Structural Characterization of Saccharomyces cerevisiae Prion-like Protein Ure2*

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Saccharomyces cerevisiae prion-like protein Ure2 was expressed in Escherichia coli and was purified to homogeneity. We show here that Ure2p is a soluble protein that can assemble into fibers that are similar to the fibers observed in the case of PrP in its scrapie prion filaments or that form on Sup35 self-assembly. Ure2p self-association is a cooperative process where one can distinguish a lag phase followed by an elongation phase preceding a plateau. A combination of size exclusion chromatography, sedimentation velocity, and electron microscopy demonstrates that the soluble form of Ure2p consists at least of three forms of the protein as follows: a monomeric, dimeric, and tetrameric form whose abundance is concentration-dependent. By the use of limited proteolysis, intrinsic fluorescence, and circular dichroism measurements, we bring strong evidence for the existence of at least two structural domains in Ure2p molecules. Indeed, Ure2p NH₂-terminal region is found poorly structured, whereas its COOH-terminal domain appears to be compactly folded. Finally, we show that only slight conformational changes accompany Ure2p assembly into insoluble high molecular weight oligomers. These changes essentially affect the COOH-terminal part of the molecule. The properties of Ure2p are compared in the discussion to that of other prion-like proteins such as Sup35 and mammalian prion protein PrP.

Keen interest has been shown during the past several years to a phenomenon of protein misfolding and aggregation (inside the cells), particularly due to studies of various amyloidosis and prion diseases (1–6). Even though it became more or less clear that conformational changes of proteins are required for the propagation of the diseases (1–6), it is not yet known how and where these conformational changes take place in vivo. There are some significant differences between prion diseases and amyloid diseases, such as transmissibility of prion diseases, but it is clear that conversion of a soluble form of a protein into insoluble aggregate is a key mechanism involved in all the cases (1–6). Such aggregates reveal high resistance to proteases, thus escaping different common degradation pathways, e.g. proteosomal complexes (7, 8). These aggregates form deposits (1–6) that could lead to cytotoxicity.

In contrast to mammalian prions, which in their aggregated form significantly damage the cells (leading finally to cell death), the so-called yeast prion-like proteins (Sup35p and Ure2p, when thought to be aggregated) do not damage yeast cells but do, however, change their phenotypes (9, 10). This change in yeast cell phenotypes could lead to an evolutionary advantage under certain conditions, e.g. giving rise to new proteins due to a readthrough of a stop codon (in case of the aggregation of Sup35p, known to be a translation termination factor (see Refs. 9 and 11)), or allow the uptake of both poor and rich nitrogen sources as happens in case of the Ure2p aggregation (Ure2p is a transcriptional factor, regulator of nitrogen metabolism in yeast (12–13)).

It is now widely accepted that formation of insoluble aggregates is a result of a shift in equilibrium between native soluble conformer of a prion protein and aggregation-competent molecules (6). Although reasons for such a shift are rather obscure, the basis for partitioning between different conformers seems to be provided (at least in case of mammalian prion proteins) by their specific structural properties (4, 14, 15). Indeed, recent structural studies demonstrated that mammalian prion proteins possess a large unstructured NH₂-terminal part (14, 15). Since synthetic peptides reproducing various regions in this domain are capable of polymerizing into amyloid fibrils (for review see Ref. 16), this part of the molecule has been suggested to govern PrP aggregation. The most widely accepted model for amyloid formation hypothesizes that primary conformational changes affect the NH₂-terminal domain of PrP and then propagate to the rest of the molecule with an efficiency depending on the local structural properties of different PrP isoforms (4, 6).

In order to understand better the features that can provide the basis for possible structural plasticity of the yeast prion-like protein Ure2, we have attempted its purification and characterization (after overexpression in Escherichia coli cells). Our data demonstrate that recombinant Ure2p is a soluble monomeric protein that can self-associate into dimers, tetramers, as well as insoluble high molecular weight oligomers. These high molecular weight oligomers are fibrillar structures that appear, when examined in the electron microscope, to be very similar to the fibers that are observed in the case of PrP in its scrapie prion filaments or that form on Sup35p self-assembly. Ure2p oligomerization is a cooperative process that is concentration-dependent. Finally, Ure2p fibers bind Congo Red as do amyloid fibers. We also bring evidence in this work for the existence of at least two structural domains in Ure2p molecules and show that only slight conformational changes accompany Ure2p aggregation. These changes affect essentially the COOH-terminal part of the molecule.

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MATERIALS AND METHODS

Reagents—EGTA, SDS, 1,4-dithiothreitol (DTT),1 and subtilisin (Carlsberg P5380) were from Sigma. Trypsin-t-1-toeylamido-2-phenyl-
ethyl chloromethyl ketone came from Worthington. Proteinase K was purchased from Stratagene. [35S]-Methionine and autoradiography
products came from Amersham Pharmacia BioTech. Acrylamide and all other electrophoresis reagents were from Bio-Rad. All other chemicals
were from ProLabo and Merck.

Construction of Ure2p Expression Vectors in E. coli—Ure2p expression
constructs were designed as described previously (17). Briefly, the open reading frame of the URE2 gene was amplified with the primer
5'-GCCGATGATTGCCTGGTTATTTATGACCTG-3' and 5'-GCCGATGAGGTTCGTCGTGTTTACGGTGTAG-3'. The amplified
cDNA was subcloned into BamHI-PstI-restricted pUHE2.2-3 and with the same enzymes generating the construct pUH-URE2* (underlined
sequences are the two CGT codons replacing the
AGA). The rare codons AGA encoding arginine residues at positions 253
and 254 were changed into CGT codons. These silent mutations were
achieved by site-directed mutagenesis using two additional oligonu-
terials were processed to monitor the time course of Ure2p cleavage by Tricine/
Dynamics).

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1 The abbreviations used are: DTT, 1,4-dithiothreitol; PAGE, poly-

acrylamide gel electrophoresis; Tricine, N-2-hydroxy-1,1-biasty-

droxymethylglycine; TFE, 2,2,2-trifluoroethanol.

pooled and adjusted to 20 mM potassium phosphate, pH 7.2, 1 mM
MgCl2, 1 mM DTT by passage through a Sephadex G-25 column (Amersham Pharmacia BioTech) and applied to a 20-mL hydroxyapatite column (Pentax, American International Chemical). Ure2p did not bind to this column and was eluted during the wash phase, whereas contamin-
ating proteins bound to the matrix. Of the material eluted from this column, 95% corresponded to Ure2p, as judged from running this ma-
terial on a 10% SDS-polyacrylamide gel. Ure2p-containing fractions were
pooled then frozen in aliquots and stored at -70 °C. The typical yield was 20 mg of Ure2p per liter of culture.

For analytical ultracentrifugation experiments, electron microscopy
obtained digests, and proteolytic peptide analysis. Ure2p was separated
from aggregated proteins by gel filtration through a Superose 6 HR
10/30 column (Amersham Pharmacia BioTech) equilibrated in 50 mM
Tris, pH 7.5, 1 mM EGTA, 1 mM MgCl2, 1 mM DTT and eluted with the
same buffer.

Sedimentation Velocity and Molecular Mass Determination—Sedi-
mentation velocity experiments were carried out by the use of a Beck-
mann Optima XLA ultracentrifuge equipped with an AN 60Ti four-hole
4 °C. Sample volumes of 100 
ml were centrifuged at 15,000 rpm. Radial
scans of absorbance at 278 nm were taken at 4-h intervals in order to
monitor the time course of proteolysis. The reaction was analyzed by yield-weight-average molecular weights by the use of the programs XLAEQ and EQASSOC supplied by Beckman. The partial specific volume was calculated from the amino acid composition to be 0.7215 cm3/g using the SEDNTERP software (John Philo), and the solvent density was 1.01 g/cm3. The
degrees of hydration of the totally unfolded protein were estimated based
on the amino acid composition by the method of Kuntz (20) according to Laue et al. (21). The degree of hydration used for all calculations, 0.3707 g of H2O/g of protein, was the result of correcting the calculated degree of hydration by a factor 0.7 (22).

The exact molecular weight of full-length Ure2p as well as Ure2p fragments was determined by matrix-assisted laser desorption-time of flight mass analysis (Bruker, Bremen, Germany) using e-cyano-4-hy-
droxyccinamic acid as matrix. Peptide sequence data for full-length Ure2p as well as Ure2p fragments were obtained by automated Edman degradation using a sequencer (model 470A or 477A; Applied Biosys-
tems, Inc., Foster City, CA) equipped with an on-line phenylthiohydan-
toin amino acid analysis system (model 120A; Applied Biosystems, In-

Electron Microscopy—Samples of soluble or aggregated Ure2p were negatively stained on carbon-coated grids (200 mesh) with 1% uranyl
acid and examined in a Philips EM 410 electron microscope.

Fluorescence and Light Scattering Measurements—The intrinsic flu-
orescence of Ure2p was recorded at 20 °C in a Aminco-Bowman series 2
spectrofluorometer in 10 x 2 mm quartz cuvettes (Hellma) containing
200 μL of Ure2p solution. The excitation monochromator was set at 290 nm, and the emission was recorded between 300 and 400 nm. Ure2p
conformational changes were monitored by the increase of tyrosine
fluorescence (excitation 290 nm, emission 335 nm).

Ure2p aggregation was monitored at 20 °C by light scattering. The
increase in the intensity of light scattered at 90° from the incident
beam was measured at 340 nm in a Aminco-Bowman series 2
spectrofluorometer.

Proteolytic Digestions—Soluble and aggregated Ure2p (0.8 mg/ml) in
50 mM Tris, pH 7.5, 1 mM EGTA, 1 mM MgCl2, 1 mM DTT were treated at 37 °C by either proteinase K (2.4 μg/ml), subtilisin (1.5 μg/ml), or
trypsin (1.5 μg/ml). Aliquots were removed at different intervals follow-
ing addition of the protease and transferred into Eppendorf tubes
maintained at 90 °C containing sample buffer (50 mM Tris-HCl, pH 6.8,
4% SDS, 2% β-mercaptoethanol, 12% glycerol 0.01% Serva Blue G and
0.01% bromophenol blue) in order to arrest immediately the cleavage
reaction. After incubation of each tube for 10 min at 90 °C, the samples
were processed to monitor the time course of Ure2p cleavage by Tricine/
SDS-polyacrylamide gel electrophoresis (22).

The 35S-labeled NH-terminal and COOH-terminal domains of
Ure2p were generated as described previously (24). Proteinase K or
trypsin (10 μg/ml) was added to the translation reaction, and the time
course of proteolysis was followed as described above. The reaction
products were visualized on the gel using a PhosphoImager (Molecular
Dynamics).

Additional Methods—Protein concentrations were determined by ei-

Expression and Purification of Recombinant Ure2p—The expression
construct pUHE-URE2* was transformed into the BMT171,18 E. coli
strain, grown on LB medium to an A660 of 0.8–1.0, induced with 0.5 mM
isopropyl-1-thio-β-galactopyranoside, and harvested 2.5 h later.
Recombinant Ure2p was found to be in the soluble fraction following lysis
in 10 mM Tris, pH 7.5, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride
and centrifugation at 25,000 × g. Tracer [35S]-labeled Ure2p was added to
the centrifuged lysate after adjusting the buffer conditions to 20 mM
Tris, pH 7.5, 20 mM KCl, 1 mM EGTA, 1 mM MgCl2, 1 mM DTT (buffer
A). The lysate was then filtered through a 0.45-μm Millipore filter and
applied to a 50-ml (2.2 × 15 cm) UNO Q column (Bio-Rad) equilibrated in
buffer A. The column was washed with 150 ml of equilibration buffer
and developed with a 400-ml linear gradient of 20–500 mM KCl. Frac-
tions emerging at salt concentrations between 150 and 185 mM KCl
were pooled. Fractions containing Ure2p were collected and the KCl
concentration adjusted to 1 M, and applied to a 25-mL phenyl-
Sepharose high performance column (Amersham Pharmacia BioTech)
equilibrated in 20 mM Tris, pH 7.5, 1 M KCl, 1 mM EDTA, 1 mM MgCl2,
1 mM DTT. The column was washed with 50 ml of equilibration buffer
and developed with a 200-ml linear gradient beginning with this buffer
and ending with the same buffer containing no KCl. Fractions emerging
from the column in the absence of KCl contained Ure2p. They were

Recombinant overexpressed Ure2p was purified to homogeneity through three successive chromatographic dimensions as described under “Materials and Methods” (Fig. 1A, lanes 3–5). The purified protein was found to have a molecular mass of 40 kDa upon analysis by SDS-PAGE (Fig. 1A) and migrated with an apparent mass of 130 kDa upon gel filtration (Fig. 1B). The molecular mass of this polypeptide (40,415 Da) was determined by mass spectrometry (Fig. 1C). It is consistent with that of the calculated mass of full-length Ure2p (40,441 Da).

Two species with apparent molecular masses of 38 and 23 kDa were also present in several purified Ure2p preparations (Fig. 1A). The molecular masses of these two additional polypeptides were measured by mass spectrometry and found to be 38,372 and 22,079 Da (Fig. 1C). The 40-, 38-, and 23-kDa polypeptides were separated by SDS-PAGE and subjected to automated Edman degradation. The polypeptide that has a molecular mass of 40 kDa corresponds to authentic full-length Ure2p. Amino acid sequencing of the two other peptides gave the following sequences, VNI-NRNSN and SHVEYSRI. The polypeptide that has a molecular mass of 38 kDa corresponds to Ure2p devoid of its 20 first amino acid residues, whereas that of 23 kDa could correspond to an internal initiation of translation occurring at Met-94, resulting in a truncated Ure2p molecule.

**Quaternary Structure of Ure2p**—We first estimated the size of recombinant Ure2p by size exclusion chromatography using a Superose 6 column. Ure2p elutes from this column as a single peak between the molecular weight markers γ-globulin (158 kDa) and ovalbumin (44 kDa) and has an apparent molecular mass of 130 kDa incompatible with the expected molecular mass of monomeric Ure2p (40.4 kDa) (Fig. 1B). Since the measured molecular mass does not fit with that of a dimer of Ure2p nor with that of a tetramer, we envisaged the possibility that Ure2p would not behave as a globular protein. Indeed, a number of proteins have been found to behave on sizing columns with apparent molecular masses very different from that derived from their primary sequences (28–30). These polypeptides are found to be either rod-shaped or to possess unstructured domains.

To investigate whether the elution behavior of Ure2p from a size exclusion column is due to its oligomerization or to its behavior as a non-globular protein, we determined the molecular mass of the protein by equilibrium sedimentation. The weight-average molecular mass of pure Ure2p (0.4 mg/ml) in aqueous solution measured by equilibrium sedimentation was found to be 75,000 (Fig. 2A), inconsistent with its behavior as a monomeric protein. In order to obtain additional insight into the oligomeric state of Ure2p, we carried out sedimentation velocity experiments, to investigate whether the elution behavior of Ure2p from a size exclusion column is due to its oligomerization or to its behavior as a non-globular protein, we determined the molecular mass of the protein by equilibrium sedimentation. The weight-average molecular mass of pure Ure2p (0.4 mg/ml) in aqueous solution measured by equilibrium sedimentation was found to be 75,000 (Fig. 2A), inconsistent with its behavior as a monomeric protein. In order to obtain additional insight into the oligomeric state of Ure2p, at increasing Ure2p concentrations, we carried out sedimentation velocity experiments and analyzed them to yield the apparent distribution of sedimentation coefficients, $g^*(s)$. Fig. 2B shows typical sedimentation boundaries at series of equally spaced times and at increasing Ure2p concentrations. Raw data (symbols in Fig. 2C) were fitted by nonlinear least squares procedures (Fig. 2C, solid line) as described by Philo (18). At low Ure2p concentrations ($\leq 0.4$ mg/ml), the data fit very well to a two-component system involving a 2.8 S and a 4.3 S species with proportions of about 19 and 80%, respectively at a Ure2p concentration of 0.4 mg/ml. Using the relation $(s_1/s_2)^3 = (M_1/M_2)^2$ and bovine serum albumin as a reference (22), one can obtain apparent molecular masses of about 40 and 80 kDa consistent with the behavior of Ure2p as a mixture of monomeric and dimeric molecules. The frictional ratio values ($f/f_0$) suggest that Ure2p is asymmetrical. At higher Ure2p concentrations (0.5 mg/ml), the monomeric species of Ure2p disappeared, and the data fit very well to a single component system involving a 4.3 S species. At even higher Ure2p concentrations ($\geq 0.6$ mg/ml), the data fit very well to a two-component system involving a 4.3 S and a 6.5 S species. The heaviest species has a sedimentation coefficient consistent with the behavior of a tetramer of Ure2p. The above sedimentation velocity data as well as the calculated hydrodynamic parameters of Ure2p are summarized in Table I.

We conclude from these data that soluble Ure2p exists in a
monomeric, dimeric, and tetrameric form in solution. The fact that Ure2p oligomerization is concentration-dependent suggests that these forms are in equilibrium.

**Ure2p Self-associates into High Molecular Weight Oligomers in Vitro—**

Ure2p assembly into insoluble high molecular weight oligomers was achieved by two means. The first consisted of inducing self-association by bringing the pH of the solution from 7.5 to 6.5. The second consisted of incubating Ure2p for prolonged periods at either 4 or 28 °C. Ure2p aggregation was followed spectrophotometrically or by measurement of light scattering. Aggregation of Ure2p was instantaneous upon adjustment of the pH to 6.5 (Fig. 3A). In contrast, Ure2p autoassembly at pH 7.5 was very slow upon incubation of the solution at 4 °C (Fig. 3B) or 28 °C (Fig. 3C). Ure2p assembly into insoluble high molecular weight oligomers is a cooperative process and follows a simple sigmoidal curve. Indeed, three phases can be distinguished in the assembly process as follows: a lag phase where nucleation occurs followed by an elongation phase where assembly accelerates preceding the onset of a plateau. The lag phase is shortened significantly upon addition of Ure2p seeds (not shown) further demonstrating the cooperative character of Ure2p autoassembly.

In all cases, the amount of Ure2p that assembles into high molecular weight oligomers and that sediments upon centrifugation at 4000 × g represents 95% of the initial concentration.

**Table I**

Species of Ure2p formed at increasing protein concentrations and their hydrodynamic parameters

Properties and relative abundance of Ure2p oligomers formed at increasing protein concentrations calculated from sedimentation profiles similar to that displayed in Fig. 2B. The conformational parameters were calculated as described under "Materials and Methods," using the molecular mass and the partial specific volume values determined from the amino acid composition of Ure2p. Numerical values of n-mer of each species were obtained by dividing experimental molecular mass of each species by the monomeric Ure2p theoretical mass. S20,w is the sedimentation coefficient; f and f0 are the friction coefficients, and R is the Stokes radius.

| Concentration and species | S20,w | f/f0 | RS | n-mer | Relative abundance |
|---------------------------|-------|------|----|-------|-------------------|
| 0.4 g/liter Form 1        | 2.8   | 1.14 | 2.30 × 10^-9 | 1   | 19               |
| Form 2                    | 4.3   | 1.57 | 2.90 × 10^-9 | 2   | 79               |
| 0.5 g/liter Form 1        | 4.3   | 1.57 | 2.90 × 10^-9 | 2   | 100              |
| Form 2                    | 4.3   | 1.57 | 2.90 × 10^-9 | 2   | 75               |
| Form 3                    | 6.5   | 1.65 | 3.66 × 10^-9 | 4   | 25               |

**Fig. 3. Assembly of Ure2p into high molecular weight oligomers.** A, kinetic of Ure2p assembly monitored by light scattering. The aggregation of Ure2p in 20 mM potassium phosphate buffer, pH 7.5, 1 mM MgCl₂, 1 mM DTT, 1 mM EGTA at 20 °C was induced at the time indicated by the arrow by adjustment of the pH of the solution to pH 6.5 by addition of HCl. B and C, time course of Ure2p autoconversion from a soluble form to an insoluble form, in 20 mM potassium phosphate buffer, pH 7.5, 1 mM MgCl₂, 1 mM DTT, 1 mM EGTA, followed by measurement of the turbidity of the solution at 4 °C (B) and 28 °C (C) in the absence (●) or the presence of 100 mM (●) and 4 M (●) GdnHCl.
of Ure2p in its soluble form.

Electron microscopy of negatively stained samples of soluble and aggregated Ure2p revealed that the soluble form of Ure2p contains structured circular particles with an outer diameter of about 12 nm (Fig. 4A). In contrast, the insoluble form of Ure2p obtained upon incubation of the protein for 70 h at 4 °C consists of fibrils that are 15–20 nm wide and their length varies between 0.5 and 10 μm (Fig. 4B). These fibers are similar to the fibers that are observed in the case of PrP in its scrapie prion filament form (31) or that form upon Sup35 self-assembly (32). Ure2p fibers are often associated laterally and in some cases twisted together (Fig. 4B, inset). Finally, the aggregated form of Ure2p obtained upon adjustment of the pH from 7.5 to 6.5 consisted of filamentous structures that are up to 0.5 μm long and devoid of any regular shape (not shown).

Time Course of Ure2p Fiber Formation—A distinctive property of amyloid fibers is their capacity to bind the dye Congo Red (33). The spectral shift that accompanies Congo Red binding to amyloid proteins (34) was used to document the time course of Ure2p assembly following the incubation of the soluble form of Ure2p. The lag time (hours) preceded the course of Ure2p fiber formation is monitored by Congo Red binding. Ure2p assembly of the soluble form of Ure2p at 30 °C is shortened at higher Ure2p concentrations (30 mM KPO₄, 150 mM KCl, D) time course of Ure2p assembly (●) upon dilution (1,000-fold) of fibers assembled from a solution of Ure2p (10 μM) into a solution containing the soluble form of Ure2p (10 μM).

Ure2p Is a Two-domain Protein—A number of secondary structure determination algorithms predict Ure2p to be a two-domain protein. The NH₂-terminal domain appears as very poorly structured unlike the COOH-terminal domain. Indeed, the COILS program (35) predicts the NH₂-terminal part of Ure2p to have a high probability of being in coiled coil conformation. These calculations are displayed in Fig. 5A. Interestingly, both parts of the gene encoding Ure2p also clearly reveal differences in codon usage (Fig. 5B). Specific features of synonymous codon distribution along mRNA were suggested and shown in certain cases to reflect domain organization of proteins (36, 37). To determine whether this is indeed the case, Ure2p NH₂-terminal and COOH-terminal domains (amino acid residues 1–94 and 94–354, respectively) were expressed as [35S]-labeled polypeptides in rabbit reticulocyte lysate and subjected to proteinase K or to trypsin treatment. The reaction products were analyzed by SDS-PAGE and visualized using a PhosphorImager. The data are presented in Fig. 6. The COOH-terminal domain of Ure2p which has a molecular mass of 29 kDa (band lettered A) is rapidly cleaved upon proteinase K treatment (Fig. 6A) into three peptides (14.5, 5, and 3 kDa, labeled B, C, and D, respectively). The 14.5-kDa peptide is then degraded into a peptide that has a molecular mass of 10 kDa (band E) and that resists protease cleavage for up to 60 min, whereas the 5.5-kDa peptide generates a polypeptide that has a molecular mass of 3 kDa. The product that has a molecular mass of 7 kDa resists proteinase K treatment for up to 60 min. Treatment of the COOH-terminal domain of Ure2p by trypsin (Fig. 6B) leads to similar observations. The full-length COOH-terminal domain resists protease cleavage for up to 60 min and generates very slowly degradation products that have molecular masses of 25 and 7 kDa (solid and open arrowheads,
were monitored by SDS-electrophoresis followed by autoradiography. 

A

\[ ^{35}S \] methionine. Aliquots were removed at the times shown on the top of each slot, and proteolysis was terminated by heat denaturation after addition of SDS-containing sample buffer. The reaction products were analyzed on Tricine-SDS gels and autoradiographed. Quantitation of the amount of polypeptides that have a molecular mass higher than 3 kDa generated following proteinase K, subtilisin, or trypsin (bottom) treatments of Ure2p NH\(_2\)- and COOH-terminal (●) parts are displayed on the left side of each panel.

respectively) which resist trypsin treatment for up to 120 min (not shown). In contrast, both proteinase K and trypsin treatment of the NH\(_2\)-terminal domain of Ure2p result in a very quick and total degradation of this domain (0.25 and 1.5 min, respectively).

We conclude from these data that the COOH-terminal domain of Ure2p, in agreement with secondary structure predictions, is much more structured and therefore compact and resistant to limited proteolysis treatments than the extended NH\(_2\)-terminal domain of the protein.

The Global Conformation of Ure2p Is Not Affected by Self-assembly—To determine whether Ure2p aggregation is accompanied by a conformational change of the protein, the soluble and aggregated forms of Ure2p were subjected to limited proteolysis. Three different proteases were used as follows: proteinase K, subtilisin, and trypsin. The digestion profiles of the soluble (left) and insoluble (right) forms of Ure2p generated upon proteinase K (Fig. 7A), subtilisin (Fig. 7B), and trypsin (Fig. 7C) treatments are shown in Fig. 7.

The soluble form of Ure2p is rapidly degraded by proteinase K (less than 1 min) into one major product that has a molecular mass of 30 kDa and four other products that have molecular masses comprised between 28 and 22 kDa. Only the products of molecular masses lower than 26 kDa persists 15 min after the onset of proteinase K treatment and evolves toward a final stable product that has a molecular mass of 22 kDa. Proteinase K treatment of the insoluble form of Ure2p generates a number of polypeptides that are similar to the ones generated upon treatment of the soluble form of Ure2p by the same protease. The final product of proteinase K treatment is also the same. However, and in contrast with the profile obtained with the soluble form of Ure2p, full-length Ure2p in its insoluble form appears much more resistant to proteinase K treatment than the soluble form (5 min compared with 30 s).

Subtilisin degrades in less than 30 s the soluble form of Ure2p into two products that have molecular masses of 22 and 20 kDa. These polypeptide chains are then degraded into two lower molecular mass products (14.5 and 7 kDa). The insoluble form of Ure2p resists subtilisin cleavage for nearly 1 min.

Intermediate products that have molecular masses of 33 and 30 kDa that barely exist when the soluble form of Ure2p is subjected to subtilisin treatment persist in the solution for up to 5 min. These intermediates then disappear in favor of two polypeptides that have a molecular mass of 22 and 20 kDa.

Finally, trypsin treatment of both the soluble and insoluble forms of Ure2p generates similar digestion profiles. The major degradation product is a polypeptide that has a molecular mass of 29 kDa. However, the insoluble form of Ure2p appear to be, once again, more resistant to proteolysis than the soluble form of the protein.

We conclude from the comparison of the digestion profiles of the soluble and insoluble forms of Ure2p by proteinase K, subtilisin, or trypsin that the products that are generated are identical and that the soluble form of Ure2p is systematically less resistant to protease treatments than the insoluble form of the protein. Because the digestion patterns for the soluble and insoluble forms of Ure2p are strictly identical, our data further indicate that no major conformational change affects Ure2p upon its aggregation.

The Structure of Ure2p COOH-terminal Domain Changes Slightly upon Self-assembly—Subtle conformational changes occurring during self-assembly of Ure2p may not affect the cleavage sites of the proteases used above. Such conformational changes could be documented by measurement of the intrinsic fluorescence of the protein in the case where aromatic residues
are evenly distributed all along the polypeptide chain. Alternatively, such conformational changes could be detected by measurement of the ellipticity of the protein.

Fig. 8A provides, by measurement of intrinsic fluorescence, an independent indicator of the conformational differences between the soluble and insoluble forms of Ure2p. Given that all tyrosine as well as tryptophan residues are located within the COOH-terminal domain of the protein, these data allow us to investigate whether the conformation of Ure2p COOH-terminal domain changes during the self-assembly process. The intensity of the fluorescence is 50% smaller when Ure2p aggregates into high molecular weight oligomers as induced by bringing the pH from 7.5 to 6.5 (Fig. 8A and B). Furthermore, a slight shift in the wavelength of the emission maximum is observed (Fig. 8A). These changes are consistent with a lesser exposure of tyrosine and tryptophan residues to the aqueous milieu due to self-assembly of Ure2p into high molecular weight oligomers. Indeed, Ure2p pH-induced aggregates scatter the light significantly (Fig. 8C).

We conclude from these observations that Ure2p aggregation is accompanied by a slight conformational change of its COOH-terminal domain. This conformational change is due both to a quenching effect due to the aggregation of Ure2p molecules as well as to a change in the exposure of tryptophan and tyrosine residues to water molecules.

To determine the secondary structure content of Ure2p, we recorded the circular dichroism spectrum of Ure2p in aqueous solution, in the low UV range (Fig. 8D, solid line). The calculated α-helical content of Ure2p is 28%. The CD spectrum of Ure2p in the presence of 60% TFE, a solvent known to stabilize α-helices, was also recorded (Fig. 8D, dashed line). The α-helical content of Ure2p in 60% TFE was found to increase by 8%.

We conclude from these observations that the α-helical content of Ure2p changes to some extent in the presence of a solvent known to stabilize α-helices. Given that the COOH-terminal domain of the protein appears to be compact and structured, the changes in the α-helical content must be due to a stabilization of the NH2-terminal domain of the protein into α-helices. Thus Ure2p NH2-terminal domain appears not only to be less structured than its COOH-terminal domain but also more flexible than the latter.

**DISCUSSION**

Yeast prions Sup35 and Ure2p do not kill the cells that harbor them and are not hazardous nor pathogenic for humans. They present, therefore, a useful model system to study the molecular mechanisms of mammalian prions advent and spread. Our capacity to express recombinant yeast prion Ure2p as a soluble protein allowed us to characterize some of its properties. The work presented here provides new insights into the structure of this protein and the mechanism of its assembly into various quaternary structures.

**Structure of Ure2p**—Soluble Ure2p was found to emerge from a sizing column with an apparent molecular mass of 130,000 incapable with that expected for a monomeric Ure2p molecule that would have a globular shape. Sedimentation velocity measurements reveal that recombinant Ure2p forms oligomers in a concentration-dependent manner. Indeed, soluble Ure2p preparations are heterogeneous mixtures of monomeric, dimeric, and tetrameric Ure2p molecules as well as higher order oligomers. Examination of soluble Ure2p preparations by electron microscopy reveals globular but heterogeneous particles. The most striking structures we observed were ring-shaped particles with an outer diameter of 10 nm composed of four arrowheads pointed toward the center of the particles. The finding that Ure2p oligomerization is concentration-dependent indicates that the monomeric, dimeric, and tetrameric forms of Ure2p as well as the higher molecular weight oligomers are in equilibrium. Over 95% of the soluble form of Ure2p was found to assemble into insoluble high molecular weight oligomers either upon incubation of the protein for prolonged periods at 4 or 28 °C or adjustment of the pH of the solution from 7.5 to 6.5. In the latter case fibrillar structures highly heterogeneous in size and shape were obtained while fibrils that are 15–20 nm wide varying in length between 0.5 and 10 μm were obtained upon incubation of the soluble form of Ure2p at either 4 or 28 °C. These fibers appear very similar in the electron microscope to PrP scrapie-associated filaments and to the fibers observed upon Sup35 autoassembly and bind Congo Red which is a characteristic of amyloid fibers.

The kinetics of Ure2p assembly are sigmoidal indicating a cooperative process. Furthermore, the lag phase preceding Ure2p assembly depends on the concentration of the protein and disappears upon addition of preformed Ure2p fibers to the soluble form of the proteins which indicates that Ure2p autoassembly is a nucleated process. Finally, the critical concentration for Ure2p assembly appears to be lower than 0.5 μM since the proportion of protein assembled into fibers represents 95% of the input soluble Ure2p (10 μM). Taken together, the properties of Ure2p in vitro can account for the propagation of Ure2p polymers in a manner similar to what occurs in the case of Sup35 and PrP.

**Conformational Changes of Ure2p**—Several genetic studies suggest that Ure2p is a two-domain protein that were recently shown by means of the two-hybrid system to interact with each other (38). Indeed, the NH2-terminal third of the molecule appears essential for the occurrence of [URE3] phenotype, whereas the catalytic activity of the protein appears to be...
located in the COOH-terminal two-thirds of the molecule (10, 39). Secondary structure calculation algorithms predict that most of the NH₂-terminal third of the protein is unstructured (i.e. essentially constituted by coils, turns, and sheets), whereas the α-helical structures are mainly located in the COOH-terminal two-thirds of the protein. Thus Ure2p is predicted to be at least a two-domain protein. These predictions are supported by the data obtained upon treatment of the COOH- and NH₂-terminal domains of the protein, expressed independently, with different proteases. Ure2p NH₂-terminal domain showed a much higher sensitivity to protease treatments than the COOH-terminal domain of the protein. Indeed, none of the peptides generated upon protease treatment of the NH₂-terminal domain was observed longer than 1 min, whereas the products of Ure2p COOH-terminal domain cleavage were stable for over 1 h. These studies revealed two fragments (22 and 14.5 kDa) highly resistant to proteinase K as well as two others (29 and 7 kDa) highly resistant to trypsin. These peptides were tentatively identified as a breakdown products of Ure2p COOH-terminal domain since they are generated upon treatment of Ure2p 94–354 fragment by the same proteases. The largest peptide (29 kDa) would correspond to Ure2p COOH-terminal domain devoid of its 8–11 NH₂-terminal amino acid residues or to the same domain devoid of its 10 COOH-terminal amino acid residues. Confirmation of the assignment will ultimately require the isolation and sequencing of the 29-kDa fragment. The same approach will lead to assignment of the 22-, 14.5-, and 7-kDa fragments.

A major conformational change occurring upon Ure2p aggregation would result in differences in the digestion profiles of the two forms of the protein. This is not what we observed. Indeed, treatment of full-length Ure2p in its soluble or aggregated forms by proteinase K or trypsin yielded peptides that are identical to that generated upon treatment of Ure2p COOH-terminal domain by the same proteases. However, the kinetics of cleavage were significantly slower for the insoluble form of Ure2p. Such results are what one expects in the case of a reduced accessibility of Ure2p (the substrate) to the protease. Furthermore, the peptides generated upon either proteinase K or trypsin treatments of full-length Ure2p were that generated upon treatment of the COOH-terminal domain of Ure2p by the same proteases. This strongly suggests that the NH₂-terminal domain is rapidly degraded whether or not the COOH-terminal domain is present. The rapid and total degradation of Ure2p NH₂-terminal domain would be a consequence of its lack of structure and compactness. Finally, our findings also indicate that the COOH-terminal domain adopts the same conformation in the presence or absence of the NH₂-terminal domain, since the same protease cleavage sites are exposed whether the NH₂-terminal domain of the protein is present or not.

Proteolytic treatment of proteins is a powerful tool to probe conformational changes that may affect the structure of the substrate polypeptides. Nevertheless, a number of tiny changes may well be overlooked. In order to detect such small changes, intrinsic fluorescence as well as circular dichroism measurements were carried out. Given that all tryptophan and tyrosine residues are located within Ure2p COOH-terminal domain, intrinsic fluorescence measurements allowed us to follow conformational changes affecting this domain of the protein. The data presented in this work clearly demonstrate that the COOH-terminal domain of Ure2p undergoes a conformational change during self-assembly process that results in a 50% decrease in exposure of tyrosine and tryptophan residues to water concomitant with a shift in the wavelength of the emission maximum. Circular dichroism data suggest that a number of amino acid residues in Ure2p are in α-helical structures. The amount of α-helices increases when the protein is in the presence of TFE, a solvent known to stabilize α-helical structure. This could be due to the transition of stretches of amino acid residues in Ure2p NH₂-terminal domain from random coils to α-helical structures. Alternatively, the increase of the content of α-helices could correspond to amino acid residues located within the COOH-terminal moiety of the protein that adopts a structure predominantly α-helical upon TFE addition. Consequently, methods allowing the specific labeling of the Ure2p NH₂-terminal domain with extrinsic fluorophores will have to be designed in order to access the effect of the solvent as well as autoassembly on the flexibility of this domain.

Present genetic, biochemical, and structural data (3–5, 9, 10, 39) allow us to make a brief comparison of some properties of mammalian prion protein PrP and yeast prions, Ure2p and Sup35p. In all cases proteins seem to consist of at least two major parts. Indeed, although the NH₂-terminal part (bearing unusual amino acids repeats, rich in Gly in the case of PrP (4, 6) and in Asn and Gln in the case of Ure2p and Sup35p (10)) seems to be in all the cases unstructured and flexible and at the same time absolutely required for the propagation of prion conditions (through an extensive aggregation of proteins), the COOH-terminal part appears to be compactly folded. We show here that Ure2p like Sup35 and PrPc can undergo extensive aggregation into highly ordered fibers. Our data demonstrate that preformed Ure2p fibers incorporate the soluble form of the protein and propagate Ure2p assembly in a manner similar to what is observed in the case of Sup35 (32, 40) and support a “protein only” seeded polymerization model for Ure2p.

Conversion of the cellular form of the prion protein (PrPc) to the scrapie isofrom (PrPSc) (leading further to its aggregation) is thought to be driven by an α-helical to β-sheet conformational transition (4, 5). The poorly structured NH₂-terminal part of the prion protein (14, 15) seems to be ultimately crucial in such a α → β interconversion, providing the plasticity required for a conformational change. This characteristic is common among PrPc, Sup35, as well as Ure2p. Nevertheless, such conformational changes are not necessarily restricted to the NH₂-terminal part of these proteins. Indeed, the majority of the mutations affecting the efficiency of propagation of prion diseases is associated with the COOH-terminal part of the protein (4, 5). Our finding that the conformation of the COOH-terminal part of Ure2p is affected during aggregation is in favor of a mechanism of assembly similar among all prions.

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