal prion domain (PrD) of Ure2 was initially defined as residues 1–65, on the basis that when this region was deleted, the [*URE3*] prion state could not be induced and conversely, overexpression of the 1–65 fragment resulted in a 6000-fold increase in the rate of prion induction [31]. In fact, smaller deletions, such as 2–20, are able to ablate prion inducing ability [31] and overexpression of larger fragments, such as 1–80, result in even higher rates of induction [69]. Sequence comparison of Ure2 homologs in different yeast species indicates that in general, the PrDs are divergent in protein sequence, but the region 10–40 is relatively conserved across different yeast species, and within *Saccharomyces* species, the conserved region extends to residues 1–43 [70]. Interestingly, the conserved region correlates with the ability of N-terminal fragments to interfere with prion propagation ("curing") in [*URE3*] strains [70]. Further, the conserved region is involved in nucleation of amyloid-like

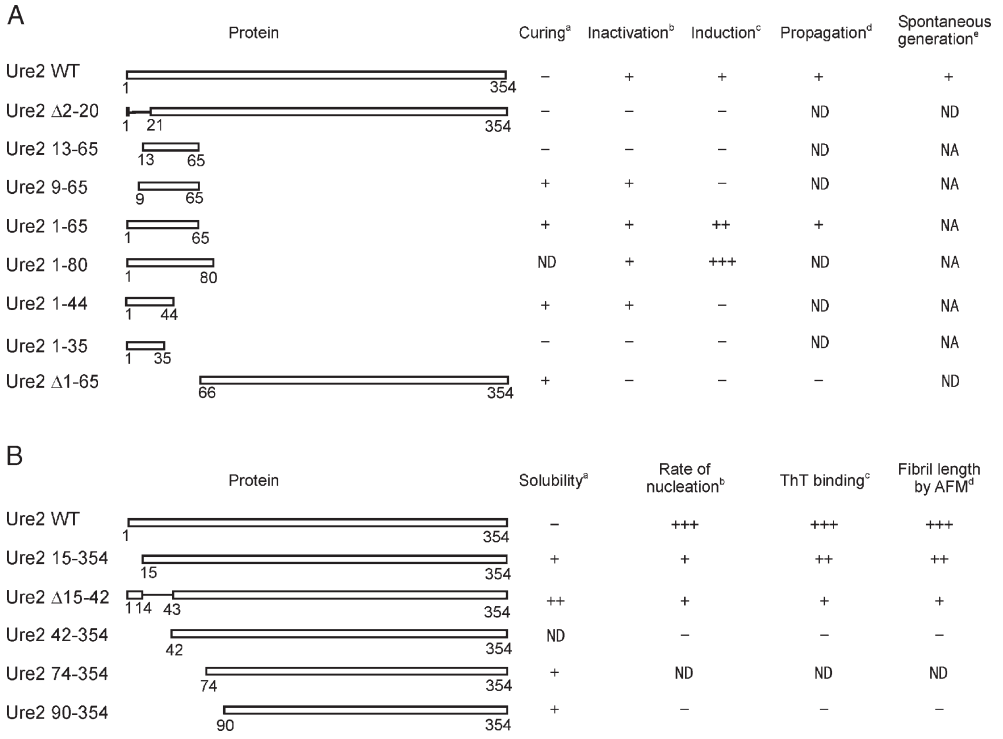

Fig. 4. (A) Mutations in the N-terminal prion domain of Ure2 that affect prion properties in vivo. Experimental details are given in the references indicated. +, observed; −, not observed (or very low rate of occurrence); ND, not determined; NA, not applicable. [a]Curing of the prion state when the gene is expressed in a WT background in an initially [*URE3*] prion strain [70,120]. [b]Inactivation of the Ure2 protein (but not necessarily induction of a stable prion [*URE3*] state) when the gene is expressed in vivo in a WT background [70]. [c]Induction of the [*URE3*] prion when the gene is expressed in a WT background in an initially non-prion strain [31]. [d]Ability to propagate the prion state when the gene is expressed in vivo in a null (*ure2*Δ) background [69]. [e]Spontaneous generation of the [*URE3*] prion when the gene is expressed in vivo in a *ure2*Δ background [69]. (This is not applicable to N-terminal fragments that lack the Ure2 functional region because they cannot show the heritable loss of Ure2 function, which is the signature of the [*URE3*] prion.) (B) Effect of N-terminal mutations in vitro. [a]Solubility in *E. coli* cell extracts or in pure solution in phosphate buffer, pH 7.5 [27]. Note that all constructs can be solubilized using Tris–HCl buffer, pH 8.4 [27], and that amyloid-like fibrils of Ure2 PrD fusion proteins have been observed in bacterial cell extracts [109]. [b]Relative rate of nucleation of amyloid-like structure, assayed by ThT binding (+++, shorter lag time; +, longer lag time; −, no increase in ThT binding observed) [34]. [c]Relative ThT binding in plateau phase [34]. [d]Relative lengths of fibrillar structures detected by AFM after incubation in phosphate buffer (+++, long fibrils; ++, short fibrils; +, rods; −, no fibrils). Note that WT and mutant fibrils show the same time-dependent changes in fibril thickness (height) under all conditions and that no differences in fibril length were observed in Tris–HCl buffer [34].

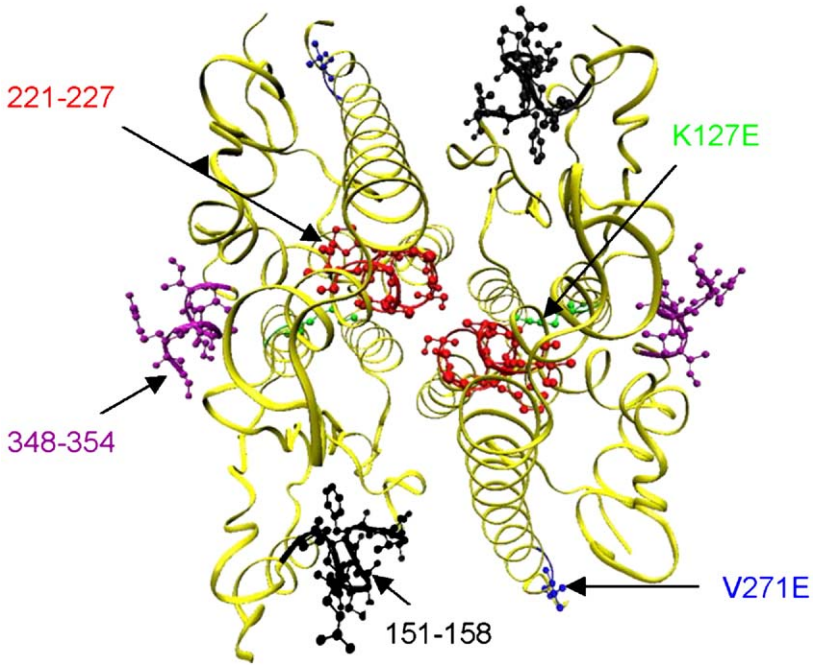

Fig. 5. Crystal structure of the C-terminal globular domain of Ure2 (PDB code 1g6w) showing the sites of mutations that affect prion function, as indicated. The mutations are the deletion of residues of 151–158, deletion of residues 221–227, deletion of the C-terminal 7 residues [72]; and the mutants K127E or V271E when combined with point mutations in the N-terminus (see text) [73]. The figure was produced using the program VMD [119].

structure and formation of the structural motif recognized by the amyloid-specific dye Thioflavin T in vitro [34]. On the other hand, a series of Ure2 variants produced by scrambling the sequence within the Ure2 PrD were found to behave as prions in vivo and form amyloid fibrils in vitro [71], suggesting that the prion properties of Ure2 principally reflect the amino acid composition of the prion domain, rather than the specific primary sequence. Therefore the reason for the conservation of sequence in the Ure2 PrD between different yeast species remains an interesting question.

Mutations affecting the prion properties of Ure2 are not restricted to the N-terminal PrD. A number of deletions in the C-terminal region have been found to increase or decrease the prion-inducing propensity of Ure2 [72]. These include deletion of residues 221–227, which decreases prion induction, and the deletions 151–158 or 348–354, which increase the rate of prion induction. Interestingly, while deletion of the prion domain (1–65) alone removes prion inducing ability, this deletion in combination with the deletion of the regions 151–158 and 348–354 produces a fragment that can once again induce prion formation. In addition to these deletions, screening of a mutagenised library identified a mutant of Ure2 containing a series of point mutations (2 in the N-terminal domain, 7 in the C-domain) that results in a 1000-fold increase in the rate of prion induction when the mutant is overexpressed in a WT background [73]. In fact, 50% of this increase in induction rate could be attributed to a pair of mutants in the N-terminus (S10L and R17C) combined with a single mutation in the C-terminus (K127E). While this triple mutant is non-functional in nitrogen regulation, a mutation pair (S10L/V271E) was identified that is functional, but is still 10× more efficient than WT at inducing the [*URE3*] prion phenotype [73]. The results of these mutagenesis studies imply not only the involvement of the C-terminal region in the mechanism of prion formation, but also that there is interplay between the N-terminal and C-terminal regions of the protein. The locations of these various mutations are scattered across the protein structure (see Fig. 5). Therefore, it is hard to predict simply from inspection of the wild-type structure the effect the mutations have on the conformation and folding of the protein. Further biophysical studies to characterize the structural and folding properties of these mutants may yield important insight into the molecular mechanism of prion induction and propagation.

3.2. Chaperones

Molecular chaperones play an important role in the cell to ensure that proteins fold correctly to their native functional conformation [74,75]. Many chaperones are also classed as heat shock proteins (Hsps), as their expression is up-regulated under conditions of cellular stress, when proteins may be particularly likely to unfold and aggregate. Genetic studies have identified a number of chaperones that modulate the behaviour of yeast prions [76]. In particular, the three best characterized *S. cerevisiae* prions, Sup35, Ure2 and Rnq1, all require normal expression levels of the chaperone Hsp104 in order to be propagated efficiently [13,77,78]. In the case of Sup35, inhibition of the ATPase activity of Hsp104 similarly interferes with prion propagation [79–81] and overexpression of Hsp104 also results in curing of the prion phenotype [77]. To account for these observations, it is proposed that Hsp104 is required to produce new seeds from existing prion aggregates; while

overexpression of Hsp104 results in dispersion of the prion aggregates [82–86]. This is consistent with the cellular role of Hsp104 in disassembly of existing protein aggregates, in cooperation with the Hsp70 chaperone system [87,88].

Hsp70 and its co-chaperone Hsp40 have also been implicated in the propagation of yeast prions. Overexpression of Ydj1–Hsp40 or Ssa1–Hsp70 (but not Ssa2–Hsp70) is able to cure [*URE3*] [78,89]. In addition, a missense mutation within the Ssa2–Hsp70 peptide-binding domain alters [*URE3*] stability [90]. Detailed genetic analysis has identified mutations in both the ATPase and peptide-binding domains of Ssa1–Hsp70 that alter stability of the [PSI^+] prion [91,92]. However, the clear predominance of Hsp70 ATPase domain mutations that alter [PSI^+] propagation suggests that modifying the rate of ATP hydrolysis is a major factor in the ability of Hsp70 to alter prion propagation [92] (H.M. Loovers and G.W.J., unpublished data). Interplay between Hsp104 and Hsp70 in maintenance of [PSI^+] is also observed [93]. Sis1–Hsp40 (but not Ydj1) is required for propagation of [RNQ^+], although immunoprecipitation experiments indicate that both proteins interact with Rnq1 [94], and overexpression of Ydj1 is able to cure [RNQ^+] [95].

The complexity of the chaperone–prion relationship is highlighted by the varying effects on prion propagation that have been observed. In addition to the apparent differences in effect on [*URE3*] of different members of the Hsp70–Ssa family, varying effects of Ssa1 upon propagation of [PSI^+] variants has also been reported [96]. Further, while overexpression of Hsp104 causes curing of [PSI^+] [77], stability of [*URE3*] appears to be unaffected [78], even though propagation of both prions is dependent on the presence of normal levels of Hsp104. These varying effects most likely reflect differences in amyloid structure of different prions or prion strains and/or subtle differences in substrate preference for related chaperone machineries.

Involvement of the Hsp70 chaperone system in yeast prion maintenance and curing is consistent with the finding that overexpression of Hsp70 suppresses polyglutamine-associated pathogenicity and reduces the formation of amyloid fibrils [97,98], and suggests that the Hsp70 family may have a universal role in suppressing the formation of pathogenic aggregates, including prions. However, to date, direct demonstration of an interaction between Hsp70 and yeast prions by in vitro biochemical analysis has not yet been reported.

4. Folding and stability of Ure2

4.1. Relationship between folding and amyloid formation

Various models have been proposed to describe the mechanism of amyloid or prion formation [99–101]. In general, amyloidogenic proteins show a sigmoidal time-course of fibril formation and the lag time can be reduced or circumvented by the addition of preformed fibril 'seeds', indicating that the process involves a nucleation step analogous to growth of protein crystals. Another commonly described feature is that the length of the lag time decreases with increasing protein concentration in an approximately exponential relationship. In fact, for Ure2 [36,102] and also for Sup35 [103], the relationship between increasing protein concentration and decreasing lag time is linear. This may reflect a variation in mechanism, the oligomeric nature of the native state, or simply the choice of reaction conditions [36,101,103]. Nevertheless, a common feature of the various models is the involvement of partially-folded (or misfolded) intermediate states, implying that the mechanism of amyloid or prion formation is related to the pathway by which the protein folds (or misfolds). Therefore, characterization of the folding behaviour of the prion proteins is an important step in elucidating the mechanism of prion formation. This is easier said than done, as the prion proteins, by definition, are prone to aggregate, which tends to interfere with the standard approaches used in protein folding studies. Nevertheless, in comparison with the other known prion proteins, the Ure2 protein represents a relatively tractable model and its folding behaviour has already been characterized in some detail.

4.2. Role of the N-terminal prion domain in Ure2 structure and folding

Genetic studies have demonstrated the involvement of the N-terminal prion domain in [*URE3*] prion induction and propagation [31], whereas the nitrogen regulatory function is conveyed by the GST-like C-terminal region [29]. Subsequent biophysical analysis of the purified Ure2 protein has shown that the structural properties of the N-terminal and C-terminal regions are also distinct. The N-terminal domain is extremely sensitive to protease digestion [26–28,104] and progressive deletion of the N-terminal domain has no effect on the dimerisation, stability or folding kinetics of the protein in vitro [27,33,35,36,105] (see Fig. 6). This implies that the N-terminal prion domain is in a relatively unstructured, flexible conformation and does not interact significantly with the C-terminal globular domain in the native state. A yeast two-hybrid analysis study detected interaction between N- and C-terminal fragments of Ure2 [106] and the presence of the N-terminal domain improves the efficiency of the nitrogen catabolite repression activity of the C-terminal domain [31,69], suggesting that these domains are able to interact in vivo. However, a more recent study failed to detect an interaction by yeast two-hybrid analysis [107]. It has also been confirmed by NMR spectroscopy that the N-terminal domain is unstructured in vitro [107]. Thus the support for direct interaction between the N-terminal and C-terminal domains of Ure2 seems to be ephemeral, but it remains likely that indirect interaction may occur via a binding partner such as Gln3 and/or a chaperone.

A common theme in the conversion from native to prion forms for both mammalian and fungal prions appears to be the transition from an unstructured to a β-rich form, which is analogous to the conformational changes observed for natively-unfolded amyloidogenic proteins such as the Parkinson's Disease-related protein α-synuclein and the Alzheimer's Disease-related protein tau [108]. The ability to form fibrils of a variety of chimeras containing the unstructured Ure2 prion domain attached to unrelated globular protein domains

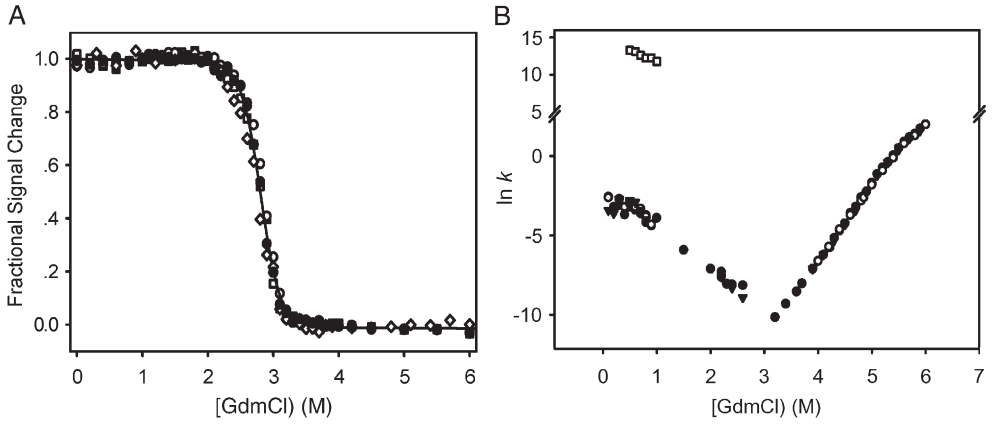

Fig. 6. Effect of deletion of the N-terminal prion domain on Ure2 stability and folding kinetics. The experimental conditions were 50 mM Tris–HCl buffer pH 8.4, 0.2 M NaCl. (A) Guanidinium chloride induced denaturation of WT Ure2 (●) and N-terminal deletion mutants (see Fig. 2) 15Ure2 (○), 42Ure2 (□) and 105Ure2 (◇). Data taken from [36] and (Y.J. et al. and S.P., in preparation). (B) Chevron plot showing the dependence of the unfolding and refolding rate constants for WT Ure2 (▼) and N-terminal deletion mutants 90Ure2 (○) and Δ15–42Ure2 (●). Second-order rate constants for the fast phase of refolding (□). Data taken from [35].

[109,110] suggests that the N-terminal prion domain is the principal driving force in Ure2 fibril formation.

4.3. Comparison of different denaturation methods

Ure2 is a remarkably stable protein and derives a significant amount of this stability from its existence as a dimer [36]. The majority of folding studies that have been carried out have used the chemical denaturant and salt, guanidinium chloride (GdmCl). This is because milder (and arguably more physiological) denaturants such as acid, alkali, urea (up to 8 M) [27] or pressure (up to 600 Mpa) [105,111] are each insufficient to unfold the protein; and temperature denaturation is irreversible [27]. In order to guarantee that the thermodynamic parameters obtained from denaturation experiments are meaningful and can be interpreted, it is important to demonstrate reversibility of unfolding; typically this is done by gradually unfolding the protein and comparing the results obtained when the experiment is done in reverse i.e. by first completely unfolding the protein and then gradually refolding it. In the case of Ure2, to obtain ideal results in this two-way experiment requires a careful balance of pH, temperature, protein concentration and choice of buffer [27]. The conditions found to be 'ideal' for folding experiments (Tris–HCl buffer pH 8.4, 0.2 M NaCl, 25 °C and 1 μM protein concentration) unsurprisingly resemble the conditions adopted for Ure2 purification in a variety of labs [26,27,32,109], and coincide with maximal stabilization of the native state of Ure2 with respect to partially-folded intermediates [36]. Nevertheless, under these conditions, the folding kinetics of Ure2 is extremely complex and a variety of folding intermediates are transiently populated during unfolding or refolding [27,35,36].

Although the N-terminal domain does not contribute to the stability of the Ure2 protein in vitro [27], or affect the pathway of folding [35], the presence of the prion domain has a marked effect on the solubility of the protein [27]. Interestingly, conditions where Ure2 is relatively destabilized, such as at lower pH, or in phosphate rather than Tris buffer, also coincide with conditions where the protein more readily forms fibrils, implying that the mechanism of fibril formation may involve partial unfolding [33,36]. However, destabilizing conditions are also associated with an increased degree of non-specific (i.e. PrD-independent) aggregation, which can compete with fibril formation and complicate the analysis [36]. Another interesting observation is that relatively small deletions in the N-terminal domain, such as deletion of residues 1–15 or 15–42 (see Figs. 2 and 4) have a marked effect on both the solubility of the protein [27] and the lag time of fibril formation [34], implying that these regions contribute to nucleation of amyloid-like structure. The lower propensity of mutants such as Δ15–42Ure2 (see Fig. 2) to undergo non-specific aggregation and to form amyloid makes them particularly useful for use in rigorous folding studies [27,35,36,105,112].

4.4. Mechanism of folding

The Ure2 protein readily populates an intermediate under equilibrium conditions at GdmCl concentrations of 2–3 M; the protein concentration dependence of the further unfolding of this intermediate demonstrates that dissociation is coupled to unfolding and hence the intermediate is dimeric rather than monomeric (see Fig. 7A). No other intermediates are readily detected under equilibrium conditions, which reflects the relative stability of the native dimer and dimeric intermediate compared to their monomeric counterparts [36]. The native state is destabilized with respect to the dimeric intermediate at pH lower than 8.5 (see Fig. 7B), or in phosphate compared to Tris buffer (see Fig. 7B, inset), and so the intermediate is more easily observed under these conditions.

Kinetic folding studies using stopped-flow methods allow the detection of further intermediates. When Ure2 is unfolded in GdmCl and then refolded by rapid dilution, three folding phases can be distinguished, implying multiple processes in refolding, such as population of folding intermediates [35]. The fastest phase is attributed to population of a monomeric intermediate, on the basis that this intermediate can be trapped by aggregation

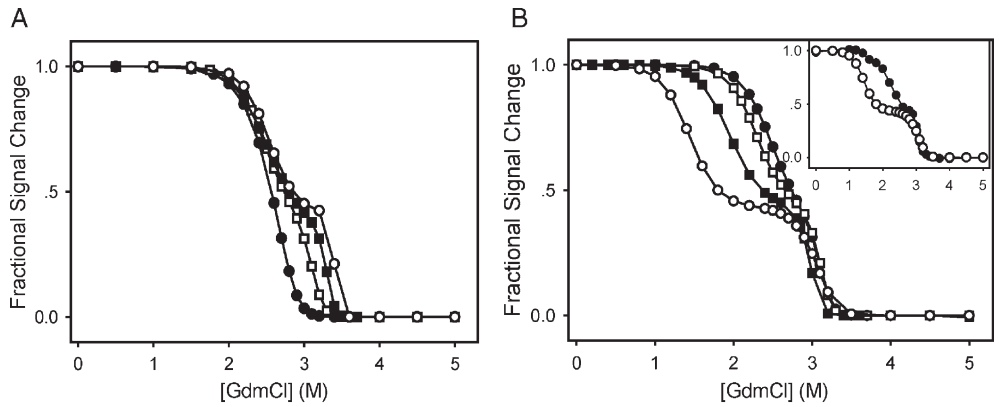

Fig. 7. Protein concentration and pH dependence of Ure2 denaturation induced by guanidinium hydrochloride. The results indicate that a dimeric intermediate is populated during denaturation; and the native state is progressively destabilized when the pH is decreased from 8.5 to 7.0. Experimental conditions were 50 mM Na–K phosphate buffer, 0.15 M NaCl and 1 μM Ure2, unless otherwise stated. Data taken from [36]. (A) Ure2 concentration 0.2 μM (●), 1 μM (□), 5 μM (■) and 20 μM (○) at pH 8.5. (B) pH 7.0 (○), pH 7.5 (■), pH 8.0 (□) and pH 8.5 (●). Inset: phosphate (○) and Tris–HCl (●) buffer at pH 7.0.

at low protein concentration or low temperature, conditions that disfavour dimerisation and correct folding [27,35]. The next observable folding phase shows protein concentration dependence consistent with a second order reaction i.e. this phase corresponds to formation of a dimeric intermediate [35]. The final observable folding phase is protein concentration independent. Unusually, it is possible to detect a lag for this folding phase; the length of the lag decreases with increasing protein concentration. This then indicates that final folding to the native state occurs only after formation of the dimeric folding intermediate and that formation of this intermediate is an obligatory step on the folding pathway [35]. In fact, structural changes continue to occur even after no further spectroscopic changes are detected and the native state of Ure2 is regained only very slowly; this slow structural rearrangement is attributed to the unusual cis conformation of Pro166 in the native state, requiring isomerisation from the dominant trans form [35]. The inability to distinguish the true native state from the spectroscopically similar pseudo-native state is a common problem in folding studies. These two states of Ure2 can be distinguished by their markedly different stabilities and unfolding rates, as detected by a 'double-jump' experiment [35]. Another method for distinguishing such states is by activity assay, and so the recent development of an in vitro assay for Ure2 enzyme activity [64] provides a useful tool for further folding studies.

A further folding intermediate can be detected during unfolding experiments and has been identified as a native-like monomer [36]. This intermediate is only populated under extreme conditions of high denaturant concentration, reflecting the extreme stability of the Ure2 dimer, even at micromolar protein concentrations [35,36]. Tethering the two halves of the Ure2 dimer by the introduction of an artificial disulphide bond prevents fibril formation under conditions where the wild-type protein forms fibrils, suggesting that dimer dissociation is necessary for Ure2 fibril formation [113], although the effect of the disulphide bond on other aspects of the stability and folding of the protein could also play a role. The relationship between population of a partially-folded intermediate and conditions which favour fibril formation also points towards partial unfolding in the process of amyloid formation [33,36]. Likewise, the ability of mutations in the C-domain to influence prion induction [72,73] implies that the mechanisms of folding and fibril formation are intertwined. However, which, if any, of the intermediates that have been described thus far are involved in this process remains an open question. Further, the propensity of these intermediates to undergo non-specific aggregation [27,33,35,36,112] presents a significant challenge to their direct characterization.

5. Conclusions

The Ure2 protein shows a number of remarkable features, not least its ability to undergo a heritable conformational change to an aggregated prion state, while still retaining the enzymatic activity of its native form. The presence of natively unfolded structure in the Ure2 N-terminal prion domain is a feature that is common to the prion proteins identified to date and may be an essential characteristic of prions. The ability of C-terminal deletions or mutations to influence prion induction suggests that there is some involvement of the C-terminal domain in the mechanism of Ure2 prion formation; but whether this is due to a direct effect (such as on the thermodynamics or kinetics of folding or dimer dissociation) or an indirect effect (such as on the recruitment and binding of chaperones or other cofactors) remains to be determined. The convenience of the yeast system to screen for factors that influence prion stability in vivo, combined with the suitability of the Ure2 protein for detailed folding analysis in vitro, makes this a promising model to shed light on the molecular mechanism of prion propagation.

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