The Cellular Concentration of the Yeast Ure2p Prion Protein Affects Its Propagation as a Prion

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The [URE3] yeast prion is a self-propagating inactive form of the Ure2p protein. We show here that Ure2p from the species Saccharomyces paradoxus (Ure2p<sub>sp</sub>) can be efficiently converted into a prion form and propagate [URE3] when expressed in Saccharomyces cerevisiae at physiological level. We found however that Ure2p<sub>sc</sub> overexpression prevents efficient prion propagation. We have compared the aggregation rate and propagon numbers of Ure2p<sub>sp</sub> and of S. cerevisiae Ure2p (Ure2p<sub>sc</sub>) in [URE3] cells both at different expression levels. Overexpression of both Ure2p orthologues accelerates formation of large aggregates but Ure2p<sub>sp</sub> aggregates faster than Ure2p<sub>sc</sub>. Although the yeast cells that contain these large Ure2p aggregates do not transmit [URE3] to daughter cells, the corresponding crude extract retains the ability to induce [URE3] in wild-type [ure3-0] cells. At low expression level, propagon numbers are higher with Ure2p<sub>sc</sub> than with Ure2p<sub>sp</sub>. Overexpression of Ure2p decreases the number of [URE3] propagons with Ure2p<sub>sc</sub>. Together, our results demonstrate that the concentration of a prion protein is a key factor for prion propagation. We propose a model to explain how prion protein overexpression can produce a detrimental effect on prion propagation and why Ure2p<sub>sp</sub> might be more sensitive to such effects than Ure2p<sub>sc</sub>.

INTRODUCTION

Prions were originally defined as a unique class of infectious agents composed solely of protein. In mammals, prions cause fatal neurodegenerative diseases, such as Creutzfeldt-Jacob disease in human, sheep scrapie, and bovine spongiform encephalopathy (Prusiner et al., 1982; Aguzzi et al., 2007; Collinge and Clarke, 2007). All these diseases are linked to abnormal self-propagating conformations of a cellular protein termed PrP. The yeast Saccharomyces cerevisiae possesses at least four different prion proteins (Sup35, Ure2p, Rnq1p, and Swi1p) that can generate phenotypes ([PSI<sup>+</sup>], [URE3], [PIN<sup>+</sup>] and [SWI<sup>+</sup>], respectively) as a direct consequence of an inherited conformational change (Wickner, 1994; Derkatch et al., 2001; Du et al., 2008). Yeast prions are denoted within brackets and by capital letters to specify that they correspond to cytoplasmically transmitted inheritable elements that are dominant in genetic crosses. The yeast prion proteins exhibit clear mechanistic similarities with PrP. In particular, mammalian and yeast prions have the ability to polymerize into amyloid aggregates (for reviews, see Baskakov, 2007; Wickner et al., 2007). Interestingly, biochemical pathways controlling prion formation and/or maintenance seem conserved from yeast to humans because antiprion drugs, isolated in yeast-based screening assays, inhibit mammalian prion propagation and vice versa (Bach et al., 2003).

Numerous results highlight the clear connection between the yeast prion phenotype and the aggregation of the protein involved in this process (Patino et al., 1996; Edskes et al., 1999; Paushkin et al., 1997; Zhou et al., 2001). Reconstitution of in vivo infectivity from amyloid fibers formed in vitro from purified protein has definitively proven the protein-only hypothesis for prion formation and established indubitably the link between amyloid fibers and the prion form propagated in cells (Tanaka et al., 2004, 2006; Brachmann et al., 2005; King and Díaz-Avalos, 2004; Patel and Liebman, 2007). Other proteins form amyloids in an autocatalytic manner, but only prion proteins are infectious. The exact nature of the self-propagating species still remains unclear. Indeed, the Ure2p aggregates isolated from [URE3] cells differ from amyloid filaments formed in vitro (Ripaud et al., 2004). Genetics studies on yeast prions have revealed the existence of cellular factors required for prion aggregation and propagation. Notably, the de novo appearance and subsequent propagation of yeast prions is highly dependent on the expression levels of a number of molecular chaperones (reviewed in Perrett and Jones, 2008). Biochemical analysis of [PSI<sup>+</sup>] propagating yeast extracts showed that Ssa1/Ssa2 chaperones were the major components interacting with infectious Sup35 aggregates (Bagrantsiev et al., 2008).

To ensure an efficient transmission of prion in actively dividing cells, prion aggregates must be continually subdivided to yield additional replication templates known as prion seeds or propagons. The disaggregating Hsp104 chaperone has the most critical and universal role in yeast prion maintenance, and it is essential to remodel existing prion complexes to generate or maintain propagons (Chernoff et al., 1995; Satpute-Krishnan et al., 2007). Then, propagons are...
partitioned to daughter cells, where they continue to direct the conversion of neosynthesized protein to the prion form and ensure inheritance of the associated phenotype.

The Ure2p prion protein controls catabolism of nitrogen sources. In the presence of a good nitrogen source, Ure2p blocks the uptake of poor nitrogen sources by sequestering the transcription factor Gln3p in the cytoplasm (Beck and Hall, 1999; Cox et al., 2000). One of these genes activated by Gln3p is the allantoate permease gene, DAL5 (Turovec and Cooper, 1987). Because of the structural similarity between allantoate and ureidosuccinate (USA), an essential intermediate of uracil biosynthesis, Dal5p can take up USA (Aigle and Lacroute, 1975). Prion conversion of Ure2p leads to its loss of function and consequently, enables the uptake of ureidosuccinate (USA) and permits growth on an ad hoc selective medium. Ure2p is a two-domain protein. The asparagine and glutamine rich N-terminal domain of Ure2p, termed prion-forming domain, is unstructured in the soluble form of the protein and is crucial for prion properties, whereas its C-terminal domain is compactly folded and sufficient to perform the cellular function in nitrogen regulation (Maison and Wickner, 1995). Although the catalytic function of Ure2p in nitrogen regulation was found to be conserved among all analyzed Saccharomyces species, controversial results were obtained concerning the conservation of prion-forming properties (Edskes and Wickner, 2002; Baudin-Baillieu et al., 2003). In particular, we did not detect [URE3] prion capacity with Ure2p from Saccharomyces paradoxus (Ure2pSp), whereas H. Edskes and coworkers reported that this protein could form a prion. The conflicting studies differed in two experimental aspects. One difference was the expression level of Ure2p in yeast cells, the other was the existence of a silent mutation in the construct used by Edskes et al. that could lead to an artificial translational read-through of URE2 (Talarek et al., 2005).

De novo prion generation is a stochastic process. Transient overproduction of the prion protein increases the frequency of prion formation (Wickner, 1994; Derkatch et al., 1996). This property was proposed by R. Wickner as one of the indicators that a heritable non-Mendelian state is due to a prion-based determinant and is now regarded as a fundamental property of such systems (for review, see Wickner et al., 2006). The rationale behind this argument is simply that a higher concentration of the protein enhances the probability of prion nucleation. However, high amounts of prion protein can also interfere with propagation (Edskes et al., 1999; Ripaud et al., 2003; Allen et al., 2005). For example, high overexpression of Ure2p leads to curing of the [URE3] prion.

A complex relationship apparently exists between prion properties and the amount of the prion protein. In the present work, we wondered whether the concentration of Ure2p could interfere with its prion properties. We report here that, when expressed at physiological level, Ure2p can convert into the prion form either spontaneously or after contact with preexisting [URE3]. But, we show that the Ure2p concentration controls the ability to induce and propagate [URE3]. We found that the number of [URE3] propagons is lower when propagated with Ure2pSc than with Ure2pSp and diminishes with increase of the cellular concentration of Ure2p. Together, our results suggest that the aggregation dynamics of Ure2p and [URE3] propagation depends both on the expression level of Ure2p and on the sequence of the prion-forming domain. This work shows that concentration of a prion protein contributes to determine its prion properties.

**MATERIALS AND METHODS**

**Nomenclature**

To avoid confusion, we used *S. cerevisiae* (Sc) or *Saccharomyces paradoxus* (Sp) in subscript to specify the gene origin. For prion nomenclature, [ure3-0] is used to describe the soluble or nonprion status of Ure2p and [URE3] is used to name the prion status.

**Plasmids and DNA Manipulations**

All plasmids used in this study are listed in Table 1. The full-length URE2 open reading frames (ORFs) of the *S. cerevisiae* and *S. paradoxus* were placed under control of the inducible GAL10-CYC1 promoter and are carried either by the monocopy plasmid (with an ARS-CEN origin of replication) pG1URE2Sc and pG1URE2Sp or by the multicopy plasmid (with a 2 μ origin of replication) pG2URE2Sc and pG2URE2Sp. The multicopy plasmids pG2URE2Sc-green fluorescent protein (GFP) and pG2URE2Sp-GFP were originally described by Ripaud et al. (2003) and Immel et al. (2007), respectively. pFL39-URE2Sc containing the URE2Sc ORF flanked by promoter and terminator sequence of URE2Sc was constructed as follows. The URE2Sc ORF was amplified using oligonucleotide 37 (5′-GTCAATTGTTTAAACTGAAATTAAGTTGATCAACAAATGGAATAACACGAAAC-3′), which introduces a sequence homologous to the URE2Sc promoter at the 5′ of the gene and oligonucleotide 41 (5′-TTTCTCTCTTCTCTTCTCTTCTTTTTAACGACGTTTATTTCTAACCAATTGGTGATCAACCAATGGAATAACACGAAAC-3′), which introduces a sequence homologous to the URE2Sc terminator at the 3′ of the gene. This fragment was cloned into the pFL39[pFL39-URE2Sc carrying a genomic fragment of URE2Sc] by using the gap repair method (Or-Werbel and Szostak, 1983).

**Strains, Media, and Genetic Methods**

All strains used in this study are disrupted for the URE2 gene. *S. cerevisiae* strain CC50 (MATa, trp1-1, ade2-1, leu2-3, 112, his3-11, 15, Δura2::HIS3) was used as the [ure3-0] parent and is termed URE2Δ in this publication (Fernandez-Bellot et al., 2000). The strain AR34, termed here URE2Sc [URE3], is isogenic to URE2Sc and carries the [URE3] strain originally described by Aigle and Lacroute (1975) that was transmitted by cytoduction. To integrate the

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on ice. Precipitates were collected by centrifugation at 14,000 rpm for 10 min on ice. Trichloroacetic acid was added to a final concentration to the loading buffer of all extracts from yeast cells propagating through exponential phase were permeabilized with 1 ml of 0.185 M NaOH, 0.2% SDS, 0.1 M Tris-HCl, pH 6.8, 4 mM EDTA, 20% glycerol, 2% 2-mercaptoethanol.

RESULTS

**Ure2sp Can Adopt a Prion Form When Expressed at Physiological Level**

To determine whether Ure2sp, can sustain [URE3] propagation when it is expressed at physiological level, we constructed an S. cerevisiae strain in which the URE2sp ORF was integrated at the URE2 locus under control of the endogenous URE2 promoter (this strain is termed URE2sp). The expression of Ure2sp was similar to that of Ure2sp, as determined by Western blotting (Figure 1A). As reported previously, Ure2sp, can perform the nitrogen metabolism repression function of Ure2sp (Figure 1B) (Baudin-Bailieu et al. 2003).

We wondered whether the Ure2sp protein could be converted into a prion form in the presence of preexisting [URE3]. For that purpose, we crossed the URE2sp strain with a URE2sp strain propagating [URE3]. The resulting URE2sp X URE2sp [URE3] diploids displayed the USA+ phenotype, whereas the URE2sp X URE2sp [ure3-0] diploid remained wild type for Ure2 function (Figure 1B). To determine whether [URE3] could be transmitted to cells expressing only Ure2sp, we analyzed the meiotic progeny of the [URE3] diploid. We observed a non-Mendelian inheritance of the USA+ phenotype (Figure 1C). This USA+ phenotype segregated independently of the URE2sp or URE2sc alleles (detected by PCR analysis; data not shown). USA+ phenotypes were cured after growth on YPD medium containing 3 mM GuHCl.
Ure2p Overexpression and Prion Propagation

Figure 1. Ure2p<sub>Sp</sub> can form and propagate the [URE3] prion. The URE2<sub>Sp</sub> open reading frame was inserted in place of the URE2<sub>Sc</sub> open reading frame at the resident URE2 locus to create a URE2<sub>Sp</sub> strain. (A) Comparative analysis of expression levels of Ure2p<sub>Sp</sub> and Ure2p<sub>Sc</sub> Quantification of Ure2p was performed by Western blot analysis with an anti-Ure2p antibody. An antibody against Ade13p was used as loading control. Molecular weights are indicated on the right side. (B) Comparative growth on SD medium of functional Ure2p in the cells makes the strain unable to grow on SD + uracil and SD + USA medium. Growth of cells was observed after 4 d at 30°C. The presence of functional Ure2p in the cells makes the strain unable to grow on SD + USA. Orthologous URE2 alleles expressed in each strain and [ure3-0] or [URE3] prion states are indicated. (C) Non-Mendelian segregation of [URE3] in tetrad from URE2<sub>Sp</sub>/URE2<sub>Sp</sub> [URE3] diploids. Meiotic progeny (spores A, B, C, and D) of five different tetrads (T1 to T5) were spotted on SD supplemented with uracil or USA. To test whether the USA<sup>+</sup> phenotype in the spore was curable, cells were streaked for single colonies on YPD medium containing 3 mM GuHCl. Single colonies were then tested for their ability to use USA (USA post-GuHCl). Frequencies of USA<sup>+</sup> colonies were determined as described in Materials and Methods. SEs are indicated.

Figure 2. Biochemical and cellular characterization of the [URE3] prion propagated by Ure2p<sub>Sp</sub>. (A) Sedimentation analysis of Ure2p<sub>Sp</sub> in a URE2<sub>Sp</sub> strain with or without [URE3]. S, supernatant fraction, P, pellet fraction. Fractions were analyzed by Western blot with an anti-Ure2p antibody. Molecular weight markers are given on the right side. (B) Fluorescence microscopy assay of Ure2p<sub>Sp</sub> aggregation status. Ure2p<sub>Sp</sub>GFP in [ure3-0] and [URE3] URE2<sub>Sp</sub> cells were observed after 2 h of growth in galactose medium.

GuHCl. We conclude from these results that Ure2p<sub>Sp</sub> can propagate the [URE3] prion.

The aggregation and resulting insolubility of Ure2p are hallmarks of the [URE3] prion state (Edskes et al., 1999; Ripaud et al., 2004). To characterize biochemically the prion form of Ure2p<sub>Sp</sub>, we analyzed the solubility of Ure2p<sub>Sp</sub> in USA<sup>+</sup> cells by cellular fractionation after ultracentrifugation of yeast extracts. We found that Ure2p<sub>Sp</sub> is specifically aggregated in [URE3] cells (Figure 2A). Furthermore, the cellular distribution of Ure2p<sub>Sp</sub> was monitored by GFP fluorescence. For that purpose, URE2<sub>Sp</sub> [ure3-0] and [URE3] strains were transformed with a plasmid expressing an inducible fusion of Ure2p<sub>Sp</sub> and the green fluorescent protein (Ure2p<sub>Sp</sub>-GFP). Induction of Ure2p<sub>Sp</sub>-GFP expression in cells containing [URE3] led primarily to a punctuate fluorescence pattern, whereas [ure3-0] cells displayed diffuse fluorescence (Figure 2B). These data indicate that the [URE3] prion of Ure2p<sub>Sp</sub> corresponds to a self-propagating aggregate.

Together, these results show that Ure2p<sub>Sp</sub> can propagate the [URE3] prion. We next wondered whether Ure2p<sub>Sp</sub> can also promote spontaneously [URE3] formation, because aggregation and propagation of yeast prion could be mediated by distinct mechanisms (Derkatch et al., 2000; Zhou et al., 2001; Bradley and Liebman, 2004). Frequency of USA<sup>+</sup> formation was determined for a URE2<sub>Sp</sub> strain. Spontaneous USA<sup>+</sup> formation occurred at about the same rate as in URE2<sub>Sc</sub> cells (~2 in 10<sup>6</sup> cells) (Figure 1D). The majority of the USA<sup>+</sup> cells were cured in presence of 3 mM GuHCl. As expected, when reselected on USA-containing medium, USA<sup>+</sup> clones occurred in such cured clones at a similar frequency as in the original cells (data not shown). We conclude from this set of experiments that when expressed at physiological level, Ure2p<sub>Sp</sub> can spontaneously form [URE3] and stably propagate it.

High Overexpression of Ure2p<sub>Sp</sub> Abolishes Its Prion Properties

In apparent contradiction with the results reported in the previous section, we had reported previously that Ure2p<sub>Sp</sub> is not able to convert into the [URE3] prion form (Baudin-Baillié et al., 2003; Talarek et al., 2005). Because the only difference between the present experiment and these previous studies was the use of different promoters, we wondered whether the capacity to support prion propagation could be dependent on Ure2p<sub>Sp</sub> cellular concentration. So, we expressed different amounts of Ure2p<sub>Sc</sub> and Ure2p<sub>Sp</sub> in URE2<sub>Sc</sub> and URE2<sub>Sp</sub> [URE3] cells, respectively. We used monocopy or multicopy plasmids carrying the URE2<sub>Sc</sub> or URE2<sub>Sp</sub> ORF under control of the GAL10 promoter. After growth in galactose medium, the amount of Ure2p was quantified by Western blotting experiments (Figure 3A). Monocopy
multicopy plasmids led to approximately a 50- and a 500-fold overexpression, respectively, compared with Ure2p expressed from URE2 at the chromosomal locus. It was noted that the amount of Ure2p_Sp in yeast, regardless of the promoter, is slightly higher than Ure2p_Sc, while S. cerevisiae and S. paradoxus share the same sequence in their globular domains, and the differences are restricted to the prion-forming domain (Supplemental Figure S1) (Edskes and Wickner, 2002; Baudin-Bailieu et al., 2003). Because this domain is essential to stabilize the catalytic domain of Ure2p (Shewmaker et al., 2007), the sequence differences in the prion-forming domain sequence between two orthologues might result in distinct stability of Ure2p.

We then tested the ability of the yeast cells to propagate [URE3] by testing their capacity to grow on galactose medium containing USA. As a control, we confirmed that the transformation per se did not impair the prion phenotype (Figure 3B, SD+USA). As published previously, a strong overexpression of Ure2p_Sp is compatible with growth on USA medium, whereas a very strong overexpression of Ure2p_Sc is not (Figure 3B, SG+USA) (Baudin-Bailieu et al., 2003). Intermediate overexpression of Ure2p_Sc or Ure2p_Sp did not affect [URE3] propagation (Figure 3B, SG+USA). The observation that Ure2p_Sp overexpression led to severe inhibition of prion propagation contrasts with the fact that transient overexpression of Ure2p increases the frequency of prion formation (Wickner, 1994; Baudin-Bailieu et al., 2003). So, we have also determined the effect of massive Ure2p_Sp transient overexpression on [URE3] formation. We transiently expressed various amounts of Ure2p_Sc or Ure2p_Sp. Prion apparition was assayed as formation of USA+ colonies on a dextrose medium (Figure 3C). As reported previously, an increase of Ure2p_Sc promoted the appearance of [URE3] at a higher frequency (Wickner, 1994). The difference in the induction rate was not statistically significant when Ure2p_Sc was overexpressed either from a multicopy plasmid or multicopy plasmid. A mild overproduction of Ure2p_Sp also led to a high induction of [URE3] formation. In contrast, such an induction rate was not observed after a high transient overexpression of Ure2p_Sp. We got the same results when transient overexpressions were performed in a URE2_Sp strain (data not shown).

Overall, our results indicate that prion formation rate is not strictly correlated with the expression level of Ure2p_Sc or Ure2p_Sp and in particular that high overexpression of Ure2p_Sp dramatically lowers [URE3] formation rate.

Ure2p_Sp and Ure2p_Sc Aggregate in [URE3] Cells in an Ordered Pathway

Given that there are clear links between the yeast prion phenotype and the aggregation of the protein involved in this process, we decided to compare the aggregation dynamics of Ure2p_Sc and Ure2p_Sp in vivo. To that purpose, we used the Ure2p-GFP protein that when induced in [URE3] cells, decorates the preexisting prion aggregates and allows their visualization (Fernandez-Bellot et al., 2002). The expression system is based on high copy number plasmids, the coding sequence of Ure2p_Sc-GFP or Ure2p_Sp-GFP being under the control of the inducible galactose promoter. Different patterns of aggregation, which were never detected in [ure3-0] cells, were observed. We grouped these patterns in four classes (Figure 4). We distinguished cells with small numerous foci quoted as the small aggregates class. We also observed cells with one large dot among smaller foci (medium aggregates class). The two other classes of aggregation are branching structures in which elongations are short (large aggregates) or long (very large aggregates). Formation of these distinct aggregates was not due to the overexpression of the Ure2p_Sc-GFP construct itself because the same results were obtained when a low expression level of the Ure2p_Sc-GFP fusion was used to decorate the Ure2p aggregates (Supplemental Figure S2).

We quantified the proportion of each of the four defined classes at different times after the induction of the expression of the fluorescent protein in a cell population (Figure 5A). The switch from dextrose to galactose medium was realized during late exponential phase. Because of the metabolic transition in this condition, less than one generation occurs during the first 24 h. We thus consider that we are dealing with a nondividing cell population. For each observation, at least two classes coexisted, but their ratio changed during the kinetics. Immediately after the induction of Ure2p_Sc-GFP or Ure2p_Sp-GFP, most cells belong to the small aggregates class. Few hours later, the proportion of this class has a clear tendency to decrease, and more cells with medium aggre-
gates class were observed. Finally, after a longer induction time, these two classes have completely disappeared and the branching structures were observed (large and very large aggregates class). In individual cells we never observed large aggregates and small aggregates together. The number of visible aggregates in each cell decreased in the course of expression of Ure2p-Gfp, suggesting a coalescence of Ure2p aggregates. The simplest interpretation of these observations is that Ure2p<sub>sc</sub> and Ure2p<sub>sc</sub> aggregate in the same ordered pathway in [URE3] cells and that the observed aggregates types derive from each other leading to aggregates of increasing size and in decreasing number.

Figure 4. Classes of Ure2p-GFP aggregates in [URE3] cells. [URE3] cells were transformed with multicopy pG2URE2<sub>sc</sub>-GFP and pG2URE2<sub>sc</sub>-GFP plasmids. The cells were grown overnight on dextrose medium supplemented with USA. In exponential phase (OD<sub>600</sub> = 1), the cells were placed in galactose medium supplemented with uracil, and different times after the induction, the fluorescent proteins were observed in the cells. Typically observed patterns are shown.

Figure 5. Aggregation dynamics of Ure2p-GFP in [URE3] cells. [URE3] cells were transformed with multicopy pG2URE2<sub>sc</sub>-GFP and pG2URE2<sub>sc</sub>-GFP plasmids. The cells were grown overnight on dextrose medium supplemented with USA. In exponential phase (OD<sub>600</sub> = 1), the cells were placed in galactose (A) or raffinose (B) supplemented with uracil. Different times after the induction, the proportion of cells (among at least 100 fluorescent cells) containing the different class of aggregates were evaluated.

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High Overexpression of Ure2p Accelerates the Aggregation Dynamics of Ure2p

We have demonstrated that the [URE3] prion properties depend on the amount of Ure2p. We therefore studied the aggregation kinetics of Ure2pSc and Ure2pSp at two distinct cellular concentrations of Ure2pSc-GFP and Ure2pSp-GFP. To this purpose, we have induced the expression either with raffinose or raffinose + galactose as the carbon source. Raffinose, contrary to dextrose, does not repress the GAL10 promoter, allowing a weak expression of the protein. In this condition, the proportion of cells containing small aggregates decreased slowly with both orthologues (Figure 5B). Indeed, \( \sim 40\% \) of the cells still correspond to the small aggregates class 24 h after the onset of induction. In contrast, when fluorescent proteins were overexpressed by inducing with galactose, this cell population had totally disappeared 9 h after the induction of overexpression. Moreover, in overexpression conditions, we observed emergence of cells with large aggregates as soon as 6 h after the onset of induction, whereas in the control, they were never formed. When Ure2pSc-GFP or Ure2pSp-GFP was expressed at low levels. These results show that the concentration of cellular Ure2p influences its aggregation dynamics in [URE3] cells.

We also observed that, in galactose + raffinose medium, there is a significant difference in the aggregation dynamic of Ure2pSc-GFP and Ure2pSp-GFP (Figure 5A). The proportion of cells exhibiting small aggregates decreases more rapidly with Ure2pSc-GFP. In fact, 6 h after galactose induction, 58% of cells expressing Ure2pSc-GFP contained small aggregates, whereas the proportion of this population was only 12% with Ure2pSp-GFP. Moreover, Ure2pSp-GFP formed large aggregates more efficiently than Ure2pSc-GFP because after 24 h, 92% of the cells expressing Ure2pSp-GFP contained these particular aggregates compared with 35% for cells expressing Ure2pSc-GFP. In fact, at all time points, aggregates were larger in Ure2pSp-GFP than in Ure2pSc-GFP-expressing cells. These results clearly demonstrated that the aggregation dynamics rely not only on the cellular concentration of the prion protein but also on its primary sequence.

The Aggregation Dynamics of Ure2p Defines the Number of [URE3] Propagons

We wondered whether the differences in aggregation dynamics of Ure2p in [URE3] cells (URE2Sc, URE2Sc, overexpressing Ure2p and URE2Sp strains) could be correlated with variations in the number of [URE3] propagons per cell. We have estimated the number of [URE3] propagons by the method described by Eaglestone et al. (2000). This analysis is based on chemical inactivation of Hsp104 by low concentrations of GuHCl that inhibits prion replication and thus leads to rapid prion loss in dividing cells by progressive dilution of the propagons (Eaglestone et al., 2001; Ferreira et al., 2001). [URE3] strains (URE2Sc, URE2Sc, overexpressing Ure2p and URE2Sp strains) were transferred during log phase into YPD medium supplemented with 3 mM GuHCl; at regular time points, cells density was determined and cultures were spread on plates to determine the percentage of [URE3] loss. In the strain overexpressing Ure2pSc, the overexpression was stopped when cells were transferred into YPD supplemented with 3 mM GuHCl.

As expected, the kinetics of [URE3] curing displayed two phases; there was a lag phase during which no curing occurred followed by a linear increase in the number of cured cells over time (Figure 6) (Eaglestone et al., 2000; Ripaud et al., 2003). The curing profile for the [URE3] URE2Sc strain was much slower than that observed in either the [URE3] URE2Sc strain overexpressing Ure2pSc or the [URE3] URE2Sp strain. For the two latter strains, the elimination of [URE3] was so fast that the lag phase was not always apparent in the four performed independent curing kinetics (Figure 6). The number of propagons in [URE3] cells can be inferred from these kinetics of elimination. From the method developed by B. Cox (Cox et al., 2003), we estimate the apparent number of propagons to be 52 for [URE3] URE2Sc strain. This number is much larger than that found for either [URE3] URE2Sc or [URE3] URE2Sc, overexpressing Ure2p (15 and 10 propagons, respectively). These results show that the number of propagons is influenced by the primary sequence of the Ure2p prion-forming domain, and remarkably, that an increase of cellular concentration of Ure2p decreases the number of propagons. In other terms, we have correlated a variation of aggregation dynamic of Ure2p with variations in the numbers of [URE3] propagons.

Infected by Ure2p Aggregates and Their Ability to Propagate as a Prion Can Be Dissociated

In a previous study, we showed that the formation of large aggregates of Ure2p correlates with [URE3] elimination (Ripaud et al., 2003). We confirm that in these experimental conditions, when only the large and very large aggregates were observed, the majority of cells lost the [URE3] prion. Indeed, when the overexpression of Ure2pSc-GFP in [URE3] cells was stopped after 24 h of induction, only \( 5 \times 10^{-4} \) cells still maintained the USA+ phenotype on glucose medium (data not shown). This strong and fast prion elimination led us to propose that curing of [URE3] occurred by inactivation of propagons through the massive aggregation of Ure2p.
Ure2p aggregates together with a plasmid bearing the yeast cells with a crude extract of a strain containing large yeast cells. To test this hypothesis, we transformed [ure3–0] to induce [URE3] apparition once introduced in [ure3-0] prion aggregates and should therefore conserve their ability. Aggregates should be structurally related to the bona fide bigger aggregates. In the latter hypothesis, the Ure2p large expression of Ure2pSc or Ure2pSp from a monocopy plasmid. If the gene is overexpressed. As a rule, it is widely accepted that "the more you express, the more prion you get." The observation of dividing cells only if, in combination, these parameters surpass a threshold to allow sustained propagation. The native yeast prion proteins change from the functional form to the prion form more frequently if the parent alleles are overexpressed. As a rule, it is widely accepted that "the more you express, the more prion you get." The observed frequency of prion formation after a transitory overexpression of Ure2p Sp in S. cerevisiae was similar to the ones allowing prion propagation in S. cerevisiae. Why [URE3] propagation from Ure2p Sp could not be detected in S. paradoxus is unclear at present. It might be that Ure2p Sp prion propagation cannot occur in that host or else that prion formation occurs at a very low frequency.

The above-mentioned finding prompted us to investigate whether the cells with large aggregates of Ure2p are able to generate new transmissible aggregates. To that purpose, the progeny of cells with the large Ure2p-GFP aggregates were analyzed for their fluorescent aggregates content. After one generation, the proportion of cells with large aggregate clearly decreased, suggesting that large aggregates are nontransmissible (Supplemental Table S1). In accordance with the observed fast prion elimination, in microcolonies, daughter cells of mothers containing large aggregates Ure2p-GFP consistently contained no aggregates (Figure 7B). In a control experiment, the daughter cells of mothers propagating [URE3] prion exhibited the same aggregation pattern (small aggregates, visualized by the expression of a low level of Ure2p Sp-GFP from the pH327 plasmid) (Supplemental Figure S3).

DISCUSSION

In this study, we showed that Ure2p of S. paradoxus possesses the capacity to switch into a [URE3] prion conformation. Our analyses demonstrated that the expression level of Ure2p Sp strongly modulates this capacity. The ability to induce the prion form and/or to propagate this self-inactivation is observed only when Ure2p Sp was not expressed at high level. In contrast, Ure2p Sp and Ure2p of S. warum orthologue exhibited these prion properties regardless of the amount of Ure2p that is expressed (Baudin-Bailieu et al., 2003).

Ure2p Sp prion propagation is possible in S. cerevisiae (Edskes and Wickner, 2002; this study). In a previous study, however, we failed to isolated [URE3] clones when Ure2p Sp was expressed in its genuine cellular context, namely, in S. paradoxus (Talarek et al., 2005). Expression level of Ure2p Sp in S. paradoxus was similar to the ones allowing prion propagation in S. cerevisiae. Why [URE3] propagation from Ure2p Sp prion propagation cannot occur in that host or else that prion formation occurs at a very low frequency.

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Together, these results suggest that the infectivity of Ure2p aggregates and their ability to propagate as a prion can be dissociated.
related to a change either in the rate of amyloid growth or in the brittleness of the aggregates. The fragmentation frequency of an amyloid fiber is defined by the intrinsic physical properties of this particular amyloid conformation. It is unlikely that an increased level of Ure2p results in a change of conformational structure of its amyloid fibers. Conversely, in vitro, the speed of Ure2p fiber growth is linearly proportional to the concentration of soluble Ure2p (Fay et al., 2003). Similarly, our in vivo analysis highlighted that the aggregation dynamics of Ure2p is faster when cellular concentration of Ure2p increases. We showed that the Ure2p aggregates isolated from cells which no longer propagate [URE3] still retain their ability to induce [URE3] once artificially introduced in wild-type [ure3-0] cells. This result suggests that the large aggregates are structurally related to the amyloid [URE3] prion aggregates and do not correspond to a distinct off-pathway aggregate. The high levels of Ure2p speed of polymerization of fibers. Consequently, it is possible that above a threshold Ure2p amount, the formed aggregates are not self-propagating because their size increase and coalescence is too rapid. In this model, rapidly growing and coalescing Ure2p aggregates would fail to be segregated properly to daughter cells during cell divisions, as shown by our microscopy observations (Figure 7B). In agreement with this model, is the fact that increasing the cellular concentration of Ure2p results in a reduction of [URE3] propagon number (Figure 6).

Here, we showed that the aggregation pathway of the two Ure2p orthologues share the same intermediates states. However, a major difference lies in the aggregation dynamics that is faster for Ure2pSp than for Ure2pSc. Consequently, we observed many more cells containing very large aggregates when Ure2pSp is overexpressed. Similarly, our in vitro analyses showed that Ure2pSp exhibits a higher propensity to aggregate than Ure2pSc (Immel et al., 2007). We have also demonstrated that Ure2pSp is able to assemble in an infectious amyloid and that this one was significantly more resistant than the Ure2pSc amyloid to mechanical shearing (Immel et al., 2007). Interestingly, our propagan counting assay indicated that the apparent number of propagons per cell in the [URE3] URE2Sc strain was much smaller than that found in [URE3] URE2Sp (Figure 7). Therefore, we can speculate that Ure2pSp would tend to form aggregates, which could resist more efficiently to fragmentation and would be unable to be transmitted to daughter cells. The most likely possibility to explain this higher stability could be the longer asparagine stretch present in the prion-forming domain (Supplemental Figure S1). Indeed, the prion aggregation properties of the described yeast proteins rely on their content in glutamine and asparagine (Osherovich et al., 2004). These stretches could stabilize the polymer by specific polar zipper structures and asparagine-ladders (Perutz et al., 2002; Nelson et al., 2005). Thus, independently of its rate of polymerization, the Ure2pSc prion aggregates would be less prone to fragmentation than prion aggregates of Ure2pSp.

In Figure 8, we have summarized all these observations and integrated them in the analytical model proposed by Tanaka et al. (2006). Our results suggest that there would be another threshold above which stable prion propagation would not be possible. In this area on the diagram (top left area), the prion particles would be too large and/or to few to allow efficient propagation at cell division. Thus, although in the Tanaka model increasing fiber growth rate favors prion stability, it might be that above a given value, the polymerization rate can become detrimental to prion maintenance.

Together, our results suggest that the concentration of a prion protein modifies a fundamental parameter (the amyloid polymerization rate), which contributes to determine its transmission ability. It is possible that variation in prion protein expression could also interfere with prion propagation in mammals. Prion accumulation occurs in specific neuronal target areas. The reasons that define this cellular tropism are still largely unknown. Interestingly, it was reported that the level of prion protein differs markedly between different neuronal types (Ford et al., 2002). It would be interesting to study whether the concentration of cellular PrP could contribute to the determination of the variable cellular tropism of prion accumulation.

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REFERENCES


