The yeast Saccharomyces cerevisiae contains in its proteome at least three prion proteins. These proteins (Ure2p, Sup35p, and Rnq1p) share a set of remarkable properties. In vivo, they form aggregates that self-perpetuate their aggregation. This aggregation is controlled by Hsp104, which plays a major role in the growth and severing of these prions. In vitro, these prion proteins form amyloid fibrils spontaneously. The introduction of such fibrils made from Ure2p or Sup35p into yeast cells leads to the growth and severing of these prions. The paradigm nevertheless remains the same: the protein undergoes a modification which leads to a change or a loss of function, and this modification occurs in an autocatalytic manner; the protein provoking its own conversion.

Yeast prions are not harmful to cells, and their presence does not lead to cellular death but rather to a loss of functional phenotype. The presence of [PSI] is usually assessed by the suppression of a premature stop codon located in the Ade1 gene. However, the presence of this prion has a broad effect and may affect positively or negatively the growth of the yeast cells, depending on the conditions used. This phenotypic plasticity led S. Lindquist to propose a role for [PSI] in the acquisition of new traits during evolution. As [PSI] is remarkably conserved in the species of yeast, this study provides an attractive and valuable model for understanding complex aspects of mammalian prion biology. The yeast system has provided the first experimental demonstration (1) of the “protein-only” concept proposed by J. Griffith (2) and developed by S. Prusiner (3) to explain the disconcerting biological properties of the infectious agent responsible for the transmissible spongiform encephalopathies.

More recently, prions of Saccharomyces cerevisiae have offered a molecular explanation for the puzzling prion strain properties (4, 5). Apart from the power of budding yeast as a model for complex events involved in prion disease, the presence and the physiological role of these prions in yeast cells is itself a matter of interest and debate.
hemiascomycetous phylum (9), this mechanism could be widely used by these unicellular organisms. In contrast, less is known about [URE3]. In previous work, we (10) and others (11) have isolated several orthologous URE2 genes from different hemiascomycetous yeasts. We have then determined the capacities of these genes to complement the URE2 function for NCR. We have also tested in S. cerevisiae the capacities of these different URE2 genes to beget a prion phenotype. Interestingly, we found that this prion formation capacity was not maintained for the S. paradoxus URE2 gene (SpURE2). S. paradoxus is an hemiascomycete yeast very closely related to S. cerevisiae. The two functional domains are identical, whereas the two PFDs exhibit only a few differences (supplemental Fig. S1). Interestingly, it was found that the SpPFD has the capacity to induce the appearance of [URE3] (generated by ScURE2). This protein represents a paradox because it indicates that SpUre2p contains a PFD that is functional in trans, but not in cis (10). The inability of SpUre2p to switch into the inactive prion isoform might be because of the lack of specific factors required for the prion formation mechanism in S. paradoxus. To gain insight into the conservation of this mechanism, we then engineered S. paradoxus by developing the genetic tools useful for the analysis of [URE3] (12). This work clearly demonstrated that only the primary sequence of SpUre2p contributes to the lack of prion properties.

Here, we characterize SpUre2p in vitro. We found that native and soluble Ure2p from S. paradoxus and S. cerevisiae have the same biochemical behavior. In both proteins, the PFD promotes efficient amyloidogenesis. The higher propensity of SpUre2p to form amyloid fibrils under more stringent conditions is the sole difference found. This characteristic reflects a less efficient fragmentation rate for SpUre2p that explains the incapacity for this protein to sustain [URE3]. To further test this hypothesis in vivo, we used GFP-tagged proteins to observe aggregates in [URE3] cells, and found clear differences in morphology, consistent with greater resistance to fragmentation of SpUre2p. Finally, we demonstrate that SpUre2p fibrils produced in vitro are infectious, confirming that the failure of SpUre2p to act as a prion stems from its inability to propagate transmissible prions in vivo, rather than a fundamental difference in amyloid structure.

**EXPERIMENTAL PROCEDURES**

**Materials**—GSH, β-NADPH, CHP, and glutathione reduc-tase were from Sigma.

**Construction of Ure2p Expression**—The URE2 open reading frame was PCR-amplified from pUHE-URE2 and cloned into pET3a resulting in pET3a-URE2. The 6× His tag was PCR-amplified and inserted at the 3′-end of URE2 resulting in pET3a-URE2-His6 (13). The full-length Ure2p Sp expression vector (pET-URE2 (Sp)-His6) was obtained by amplifying by PCR a pUHE-URE2 (Sp) construct that encodes for Ure2p (Sp), using the following primers: 5′-CGGCGATATGATGAAATAC-AACGGGCAACC-3′ and 5′-CCGCTGAGTCACTTACCAC-GCAATGCC-3′. The amplified fragment was digested with Ndel and ligated into pET-URE2 (Sc)-His6 digested using Ndel and NcoI.

The pY2T-scURE2GFP plasmid was originally described by Ripaud et al. (32). The pY2T-spURE2GFP was derived from the plasmids pY2T-scURE2GFP and pY2T-spURE2 originally described by Baudin-Bailieu et al. (10): a fragment coding the PFD domain of spUre2p was amplified by PCR using oligonucleotides (5′-TACATAGTGCTTTTGTAGC-3′), binding the GAL10 promoter, and (5′-TGTGTTCCAGCTGAGTGTGCT-3′), which introduces a Bsu36I restriction site a 3′-end of the fragment. The PCR fragment was inserted into pY2T-scURE2GFP, between BamHI and Bsu36I sites, in place of the coding sequence of the spUre2p PFD domain.

**Microscopy Techniques**—AB34 cells (MAT alpha, trp1-1, ade2-1, leu2-3, 112, his3-11, 15, ura2::HIS3 [URE3]) were transformed with pY2T-scURE2GFP or pY2T-spURE2GFP plasmids. Cells were grown overnight on YNB medium (0.67%) supplemented with rifampicin (2%), appropriate amino acids, and USA (15 mg/liter). 2% galactose was then added to induce the Ure2pGFP expression. Aliquots were plated at different times after galactose addition, and resuspended in DABCO solution (218 nm diazabicyclo 2-2-2 octane (Sigma), 25% (v/v) phosphate-buffered saline buffer, 75% (v/v) glycerol). Cells were observed and photographed with a DMRB microscope (Leica, Germany) with a PL APO 63 objective.

**Ure2p Expression, Purification, and Fibril Formation**—Recombinant full-length Ure2p(Sc)-His6 and Ure2p(Sp)-His6 were overexpressed as soluble proteins in Escherichia coli BL21 codon + DE3 strain (Stratagene) in LB medium containing 50 μg/ml ampicillin, 30 μg/ml chloramphenicol. Cells were grown overnight at 18 °C until the A600 nm reached 0.8–1.0. Recombinant full-length Ure2p(Sc)-His6 was purified as previously described (14) with a modified buffer (20 mM Tris-HCl, pH 8, 300 mM NaCl) for the sonication.

**In Vitro Analysis of SpUre2p**

The sonication of cells expressing the recombinant Ure2p(Sc)-His6 was achieved in 20 mM Tris-HCl, pH 8, 50 mM NaCl + protease inhibitor mixture (complete EDTA-free, Roche Applied Science). The insoluble material was removed by centrifugation for 15 min at 20,000 × g, and the lysate was incubated with Ni-NTA resin (Qiagen) for 1 h at 4 °C. The resin was then washed with 50 mM NaCl, 20 mM Tris-HCl, pH 8, 20 mM imidazole. Protein elution was carried out in the same buffer containing 250 mM imidazole. The fractions containing Ure2p (Sp) were pooled, the NaCl concentration adjusted to 1 M, and applied to a HiTrap Phenyl HP (Amersham Biosciences) equilibrated in buffer A (20 mM Tris-HCl, pH 8, 1 mM NaCl). The column was washed with equilibration buffer (buffer A) and developed with a first step of 35% buffer A, a second step of buffer B (20 mM Tris-HCl, pH 8), and finally a step of the buffer 20 mM Tris-HCl, pH 8, 10% glycerol.

Fractions emerging from the column in buffer B were pooled and concentrated with the help of a Centriplus Plus-20 (Millipore). The typical yield was 30–40 mg of Ure2p(Sc)-His6 and Ure2p(Sc)-His6 per liter of culture. Alternatively, ScUre2p was produced with an N-terminal 6× His tag, using the vector mini-pRSETa, and purified under native conditions as described previously (15). The SpUre2p gene was also transferred from pET-URE2 (Sp)-His6 to the mini-pRSETa vector to produce...
In Vitro Analysis of SpUre2p

SpUre2p with an N-terminal His tag. This was achieved using PCR using the following primers: 5′-gccgccggtcatgatattaacacccgtaaacaagta-3′ (5′ primer containing BamH1 site) and 5′-gctcccggcgcctactaacacccgtaaacaagta-3′ (3′ primer containing Kpn1 site). The amplified fragment was digested with BamH1 and Kpn1. The digestion product was then ligated into mini-pRSETa vector, digested with the same enzymes. The mini-pRSETa-URE2 (Sp) plasmid was transformed into E. coli C41 strain. The SpUre2p protein was then produced and purified as described above. The presence of N- or C-terminal His tags had no effect on the fibril formation behavior of either protein.

Size Exclusion Chromatography—The molecular size of the proteins was analyzed by chromatography on a FPLC Superose 12 column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl, pH 8, 50 mM NaCl.

Filament Assembly—Fibrils were assembled for EM and PK analysis by incubating proteins (final concentration 75 μM) at 4 °C for 1–2 weeks without shaking in 20 mM Tris-HCl, pH 8, 300 mM NaCl for ScUre2p or 20 mM Tris-HCl, pH 8, 50 mM NaCl for SpUre2p. Filaments were purified by centrifugation (25,000 × g, 30 min) and washed at least two times with buffer before PK analysis.

Filaments were obtained in various buffers (20 mM Tris-HCl, pH 8, 50 mM NaCl, 50 mM Trehalose; 20 mM Tris-HCl pH 8, 50 mM NaCl, 1 mM glutathione; 20 mM Tris-HCl pH 8, 50 mM NaCl, 1 mM TMAO; 20 mM Tris-HCl, pH 7, 50 mM NaCl). The assembly of soluble full-length Ure2p was monitored by the measurement of sample turbidity expressed as optical density at 400 nm using a Cary 50 (Varian) spectrophotometer.

Electron Microscopy—Protein samples were loaded onto a copper grid coated with a Formvar film. To avoid rapid desiccation, sedimentation was allowed during a 10–30-min period in a moist Petri dish. Grids were then rinsed with 15–20 drops of freshly prepared 1% uranyl acetate in water (passed through a 0.22-μm filter, Millipore), dried with filter paper, and observed with a Phillips Tecnai 12 or Tecnai 20 electron microscope at 100 kV.

Limited Proteolysis—8 μl of a protein solution at 75 μM in 20 mM Tris-HCl, pH 8.0, 50 mM NaCl was mixed with 8 μl of appropriate dilutions of proteinase K (Promega), and incubated at 37 °C for 15 min. Digestion was stopped by the addition of electrophoresis sample buffer pH 6.8, containing 4% SDS, 2% mercaptoethanol (v/v), 12% glycerol (w/v), 0.01% Serva Blue G, and phenylmethysulfonyl fluoride to a final concentration of 1 mM and immediately incubated at 100 °C for 5 min.

Digestion of amyloid fibrils was stopped by the addition of electrophoresis sample buffer, phenylmethysulfonyl fluoride, and urea (to a final concentration of 8 M) and immediately incubated at 100 °C for 5 min.

Samples were analyzed by 15% SDS-PAGE. Gels were stained with Coomassie Blue or blotted to a nitrocellulose membrane (Optitran BA-S83, Schleicher & Schuell). Membranes were probed with specific affinity-purified polyclonal antibodies raised against full-length Ure2p.

Circular Dichroism Spectroscopy—The native conformation of soluble Ure2p(Sp)-His<sub>6</sub> and Ure2p(Sp)-His<sub>6</sub> was compared by circular dichroism spectroscopy. CD spectra were recorded in a 1-mm path quartz cell with a Jasco (Easton, MD) J-810 spectropolarimeter. All spectra were measured at 20 °C in 20 mM Tris-HCl, pH 8, 50 mM NaCl. Protein concentrations were 0.25 mg/ml, determined by absorption at 280 nm.

GdmCl Denaturation—1 μM protein was incubated overnight at 25 °C in 50 mM Tris-HCl, pH 8.4, 200 mM NaCl containing different concentrations of GdmCl before measuring the intrinsic fluorescence spectrum between 300 and 400 nm after excitation at 280 nm on a Shimadzu RF-5301PC spectrophotometer. The maximum change in fluorescence on denaturation was observed at 327 nm for both proteins. The plots were fitted to a three-state model as described (16).

ThT Assay—Fibril growth experiments and assay of ThT binding were performed essentially as described previously (16). 30 μM S. paradoxus and S. cerevisiae Ure2 were incubated in 50 mM Tris-HCl, pH 8.4, 200 mM NaCl at 10 °C on a MS2 Minishaker (IKAB<sup>®</sup>) in a cold cabinet or at 37 °C in an Innova 4230-refrigerated incubator shaker (New Brunswick Scientific). (The stated pH of the buffer is correct at 25 °C.) A glass bead was added if the sample was shaken. Shaking speeds were as indicated in the figure legends.

Enzyme Assay and Steady-state Kinetic Analysis—The GPx activity of Ure2p was determined using GSH and CHP as substrates using a coupled spectrophotometric assay (17, 18). The assay was carried out at 25 °C in a 1-ml reaction volume containing 100 mM sodium phosphate buffer, pH 7.5, 0.15 mM β-NADPH and 0.24 units of glutathione reductase. Steady-state kinetic analysis was carried out. We measured k<sub>cat</sub>, K<sub>m</sub>, and V<sub>max</sub> for Sc and SpUre2p to compare their activity. The k<sub>cat</sub> value was measured using 1 mM GSH, and the Ure2p protein concentration was varied between 0.3 μM and 3.0 μM. The K<sub>m</sub> and V<sub>max</sub> values for 1 μM Ure2p were determined by varying the GSH concentration between 0.33 mM and 2.5 mM. The reaction mixture was preincubated at 25 °C for 4 min, after which the reaction was started by the addition of CHP to a final concentration of 1.2 mM. The progress of reactions was monitored continuously by following the decrease in NADPH absorbance at 340 nm on a Unico UV4802 spectrophotometer. Initial rates were determined from the linear slope of progress curves obtained with an extinction coefficient for NADPH of 6220 M<sup>−1</sup> cm<sup>−1</sup> after subtracting the non-enzymatic velocities caused by the auto-oxidation of GSH by the hydroperoxide determined from the corresponding blank. The data were fitted to the Michaelis-Menten equation. Single or global fitting was carried out using the regression wizard of SigmaPlot.

Assay of Ure2 GPx Activity during the Time Course of Amyloid-like Fibril Formation—The initial sample was centrifuged at 10,000 × g for 30 min at 4 °C to remove any preexisting aggregates, and 300 μl of the supernatant was transferred into each of a series of tubes, one for each time point. The reaction mixture contained 46 μM full-length S. paradoxus Ure2 in 50 mM Tris-HCl buffer, pH 8.4, containing 0.2 mM NaCl. The samples were incubated in parallel at a constant temperature of 25 °C with shaking as described previously (16). Under these conditions, the increase in fluorescence caused by ThT binding correlates directly with the appearance of amyloid aggregates of Ure2. At each time point, one of the samples was placed on ice. Two 50-μl aliquots of the complete reaction mixture were
removed and assayed for GPx activity using 1 mM GSH and 1.2 mM CHP as substrates, as described above. A further 10-μl aliquot of the reaction mixture was removed to assay for ThT binding, as described previously (16). After centrifugation of the remaining 240 μl of sample, two 50-μl aliquots of the resulting supernatant were assayed for GPx activity. A further 10-μl aliquot of supernatant was used for protein concentration determination using the method of Bradford. The precipitate was resuspended in 240 μl of the same buffer, and then two 50-μl aliquots were assayed for GPx activity. Thus, the final protein concentration in the GPx assays was 2.3 M for the total reaction mixture and a maximum of 2.3 M in either the supernatant or the pellet fraction, depending on the relative distribution of protein between the fractions during the course of fibril formation. The values of GPx activity are mean values of two independent assays.

RESULTS

Purification of the Soluble Form of SpUre2p—After purification of the SpUre2 protein (see “Experimental Procedures”), a single species was observed that migrates with an apparent molecular mass of 42 kDa in SDS-PAGE (Fig. 1A). We next compared the behavior of recombinant Sp and ScUre2p by size exclusion chromatography (SEC). Both proteins eluted from the column in the same fraction indicating the same apparent molecular mass (Fig. 1B). The precipitate was resuspended in 240 μl of the same buffer, and then two 50-μl aliquots were assayed for GPx activity. Thus, the final protein concentration in the GPx assays was 2.3 M for the total reaction mixture and a maximum of 2.3 M in either the supernatant or the pellet fraction, depending on the relative distribution of protein between the fractions during the course of fibril formation. The values of GPx activity are mean values of two independent assays.

In Vitro Analysis of SpUre2p

FIGURE 1. Purification and characterization of recombinant SpUre2p. A, expression and purification of Ure2p. Analysis on 12% SDS-PAGE at different steps of Ure2p purification. Lane 1, crude extract (soluble fraction). Lane 2, flow-through after binding on a Ni$^{2+}$ column. Lane 3, wash. Lane 4, recombinant Ure2p emerging from the affinity chromatography column. Lanes 5 and 6, wash. Lane 7, recombinant Ure2p emerging from the hydrophobic chromatography column. B, size exclusion chromatography analysis of pure recombinant Ure2p. Elution profile of pure recombinant Ure2p from a Superose 12 column. Arrowheads show the location of molecular size markers: (lane 1, catalase, 232 kDa; lane 2, aldolase, 158 kDa; lane 3, bovine serum albumin, 67 kDa; lane 4, chymotrypsinogen, 25 kDa; lane 5, cytochrome c, 12.3 kDa).

FIGURE 2. Structure and stability of SpUre2p. A, CD spectra of soluble S. cerevisiae and S. paradoxus Ure2p in 20 mM Tris-HCl buffer, pH 8, 50 mM NaCl. The protein concentrations were 0.25 mg/ml. B, GdmCl denaturation of S. cerevisiae and S. paradoxus Ure2p. Conditions were 1 μM protein concentration in 50 mM Tris-HCl, pH 8.4, 0.2 M NaCl containing different concentrations of GdmCl at 25 °C. Fitting of the data to a three-state model (16) gives the parameters shown in supplemental Table S2.

160-kDa species, consistent with the previous results obtained with an untagged version of ScUre2p (14). This species has been shown to represent the dimeric form of the protein and was also observed for a His-tagged version of Ure2p (15). (The additional peak observed in the ScUre2p sample (Fig. 1A) corresponds to a contaminant polypeptide that is not systematically present in the ScUre2p-purified fraction and absent in the purest fraction of SpUre2p.) These data indicate that both Sc and SpUre2p assemble in the same way and show the same discrepancy between theoretical and experimental molecular mass by SEC.

Structure, Activity, and Stability of SpUre2p—Far-UV CD spectra (190–250 nm) of both proteins were then recorded (Fig. 2A). The two spectra clearly overlap, indicating a similar content of α-helix and the β-sheet in the two proteins. As the only differences between Sc and SpUre2p are found in the unstructured (1–94) part of protein, this means that there is no significant difference in the structure of the functional domains. The ScUre2p C-domain shows glutathione-dependent peroxidase activity, which is retained on formation of amyloid-like fibrils
Here we compared the enzymatic activity of Sc and SpUre2p as a function of Ure2p concentration (supplemental Fig. S2), indicating that SpUre2p, like ScUre2p, shows Gpx activity and displays typical Michaelis-Menten kinetics (Fig. S3). The steady-state kinetic parameters obtained for the two proteins are the same within error (Figs. S1 and S2, and Table 1). These results indicate that both proteins are purified in a functional state and share the same enzymatic activity. We next examined the stability of these two proteins in the presence of the chaotropic agent guanidium hydrochloride (Fig. 2B). The equilibrium unfolding of Ure2p was recorded by following intrinsic fluorescence as a probe, under conditions where the stability of ScUre2p has already been studied in detail (15). Sc and SpUre2p showed identical equilibrium denaturation behavior (Fig. 2B) and fitting of the data to obtain thermodynamic parameters gave the same values within error (supplemental Table S1). These results suggest that the prion domains of Sp and ScUre2p contribute in the same way to the folding of the proteins.

**Aggregation Propensity of Sc and SpUre2p**—ScUre2p forms amyloid structures spontaneously that recapitulate all the properties considered typical of amyloids, namely: fibrillar morphology (19, 20), apple-green birefringence when stained with Congo Red (20), Thioflavin T binding (16, 21), proteinase K resistance of the fibril core (corresponding to the N-terminal domain (22)), and cross-beta structure of the fibril core (23). When incubated under standard conditions (20 mM Tris-HCl, pH 8, 50 mM NaCl, 20 °C without agitation), both proteins form macromolecular entities monitored by turbidity (Fig. 3). This technique is generally used to follow heat denaturation (24). Surprisingly, SpUre2p exhibits a higher propensity to aggregate than ScUre2p. These aggregates may stem either from ordered or disordered mechanisms. The use of electron microscopy definitively showed that the aggregates formed upon incubation correspond to fibrillar aggregates. As both proteins form such structures under standard conditions, we investigated different parameters that might affect specifically the fibrillation capability of one isoform. In the presence of trehalose (a

### Table 1

<table>
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<th>$K_{\text{m(app)}}$ (mM)</th>
<th>$V_{\text{max(app)}}$ (s$^{-1}$)</th>
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<td><em>S. paradoxus</em> Ure2</td>
<td>2.4 ± 0.4</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> Ure2</td>
<td>2.4 ± 0.3</td>
<td>0.11 ± 0.01</td>
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*The errors shown are the S.E. of the fit.

*The values shown are the mean of three independent experiments, and the errors shown are the S.E.*

**FIGURE 3.** Assembly of soluble full-length wild-type SpUre2p and ScUre2p in different buffers and identification of amyloid fibers. Kinetics of protein assembly was monitored by turbidity measurement of the solution (expressed as optical density at 400 nm) at 20 °C in 20 mM Tris-HCl pH 8, 50 mM NaCl. The formation of amyloid fibrils from soluble Sc or SpUre2p in the following buffers was checked by negative staining electron microscopy. Lane 1, 20 mM Tris-HCl, pH 8, 50 mM NaCl. Lane 2, 20 mM Tris-HCl, pH 8, 50 mM NaCl, 50 mM Trehalose. Lane 3, 20 mM Tris-HCl, pH 8, 50 mM NaCl, 100 mM NDSB-201. Lane 4, 20 mM Tris-HCl, pH 7, 50 mM NaCl, 1 mM glutathione. Lane 5, 20 mM Tris-HCl, pH 8, 50 mM NaCl, 1 mM TMAO. Lane 6, 20 mM Tris-HCl, pH 8, 50 mM NaCl, 1 mM glutathione. Lane 7, 20 mM Tris-HCl pH 8, 300 mM NaCl. Bottom, macroscopic observation of the final aggregates made with Sp or Sc Ure2p.
sugar that acts as a stabilizing agent (25)), non-detergent sulfo-betaines NDSB-201 (a mild solubilization agent used for protein purification and renaturation (26)), the osmolyte trimethylamine N-oxide TMAO (a chemical chaperone that helps to maintain the structure of proteins (27)), glutathione (a tri-peptide substrate of Ure2p (17) that binds to the protein (28)) and at various values of pH and salt concentrations, SpUre2p still formed fibrillar structures in the same way as ScUre2p (Fig. 3). These structures appeared to be quite similar when observed on electron micrographs. However, the SpUre2p fibers were sometimes longer than ScUre2p fibers (see Sp + glutathione). Moreover SpUre2p typically formed cloudy aggregates, whereas this is less readily observed for ScUre2p (see Fig. 3).

Susceptibility to Proteolysis of Soluble and Fibrillar SpUre2p—ScUre2p fibrils formed in vitro are clearly related to [URE3] because their introduction into yeast cells leads to the phenotypic switch characteristic of [URE3] (29). SpUre2p, although not competent to propagate this switch in yeast cells, is prone to form protein filaments that cannot be distinguished from ScUre2p infectious filaments by EM. We next examined in detail the main characteristics of these filaments. When SpUre2p filaments were subjected to proteinase K digestion used previously for digestion of ScUre2p filaments (13), they gave the same pattern on SDS gel with Coomassie Blue staining (Fig. 4A). We also compared this pattern to the profile obtained when soluble SpUre2p is used as the protease substrate under the same conditions. Interestingly, the profile obtained (Fig. 4B) is roughly the same and is consistent with the profile previously obtained when ScUre2p was subjected to limited proteolysis under identical conditions to those used here (19, 30, 31). The main species observed is a 30-kDa species that corresponds to the functional domain of Ure2p (13, 19). Overall, the different species generated upon PK treatment of either soluble or aggregated Sc or SpUre2p are populated in the same quantity under the same conditions for the two proteins. It has been reported that extensive digestion of ScUre2p fibers with proteinase K leads to the production of a fuzzy band of low molecular weight corresponding to the prion domain (22). In our hands, we could not detect such a smear, even after adding additional proteinase K or incubating Sc or SpUre2p filaments for a longer period (13) (and data not shown). In an attempt to detect this prion domain, the proteinase K fragments were also examined by a Western blot analysis (Fig. 4C). The antibodies used to reveal the different fragments generated by the digestion of SpUre2p filaments with proteinase K permit the identification of the fragments previously identified by the staining procedure, but could not permit the identification of the prion domain.

Comparison of Time Course of Amyloid Fibril Formation for Sc and SpUre2p—Fibril formation for Sp and ScUre2p was monitored by ThT fluorescence under various conditions of temperature, with and without agitation. Like ScUre2p, SpUre2p showed an increase in ThT fluorescence, indicating formation of amyloid-like structure, with a sigmoidal time course (Fig. 5). When GPx activity of SpUre2p was monitored during the course of fibril formation, the enzymatic activity of the sample was observed to disappear from the supernatant fraction and appear instead in the pellet fraction, concomitant with the formation of amyloid-like fibrils as monitored by ThT (supple-
aliquots together with a LEU2 plasmid. Leu-otrophic for leucine, were co-transformed with the sonicated lysed for their capacity to induce [URE3] once introduced in the presence of USA as sole precursor of UMP. The prion formation capacity is therefore clearly restricted to the N-terminal poorly structured domain and the rest of the protein.

character was further assessed by the phenotypic reversibility of the growth on USA medium after having treated the cells with 5 mM GdnHCl. The results (supplemental Fig. S5) clearly demonstrate that SpUre2p amyloid is capable of infectivity, suggesting that its inability to propagate the prion state stems from an inability to generate new transmissible seeds rather than a fundamental difference in amyloid structure. Because Sc and Sp Ure2p amyloids made in vitro are both infectious, it is of interest to analyze whether any difference in aggregate structure is observed in vivo. Overexpression of ScUre2-GFP in [URE3] yeast cells permits the aggregation process to be followed. As previously shown (32), this process leads to the formation of a large globular aggregate (Fig. 6). When SpUre2-GFP is expressed instead of ScUre2-GFP, the kinetics of aggregation were more rapid. After 3 h, most of the cells show one large punctate focus (Fig. 6). A more dramatic difference was found after 24 h of SpUre2-GFP expression. In more than 80% of the yeast cells, a jellyfish-like structure was observed instead of the globular aggregate found when ScUre2-GFP is expressed. This branching structure is indeed compatible with a higher capacity for SpUre2p to form fibrillar structures and to resist fragmentation under these experimental conditions.

DISCUSSION

The conservation of prion properties through evolution is of interest to analyze the role of this mechanism in the living world. The two most intensively studied yeast prions [PSI] and [URE3] are not conserved in the same way. [PSI] can be propagated in S. cerevisiae by expressing Sup35 from distantly related hemiascomycete yeasts (9, 33–35), whereas [URE3] failed to be propagated by expressing Ure2p of K. lactis, a milk-loving yeast, believed to have diverged from the Saccharomyces clade at least 150 million years ago (10). A closer yeast species (S. paradoxus) possesses an intriguing URE2 gene. When overexpressed in S. cerevisiae, the corresponding protein cannot induce [URE3] although its N-terminal domain expressed alone increased dramatically the rate of appearance of [URE3] in the same way as a bona fide “prion forming domain” (10). When S. paradoxus is used as the host for S. cerevisiae Ure2p expression, we were able to observe the [URE3] phenotype whereas this prion phenotype could not be observed at the same frequency when S. paradoxus Ure2p was expressed (12).

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The prion formation capacity is therefore clearly restricted to the primary sequence of the protein rather than to species-specific cellular factors involved in this mechanism.

The prion phenotypes [PSI] and [URE3] observed in vivo can be induced by transforming yeast cells with amyloid fibrils made in vitro from Sup35 or Ure2p, respectively (4, 5, 29, 36). The amyloidogenic properties of these proteins are encoded into their N-terminal “prion forming domains” which are rich in asparagine and glutamine. Because SpUre2p cannot form a prion in vivo (although it contains an efficient PFD), it was therefore of interest to analyze its biochemical properties in vitro.

In Vivo Comparison of Sp and ScUre2p Aggregates—During the course of amyloid formation, aliquots of SpUre2p were analyzed for their capacity to induce [URE3] once introduced in wild-type CC30 yeast cells. Yeast spheroplasts, which are auxotrophic for leucine, were co-transformed with the sonicated aliquots together with a LEU2 plasmid. Leu+ transformants were tested for the presence of [URE3] by their capacity to grow in the presence of USA as sole precursor of UMP. The prion
Genetic (37) and biochemical (38, 39) experiments have suggested the existence of such an interaction in ScUre2p. However, direct experiments based on NMR and surface plasmon resonance (40) failed to confirm the existence of such an interaction. Our current work will not help to elucidate this conundrum because the behavior of the orthologous SpUre2 protein is indistinguishable from the genuine ScUre2p. The loss of prion function of SpUre2p in vivo is therefore probably not caused by a particular structure adopted by the PFD in the soluble form of the protein. This is in agreement with previous published results because these two proteins are identical in their functional domain (from Met\textsuperscript{25} to the end), while the remaining PFD (in which very few differences are found, supplemental Fig. S1) does not play any role in the overall structure and stability of the protein (14, 15). We next analyzed the stability of the protein. In the presence of increasing concentrations of guanidium hydrochloride, both Sp and ScUre2p exhibit the same denaturation propensity. All these experiments clearly show that the difference in prion forming behavior of Sc and SpUre2p is not caused by a difference in the folding of the soluble forms of the proteins. We therefore continued to apply further approaches to highlight subtle differences that must exist because these two proteins behave differently in vivo.

Next, we examined the amyloid propensity of SpUre2p. A priori, we expected a complete inability of SpUre2p to form any amyloid structure because this property is intimately linked to the prion formation mechanism. Unexpectedly, we found that the protein switches spontaneously from the soluble state to a largely aggregated structure. EM observation of these aggregates allowed the identification of the typical filamentous structures that are observed during ScUre2p aggregation. Moreover, SpUre2 fibril formation could be observed under many different conditions. This result indicates an efficient propensity for both Sp and ScUre2p to form filamentous amyloid-like structure.

An interesting feature of ScUre2p fibrils is that native-like globular structure and enzyme activity is maintained within the fibrils (17, 41, 42). To examine whether this is also the case for SpUre2 fibrils, we compared the susceptibility to limited protease digestion and the enzyme activity of the soluble and fibrillar forms of the protein. As found previously for ScUre2p under identical conditions the soluble and aggregated forms showed remarkably similar protease digestion patterns, and enzyme activity was maintained in the fibrillar state. At this level of observation, Sp and ScUre2p behave identically. The functional C-terminal domain appears to have a common structure in both soluble and fibrillar states. However, these proteins profoundly differ in vivo in their prion formation properties. This could be simply explained if SpUre2p amyloid fibrils were not capable of infectivity. However, we demonstrated that when the protein assembled in vitro into amyloid structure is introduced in wild type yeast cells, it has the capacity to switch endogenous ScUre2p into its prion shape. This experiment establishes unambiguously the infectious capacity of ScUre2p amyloid and suggests that the lack of prion behavior for SpUre2p results not from any fundamental difference in structure, but rather from an inability to reliably propagate the prion state. Recently, a model has been proposed to describe prion strains (43). This model highlights the importance of the kinetics of amyloid formation and in particular its rate of growth and fragmentation. The capacity to be infectious is therefore because of the rate of synthesis, but also to the fragility of the aggregates formed. Possible explanations for the particular behavior of SpUre2p could therefore be a difference either in the rate of amyloid growth or in the brittleness of the aggregates. The kinetics of Sp and ScUre2p fibril formation based on ThT fluorescence shows a classical sigmoidal curve. Interestingly, without agitation, both curves overlap, indicating a similar propensity for the proteins to switch from the soluble to the amyloid state. Upon mild agitation, the fragmentation of amyloid fibrils formed early generates new seeds and decreases the lag time. In contrast, when incubated under strong agitation, the specific signal because of the interaction between ScUre2p amyloid fibrils, and ThT was significantly reduced.

In Vitro Analysis of SpUre2p

**FIGURE 6. Dynamics of the fluorescent aggregates in [URE3] cells during Sc or SpUre2-GFP overexpression.** [URE3] yeast cells were transformed with pYe2T-scURE2GFP or pYe2T-spURE2GFP plasmids. Cells were grown overnight on raffinose medium. 2% galactose was then added, to induce the Ure2-GFP expression, and cells were observed at different times after galactose addition.
In Vitro Analysis of SpUre2p

Direct observation by EM links this failure to the reduced ability to maintain fibrillar structure under conditions of strong agitation. SpUre2p shows quite different behavior under these conditions and a clear increase in ThT fluorescence over the time course is observed, which corresponds with the ability of the protein to form long fibrils, as observed by EM. The Sp fibrils seem to be significantly more resistant to mechanical force. This then suggests a mechanism for the inability of the SpUre2p to act as a propagatable species in yeast: if the prion aggregates are too robust to be fragmented, then there will be insufficient seeds to allow efficient propagation at cell division, and so the prion state would always be lost before it could be detected.

organization. However, it is tempting to postulate that they may involve in this non-infectious effect. Elucidating the mechanism of Ure2p prion formation.

In vivo, the formation of [URE3] requires the presence of active chaperones and is completely dependent on the system has permitted clarification of the role of this protein on prion formation and infectivity (36). If the higher propensity of SpUre2p to form amyloid makes it a “cul-de-sac” for prion formation, we wonder whether overexpression of HSP104 might rescue the prion mechanism. However, when HSP104 is overexpressed in CC30 strains, together with SpUre2p (under the conditions previously used, Ref. 10), the yeast cells did not gain the capacity to form [URE3] (data not shown). Of course, ScUre2p in the [URE3] form is also insensitive to overexpression of HSP104 (44), therefore this lack of effect is perhaps unsurprising. We now have to identify the amino acids that are specifically involved in this non-infectious effect. Elucidating the mechanism by which these amino acids contribute to this effect remains also an exciting challenge and may shed light on the mechanism of Ure2p prion formation.

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