Structure of the Prion Ure2p in Protein Fibrils Assembled in Vitro

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The Ure2 protein from the yeast Saccharomyces cerevisiae has prion properties. In vitro and at neutral pH, soluble Ure2p spontaneously forms long, straight, insoluble protein fibrils. Two models have been proposed to account for the assembly of Ure2p into protein fibrils. The “amyloid backbone” model postulates that a segment ranging from 40 to 70 amino acids in the flexible N-terminal domain from different Ure2p molecules forms a parallel superpleated β-structure running along the fibrils (Kajava, A. V., Baxa, U., Wickner, R. B., and Steven, A. C. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 7885–7890). The second model hypothesizes that assembly of full-length Ure2p is driven by limited conformational rearrangements and non-native inter- and/or intramolecular interactions between Ure2p monomers (Bousset, L., Thomson, N. H., Radford, S. E., and Melki, R. (2002) EMBO J. 21, 2903–2911). Here, we performed a cysteine scan on residues located in the N- and C-terminal parts of Ure2p to determine whether these domains interact. Amino acid sequences centered around residue 6 in the N-terminal domain of Ure2p and residue 137 in the C-terminal moiety interacted at least transiently via intramolecular interactions. We documented the assembly properties of a Ure2p variant in which a disulfide bond was established between the N- and C-terminal domains and showed that it possesses assembly properties indistinguishable from those of wild-type Ure2p. We probed the structure of Ure2pC6C137 within the fibrils and demonstrate that the polypeptide is in a conformation similar to that of its soluble assembly-competent state. Our results constitute the first structural characterization of the N-terminal domain of Ure2p in both its soluble assembly-competent and fibrillar forms. Our data indicate that the flexibility of the N-terminal domain and conformational changes within this domain are essential for fibril formation and provide new insight into the conformational rearrangements that lead to the assembly of Ure2p into fibrils and the propagation of the [URE3] phenotype in yeast.

Bakers’ yeast cells exhibiting the non-mendelian genetic element [URE3] (1) show altered negative regulation of nitrogen metabolism (2–4). The [URE3] phenotype is due to the prion properties of the Ure2 protein (5). In a manner similar to other proteins with prion properties from Saccharomyces cerevisiae, Ure2p is a two-domain protein. The physical boundary between the two domains is residue 94 (6). The N-terminal domain, rich in asparagine, glutamine, serine, and threonine residues (62% of the total residues), is crucial for prion propagation and is therefore referred to as the prion domain (7). The compactly folded, mainly α-helical (8, 9) C-terminal domain binds glutathione (10) and has glutathione peroxidase activity (11).

In the soluble form of natural dimeric Ure2p, the prion domain is believed to be poorly structured (6, 12, 13). We have shown recently, however, that it certainly contains some elements of secondary structure (14). A number of studies suggest a potential interaction between the N- and C-terminal domains of Ure2p. Indeed, evidences for an interaction between regions 1–63 and 152–354 were found using the two-hybrid test (15). Region 151–158 was later described as inhibiting prion propagation (16). Finally, an increased rate of [URE3] occurrence was described for a Ure2p variant (termed km 1,2,3) when amino acid substitutions in region 10–17 and the region centered around residue 127 were associated (17). The observations made using the two-hybrid system do not allow distinguishing between inter- or intramolecular interactions between the N- and C-terminal domains of Ure2p. The finding that region 151–158 has prion-inhibiting properties, together with the fact that the N-terminal domain is strictly required for prion propagation, suggests that regions within the C-terminal domain of Ure2p interact with the N-terminal domain. As a consequence, the protein remains in a state that has low aggregation propensity. Conversely, the prion-promoting activity of region 221–227 also suggests an interaction between the N- and C-terminal domains of Ure2p (16). In the latter case, the protein has high aggregation propensity. Last, the simplest explanation for the observation that, when associated, amino acid substitutions in the N- and C-terminal domains of Ure2p increase the rate of [URE3] occurrence is that these substitutions favor a state that has high aggregation propensity, possibly by impairing the interaction between the two domains.

To determine whether the N- and C-terminal moieties of Ure2p interact, we performed a cysteine scan. A number of Ure2p variants in which Gly, Ser, Arg, and Phe residues located in the N- and C-terminal parts of the protein were replaced with cysteine residues were generated. Several such Ure2p variants were soluble. To determine whether the N- and C-terminal domains interact, Ure2p variants with cysteine residues in the N- and C-terminal domains were generated. The combination of Cys6 and Cys137 was interesting. Using this variant, we show here that the N-terminal domain of Ure2p interacts with its C-terminal moiety at least transiently via intramolecular interactions. We document the assembly properties of a Ure2p variant in which a disulfide bond was established between Cys6 and Cys137 and show that it possesses assembly properties indistinguishable from those of wild-type Ure2p. Finally, we probe the structure of Ure2pC6C137 within the fibrils and demonstrate that the polypeptide is in a conformation similar to that of its soluble state. These results strengthen the model we proposed for Ure2p assembly in which Ure2p fibrils obtained under phys-
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Iologically relevant conditions are built of native-like units in a manner reminiscent of the assembly of other biological polymers (18).

MATERIALS AND METHODS

Protein Purification and Construction of Ure2p Variant Expression Vectors in Escherichia coli—Recombinant full-length Ure2p (amino acids 1–354) and the variant Ure2p91EGR94 (Ure2pFX) were overexpressed as soluble proteins in E. coli, purified as described previously (6, 14, 19), and stored at −80 °C in buffer A (20 mM Tris-HCl (pH 7.5), 250 mM KCl, 1 mM dithiothreitol (DTT), and 1 mM EGTA) at a concentration of 5–10 mg/ml. Protein concentrations were determined spectrophotometrically (Hewlett-Packard HP 8453 diode array spectrophotometer) using an extinction coefficient of 0.67 mg/cm−2 at 280 nm and a molecular mass of 40.2 kDa.

A number of Ure2p variants in which Gly, Ser, and Phe residues located in the N- and C-terminal parts of Ure2p were replaced with cysteine residues were generated. The variants Ure2pC6 and Ure2pC137 were of interest for this study. The Ure2pC6 and Ure2pFXC6 expression vectors were obtained by site-directed mutagenesis by replacing the GGC codon encoding Gly6 with a TGC codon encoding a cysteine residue. Mutagenesis was performed using the QuikChangeTM site-directed mutagenesis kit (Stratagene Europe, Amsterdam, The Netherlands), the pET-URE2FX expression vector (14), and primers 5′-CATATGATGAATAACAACTGCAACCAAG-TGTTCGAATA-3′ and 5′-ATTCGACACTTGGTTGCAGTTGTTAT-TCCATCATATG-3′. Ure2pC6C137 and Ure2pFXC6C137, in which the TTD codon encoding Phe137 was replaced with a TGT codon encoding a cysteine residue, were generated in the same manner using the pET-URE2C6 and pET-URE2FXC6 expression vectors, respectively, and primers 5′-CTAAAGTGAACCTTGGATGTCATTATAACACAATC-3′ and 5′-GATTGTGTTATAATGACATCCAAGTTCACTTAG-3′.

Covalent Modification of Ure2pC6 and Cross-linking of Soluble and Fibrillar Ure2pC6 and Ure2pC6C137—Ure2pFXC6 (50 μM) in buffer B (20 mM Tris (pH 7.5), 250 mM KCl, and 1 mM EGTA) supplemented with 55 μM DTT was incubated with 150 μM 6-bromoacetyl-2-dimethylaminonaphthalene (Molecular Probes, Inc., Eugene, OR) for 2 h at 4 °C to allow the formation of a thioether bond between the sulfhydryl group of Ure2pC6 and the probe. The acetyl-2-dimethylaminonaphthalene (ADAN)-Ure2pC6 complex was separated from unreacted dye by dialysis, the proteins were either incubated for 12 h at 4 °C or washed with 50 mM Tris-HCl (pH 6.8), 4% SDS, 12% glycerol, and 0.01% bromphenol blue and then analyzed and then reduced directly on the MALDI target by addition of 0.5 M DTT in 50 mM ammonium bicarbonate. The latter treatment was achieved at an enzyme/substrate ratio of 1:20 (w/w).

The reaction was performed at 37 °C in 20 mM Tris (pH 7.5) and 75 mM KCl for 5 h. The trypsin used was sequencing grade from Roche Diagnostics. All cleavage reactions were stopped by addition of 1 mM phenylmethylsulfonyl fluoride together with protease inhibitor mixture (Complete™, Roche Diagnostics) following the manufacturer’s recommendations. The samples to be used for SDS-PAGE analysis were immediately mixed (1:1 volume ratio) with denaturing buffer (50 mM Tris-HCl (pH 6.8), 4% SDS, 12% glycerol, and 0.01% bromphenol blue) with or without 2% β-mercaptoethanol (depending on whether the disulfide bond needed to be maintained or not), preheated at 95 °C, and further incubated at the same temperature for 4 min.

SDS-PAGE and Western Blotting—SDS-PAGE was performed on 12% polyacrylamide gels (14 × 15 × 0.15 cm) following the standard method described by Laemmli (21). To sequence the proteolytic products observed on the SDS gels, the polypeptides were transferred onto polyvinylidene difluoride membranes (Applied Biosystems) following the manufacturer’s recommendations (30). For other purposes including quantification, the gels were stained with Coomassie Blue, destained, imaged using a Sony CCD camera, and further analyzed on a Macintosh computer (Apple Computer, Inc., Cupertino, CA) using NIH Image software (available at rsb.info.nih.gov/nih-image/).

Matrix-assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) Analysis—Soluble and fibrillar Ure2p and their respective fragments were subjected to MALDI-TOF-MS analysis. Sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid; Aldrich) was used as matrix for long polypeptides, whereas α-cyano-4-hydroxycinnamic acid (Aldrich) was used as matrix for short peptides. Both matrices were prepared as saturated solutions in 30% acetonitrile, 70% water, and 0.1% trifluoroacetic acid. The spectra were acquired on a MALDI-TOF mass spectrometer (Voyager-DE STR, Applied Biosystems) equipped with a nitrogen laser beam (337 nm) and a delayed extraction device. Mass measurements were performed in positive and linear modes. For protein samples, the accelerating voltage was 25 kV; the grid voltage was 90%; the guide wire was 0.3%; and the delayed extraction was 1600 ns. For tryptic peptides, the acceleration voltage was 20 kV; the grid voltage was 90%; the guide wire was 0.1%; and the delayed extraction was 750 ns. For trypsin peptides, the acceleration voltage was 20 kV; the grid voltage was 93%; the guide wire was 0.1%; and the delayed extraction was 300 ns. Mass calibration was achieved using enolase, carbonic anhydrase, cytochrome c, adrenocorticotropic hormone (ACTH) (1–18), ACTH (1–39), ACTH (1–39), ACTH (1–17), neurotensin, [Tyr4]substance P, and des-Arg-bradykinin. To confirm the establishment of disulfide bonds, samples were analyzed and then reduced directly on the MALDI target by addition of 5 mM DTT in 50 mM ammonium bicarbonate. The latter treatment was carried out for 10–20 min at room temperature, the time necessary to obtain a dry preparation. The samples were then washed with 5 μl of 1% trifluoroacetic acid, supplemented with 0.5 μl of matrix, and further analyzed by mass spectrometry.

Protein Sequencing—N-terminal sequences were obtained by automated Edman degradation using an Applied Biosystems sequencer equipped with an on-line phenylthiohydantoin-derivative analysis system (Model 120A, Applied Biosystems).

RESULTS

The N-terminal Domain of Ure2p Interacts with Its C-terminal Moiety—The N-terminal domain of Ure2p is widely believed to be poorly structured (6) and highly flexible (13). We recently found evi-

The abbreviations used are: DTT, dithiothreitol; ADAN, acetyl-2-dimethylaminonaphthalene; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; ACTH, adrenocorticotropic hormone.
dence suggesting that this important part of Ure2p is not fully unstructured (14). To determine whether the N- and C-terminal moieties interact, we generated a number of Ure2p variants in which Gly, Ser, Arg, and Phe residues located in the N- and C-terminal parts of the protein were replaced with cysteine residues. In a number of cases, single mutations led the protein to misfold and aggregate. Other variants such as Ure2pC6, Ure2pC17, Ure2pC137, Ure2pC221, Ure2pC276, Ure2pC283, Ure2pC290, Ure2pC292, and Ure2pC353 were soluble. All of these variants have the same α-helical content as wild-type Ure2p and are mainly dimeric as determined by circular dichroism measurements and analytical ultracentrifugation, respectively. To determine whether the N- and C-terminal domains interact, Ure2p variants in which each cysteine residue in the N-terminal domain was replaced with cysteine resulted in the establishment of an intermolecular disulfide bond between Ure2pC6 monomers.

This was confirmed by analyzing the degradation products of the Ure2pFXC6 dimer following treatment with the site-specific protease factor Xa by both SDS-PAGE and MALDI-TOF-MS (Fig. 1, D and E). Treatment of reduced Ure2pFXC6 (Fig. 1, D, lane 3) yielded two polypeptides corresponding to Ure2p-(1–94) and Ure2p-(95–354) with apparent molecular masses of 14 and 30 kDa, respectively (measured masses of 10,497 and 29,764 Da, respectively). Treatment of cross-linked Ure2pFXC6 (Fig. 1, D, lanes 4 and 5; and E) yielded two additional species with apparent molecular masses of 50 and 23 kDa (MH+ + 20,139 Da) in addition to the 30-kDa species. The 50-kDa species corresponds to Ure2p/Ure2p-(1–94), whereas the 23-kDa species corresponds to the Ure2p-(1–94) dimer.

Most interesting, however, under mild oxidative conditions, an additional band with an apparent molecular mass of 37 kDa was observed on the gel (Fig. 1, A, lane 3). The latter species was not observed when the Ure2pC6 or Ure2pC137 variant was incubated under the same experimental conditions. The lower molecular mass of the latter species indi-
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TABLE ONE

| Ure2p molecular species that form under reducing, partially, and fully oxidative conditions |
|---------------------------------------------------------------|
| The proportions were determined using NIH Image software by SDS-PAGE (Fig. 1, A and D). |
| Experimental conditions | Ure2pC6 | Ure2pC6C137 |
|-------------------------|---------|------------|
| Reducing                | 100% monomer | 100% monomer |
| Mild oxidative          | 50 ± 2% dimer, 50 ± 2% monomer | 45 ± 3% dimer, 30 ± 3% monomer, 25 ± 3% monomer with intramolecular disulfide bridge |
| Strong oxidative        | 100% dimer | 100% dimer |

cates that the Ure2p monomer was more compact after treatment and suggests internal cross-linking. Such a species can indeed occur, as the substitution of Phe137 with Cys leads to the partial exposure of the side chain of Cys137 to the solvent (supplemental Fig. 2) according to the crystal structure of Ure2p-(95–354) (8). It is important here to note that Cys137 needs to be partially exposed to avoid the establishment of disulfide bonds between two Cys137 residues. The proportions of the observed species under the different experimental conditions were determined and are summarized in TABLE ONE. Treatment of the slow and fast migrating Ure2pFXC6 species after cleavage by factor Xa with β-mercaptoethanol yielded a polypeptide with the molecular mass of authentic Ure2p (Fig. 1A, lane 5), suggesting that the observed species with an apparent molecular mass of 37 kDa is not a truncated form of Ure2p. This is further supported by the observations that (i) no additional species with lower molecular mass were detected by MALDI-TOF-MS and (ii) sequencing of the N-terminal amino acids of the fast migrating species yielded the expected sequence for authentic Ure2p, MMNNN.

The Ure2pC6C137 forms generated under mild oxidative conditions were characterized by MALDI-TOF-MS following tryptic digestion of the reaction products. The peptide mass fingerprints of reduced and partially oxidized Ure2pC6C137 are presented in Fig. 2. Monoprototated ions corresponding to peptides 1–17 and 128–152 were detected in the peptide mass fingerprint of reduced Ure2pC6C137 (average MH+ of 1924.2 and 2862.3 Da, respectively) (Fig. 2A). The ion corresponding to peptide 128–152 disappeared from the peptide mass fingerprint, and that of peptide 1–17 decreased significantly upon incubation of Ure2pC6C137 under mild oxidative conditions, whereas the ion corresponding to peptide 1–17 cross-linked through a disulfide bond ((1–17)–S–S–(1–17)) and the ion corresponding to peptide 1–17 cross-linked to peptide 128–152 ((1–17)–S–S–(128–152)) (MH+ of 3846.4 and 4784.5 Da, respectively) were detected (Fig. 2B).

To document the specificity of the interaction between the regions centered around residues 6 and 137, the behavior of the Cys137/Cys137 and Cys6/Cys283 combinations were examined. No species with a lower molecular mass were obtained under strong or weak oxidative conditions for either variant. Instead, dimeric forms were generated (data not shown). This suggests that the range of conformations that the N-terminal domain of Ure2p can adopt is limited and that the interaction of the regions centered around residues 6 and 137 is stringent.

Based on these observations, we conclude that the N-terminal domain of Ure2p is flexible, as it establishes intermolecular interactions with the N termini from other Ure2p molecules. Most important, however, we also conclude that it establishes at least transient, but specific intramolecular interactions with the C-terminal moiety of the polypeptide chain.

Assembly Properties of Ure2pC6 in Its Reduced and Oxidized Forms—

The ability of the Ure2pC6 variant to assemble into fibrils under reducing and oxidative conditions was next examined by thioflavin T binding and negative stain electron microscopy. The assembly kinetics of Ure2pC6 under reducing conditions and wild-type Ure2p were very similar (Fig. 3A). Although the assembly of wild-type Ure2p was unaffected by the strong oxidative conditions used in this study, that of Ure2pC6 was totally inhibited (Fig. 3A). Fibrils assembled from wild-type Ure2p and Ure2pC6 under reducing conditions were indistinguishable on negative stain electron micrographs (Fig. 3, B and C). By contrast, oxidized Ure2pC6 remained in a soluble oligomeric form (Fig. 3D), which has been described previously (6, 14).

Under the mild oxidative conditions used, when 50% of Ure2pC6 was cross-linked, the protein assembled into fibrils (Fig. 3A) with an
increased lag phase, a decreased elongation rate, and thioflavin T fluorescence intensity at steady state. Ure2p assembly is highly cooperative (6, 22). Fig. 3A clearly shows that the assembly kinetics of 50% oxidized Ure2pC6 (41 μM) overlapped perfectly with that of wild-type Ure2p (20 μM). To strengthen our observations, we performed an SDS-PAGE analysis of the polypeptide content of the supernatant and pellet fractions of the assembled forms of wild-type Ure2p and reduced, 50% oxidized, and 100% oxidized Ure2pC6. The data are presented in Fig. 3E. They clearly demonstrate that cross-linked Ure2pC6 neither aggregated (s4 and p4 lanes) nor assembled into fibrils (s3 and p3 lanes).

To further characterize the assembly properties of dimeric Ure2pC6, the elongation rates of preformed Ure2p fibrils were measured at a constant overall Ure2p concentration with increasing proportions of dimeric Ure2pC6. Control measurements in which the concentration of wild-type Ure2p or the reduced form of Ure2pC6 varied from 0 to 20 μM were run in parallel. The data presented in Fig. 3F clearly demonstrate that dimeric Ure2pC6 did not interfere with the assembly reaction.

The decreased assembly propensity of Ure2p upon establishment of intermolecular disulfide bonds may be the consequence of (i) the tightening of the Ure2p N-terminal domain in a given conformation; (ii) the reduction of its flexibility and, as a consequence, the number of conformations it can adopt; or (iii) the disruption of a potential intramolecular interaction between the N- and C-terminal domains of the protein. To distinguish among these three hypothesis, a large hydrophobic group (ADAN) was attached to the reactive Cys6 residue to hinder a potential intramolecular interaction between the N- and C-terminal domains of the protein, and the assembly properties of the modified Ure2pC6 variant was documented by thioflavin T binding and negative stain electron microscopy. Fig. 4A clearly demonstrates that the ability of reduced Ure2pC6 to assemble into fibrils was abolished upon modification of Cys6 by the hydrophobic ADAN group. Large amorphous aggregates formed instead (Fig. 4B), although the protein remained monomeric upon nonreducing SDS-PAGE (Fig. 4C).

We conclude from these observations that hindering a potential intramolecular interaction between the N- and C-terminal domains...
of the protein or a conformational change induced within the N-terminal domain of Ure2p by the attachment of the hydrophobic ADAN group prevents Ure2p from assembling into fibrils. We further conclude that the N-terminal domain is crucial for assembly, in agreement with previous reports from our group (19) and others (23).

Intramolecular Interactions between the N- and C-terminal Domains of Ure2p in the Precursor of the Fibrillar Form of the Protein—In a manner similar to Ure2pC6, the assembly of the Ure2pC6C137 variant into fibrils was very similar to that of wild-type Ure2p (Fig. 5A), and the fibrils were indistinguishable from those made of wild-type Ure2p under the electron microscope (Fig. 5B). Under the strong oxidative conditions used in this study, Ure2pC6C137 dimerized into a species indistinguishable from that generated upon oxidation of Ure2pC6.

This dimeric form, similar to that of dimeric Ure2pC6, was unable to assemble into fibrils (Fig. 5A). Under the mild oxidative conditions used in this study, three products with apparent molecular masses of 72, 40, and 37 kDa were observed upon SDS-PAGE, representing 50, 30, and 20% of the material, respectively (Fig. 1A and TABLE ONE).

When the ability of the latter mixture to assemble into fibrils was assayed using the thioflavin T binding assay, the assembly kinetics overlapped perfectly with (i) that of 50% oxidized Ure2pC6, (ii) that of a 1:1 mixture of wild-type Ure2p and fully oxidized Ure2pC6 (compare Figs. 3A and 5A), and (iii) wild-type Ure2p (20 µM) compared with the control reaction, in which the overall wild-type Ure2p or reduced Ure2pC6C137 concentration was twice as high (41 µM) (supplemental Fig. 3). This suggests that the Ure2p variant with its N- and C-terminal domains bound by a covalent intramolecular interaction assembles into fibrils.
To characterize the assembly properties of the form of Ure2p in which a disulfide bond was established between Cys6 and Cys137, we compared the elongation rates of preformed Ure2p fibrils at increasing concentrations of partially oxidized and reduced Ure2pC6C137 and wild-type Ure2p (0–20 µM). The data are presented in Fig. 5C. They show that the Ure2p species in which the N- and C-terminal domains were bound through an intramolecular bond behaved in a manner indistinguishable from wild-type Ure2p.

To further demonstrate that the Ure2p species in which Cys6 and Cys137 are linked through an intramolecular bond incorporated into the fibrils, we performed an SDS-PAGE analysis of the polypeptide content of the supernatant and pellet fractions of the assembled forms of wild-type Ure2p and reduced, partially oxidized, and fully oxidized Ure2pC6C137. The data clearly demonstrate that the cross-linked Ure2pC6C137 species assembled into fibrils together with the reduced form of Ure2pC6C137 (Fig. 5D, p2 and p3 lanes), unlike the fully oxidized form of this Ure2p variant (s3 and s4 lanes). From these observations, we conclude that the reduction of the flexibility of the N-terminal domain of Ure2p by the establishment of intramolecular disulfide bonds does not influence the assembly properties of Ure2p.

Interaction between the N- and C-terminal Domains of Ure2p in the Fibrillar Form of the Protein—To determine whether the N-terminal domain of Ure2p adopts a conformation similar to that in the soluble form that assembles into fibrils, the Ure2pC6C137 variant was assembled under reducing conditions, and the fibrils were sedimented, incubated under strong oxidative conditions, and processed for SDS-PAGE analysis. Under strong oxidative conditions, soluble Ure2pC6C137 dimerized into a product with an apparent molecular mass of 72 kDa (Fig. 1A). No such species formed upon incubation of the fibrillar form of the protein under the same experimental conditions (Fig. 6A). Instead, the fast migrating species (37 kDa) formed, representing ~50% of the total amount of fibrillar Ure2pC6C137. Furthermore, when fibrillar Ure2pC6 was fully oxidized, no dimeric Ure2pC6 was generated, in contrast to what was observed for the soluble form of the protein (compare Figs. 1A and 6B). We conclude from these observations that the degree of freedom of the N-terminal domain of Ure2p decreases significantly upon assembly as the intermolecular interactions that can be established when the protein is in its soluble state are abolished. We further conclude that the N- and C-terminal parts of Ure2p that establish intramolecular interactions are within the fibrillar scaffold in a conformation compatible with the establishment of a disulfide bond under oxidative conditions, i.e., in a state close to that adopted in soluble Ure2p.

**DISCUSSION**

The prion Ure2p assembles in vitro into protein fibrils (6). The N-terminal domain of Ure2p plays a critical role in the assembly reaction (19, 23). Although there is general agreement on an assembly reaction driven by a conformational change occurring in the flexible N-terminal domain of the protein, there is disagreement on the extent of the conformational change that leads to Ure2p assembly. Indeed, two models have been proposed to account for the assembly reaction. The first hypothesizes that assembly is driven by a significant conformational change in a fragment ranging from 40 to 70 amino acids (of 94) in the N-terminal domain of the protein, leading to its organization into a cross-β-core running along the fibrils (24, 25). The second model hypothesizes that the assembly of full-length Ure2p is driven by limited conformational rearrangements and non-native inter- and/or intramolecular interactions between Ure2p monomers (18). We showed recently that the C-terminal domain of Ure2p is tightly involved in the fibrillar scaffold (14). We also showed that assembly of Ure2p into protein fibrils under physiologically relevant conditions is accompanied by changes in the exposure of protease cleavage sites located in sequences Leu12–Phe37 and Arg56–Arg66 to the solvent (14). The exposure of sequence Leu12–Phe37 to the solvent decreases, whereas that of sequence Arg56–Arg66 (which links the N-terminal domain to the C-terminal domain of the protein) increases upon assembly.

To further document the structure of the N-terminal domain of Ure2p upon assembly, we generated Ure2p variants in which cysteine residues were introduced in the N-terminal, C-terminal, and both the N- and C-terminal domains of Ure2p to probe the effect of reducing the flexibility of the N-terminal domain on the protein. When the N-terminal domain of Ure2p was stabilized in a dimeric form, the protein lost its propensity to assemble into fibrils, thus demonstrating the importance of the flexibility of this moiety in the assembly process. Under mild oxidative conditions, a disulfide bond was established between residues 6 and 137. No such bonds were established using the other combinations we generated. This indicates that these two amino acids face each other at least transiently in a conformation compatible with the establishment of the disulfide bond.

Previous studies suggested an interaction between the N- and C-terminal domains of Ure2p that modulates the prion properties of the protein. These indirect suggestions were based on the use of the two-hybrid test (15) and on genetic evidences (16) and were unable to distinguish between inter- or intramolecular interactions between the N- and C-terminal domains of Ure2p. Here, we provide direct evidences for an intramolecular interaction between these two domains. The Ure2p species with a covalent bond between residues 6 and 137 assembled in a manner indistinguishable from wild-type Ure2p. We conclude from this observation that the flexibility of the N-terminal domain of Ure2p, which is crucial for the assembly of Ure2p into protein fibrils, is not affected by bridging residue 6 to residue 137. Indeed, it is important to bear in mind that such a covalent bond may allow an important degree of freedom for amino acids 7–94. We reported previously that the exposure of sequence Arg56–Arg66 to the solvent increases upon assembly (14). It is reasonable to envisage that the precursor of the fibrillar state of Ure2p is a form in which a conformational change is limited to sequence...
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Asn$^7$–Ala$^{90}$. This is consistent with the finding that deletion of amino acids 15–42 affects very significantly the assembly properties of Ure2p (23). Indeed, in marked contrast with what one would expect if the assembly of Ure2p were driven mainly by the N-terminal poly-Gln/Asn extension, the assembly of the Ure2pΔ15–42 variant, in which the Gln and Asn proportion in the N-terminal domain is 54% (35 amino acids of 65) compared with 46% (43 amino acids of 93) in full-length Ure2p, is very significantly diminished, thus strongly suggesting that assembly is driven mainly by the conformation of sequence Asn$^7$–Ala$^{90}$.

More interesting, a disulfide bond can be established between amino acids 6 and 137, but not between amino acid 6 in one Ure2p and another one in the fibrillar form of Ure2p. This indicates that amino acids 6 and 137 are in close vicinity in Ure2p fibrils, unlike the two amino acids at position 6 from two Ure2p molecules, and provides the first structural information on the conformational state of the N-terminal domain of Ure2p within the fibrils at the molecular level. Our findings are incompatible with the parallel superpleated β-structure model that has been proposed recently to account for the structure of the N-terminal domain of Ure2p in the fibrillar form of full-length Ure2p (25). Indeed, according to this structural model, the residues at position 6 from six different Ure2p molecules are stacked within a structure rich in β-strands (Fig. 4 in Ref. 25). If Ure2p N termini were indeed stacked within the fibrils in this way, intermolecular disulfide bonds would be established between Ure2p molecules upon incubation of fibrils made of Ure2pC6 or Ure2pC6C137 under oxidizing conditions. Moreover, Ure2p surface-exposed within the parallel superpleated β-structure model to the structure rich in β-strands is opposite the area where amino acid 137 is located. Thus, the latter structural model predicts the potential establishment of a disulfide bond between amino acids 6 or 17 and 283. Such disulfide bonds are never observed.

The parallel superpleated β-structure model was proposed to account for the recently reported reflections at 4.7 Å observed in x-ray fiber diffraction images of Ure2p fibrils (26). Using the same technique, we did not observe such reflections, but we did observe reflections at 25, 47, and 52 Å for native Ure2p fibrils (27). These reflections come from the Ure2p molecules stacked within the fibrils, the size of which can be calculated from the crystal structures we (8) and others (9) determined. They are orthogonal, indicating that the fibrils are oriented. It is only when Ure2p loses its native structure within the fibrils, e.g., upon heating above 60 °C, incubation at pH 3.0 (27), or extensive drying, 6 that the fibrils acquire the amyloid characteristics: (i) reflections at 4.7 Å and (ii) increased absorbance at 1620 cm$^{-1}$ in Fourier transform infrared spectroscopy. Unlike native fibrils, the latter fibrils do not promote Ure2p assembly in a catalytic manner. One reason for the discrepancy reported using the same technique (x-ray fiber diffraction) and experimental conditions that are relatively close may reside in the fact that a synchrotron microbeam and hydrated samples exposed to the x-ray beam for <20 s were used to collect our x-ray fiber diffraction images (27), and those published recently were recorded using x-rays generated by a classical rotating anode upon exposure of dehydrated samples for 5–15 min to x-ray beams (26). The latter treatment (dehydration and potential free radical generation) may lead to loss of the native structure of Ure2p within the fibrils. It is worth noticing that the parallel superpleated β-structure model predicts reflections coming from the globular native C-terminal domain of Ure2p. Such reflections are lacking in the x-ray fiber diffraction patterns published recently (26).

Our previous findings that the C-terminal domain of Ure2p is tightly involved in the fibrillar scaffold, together with those we have reported here demonstrating that amino acids 6 and 137 from the same polypeptide chain are in close vicinity within the fibrils, allow us to refine the models for assembly we proposed 3 years ago (18). The data reported here are inconsistent with the scenario in which assembly of Ure2p into fibrils is driven solely by intermolecular interactions between the N- and C-terminal domains of Ure2p. The scenario in which assembly is driven by intramolecular followed by intermolecular interactions between Ure2p molecules is also inconsistent with the data reported here. Indeed, in our previous model, the N-terminal domain binds to the hydrophobic surface interface between the two monomers that constitute the Ure2p dimer and forms a surface that favors its interaction with the flexible region extending from Leu$^{224}$ to Leu$^{306}$.

A refined model for the assembly of Ure2p into fibrils needs to integrate the following structural information. 1) The soluble form of Ure2p in which amino acid 6 is covalently bound to amino acid 137 assembles into fibrils. 2) Amino acids 6 and 137 are in close vicinity in Ure2p fibrils. 3) Amino acids 6 and 137 from two neighboring Ure2p molecules within the fibrils establish neither 6–6 nor 137–137 covalent bonds. 4) Amino acids 15–42 are essential for assembly (21). 5) Cleavage sites located in sequence 12–37 are not exposed to the solvent (14). 6) Dimeric Ure2p is in equilibrium with the monomeric form of the protein (6, 19). The dissociation constant determined by analytical ultracentrifugation is in the micromolar range. 7) monomeric Ure2p is the precursor of the fibrillar form of the protein, as the Ure2pC221 variant, which can be cross-linked as a native dimer, loses its ability to assemble into fibrils upon cross-linking (18). 8) The C-terminal domain of the protein is tightly involved in the fibrillar scaffold (14). Finally, 9) Ure2p binds its substrate glutathione in its fibrillar form (18) and exhibits enzymatic activity (11).

A model for Ure2p assembly taking into account all the structural information listed above is presented in Fig. 7. Our model is based on the observation that the precursor of fibrillar Ure2p, besides being monomeric (18), is in a state in which the flexible N-terminal domain interacts with the C-terminal domain of the protein. Indeed, in its extended form, the N-terminal domain of Ure2p can interact with that of another polypeptide, thus yielding a covalent non-native Ure2p dimer that is not able to assemble into fibrils or to incorporate into preformed polymers. More precisely, the soluble precursor of fibrillar Ure2p is a form in which the regions located around amino acids 6 and 137 interact. In our model, we assume that a conformational rearrangement of the poorly structured sequence 7–90 occurs following the interaction of the regions centered around amino acids 6 and 137. This limited conformational change may generate a surface area that can interact either with an identical surface or with the relatively hydrophobic interdimer interface exposed to the solvent upon dissociation of soluble native dimeric Ure2p. In either case, this would lead to strong intermolecular linkages by the establishment of a large number of hydrogen bonds. However, although the interaction between non-overlapping areas can proceed indefinitely, the interaction between identical surfaces cannot. The aggregation process involving the interaction of non-overlapping areas on the surface of Ure2p could lead to the formation of the nucleation units we characterized at the early stages of assembly (14, 22), which would then either condense into fibrils, as proposed by Xu et al. (28) for the assembly of the Sup35pNM fragment into fibrils by analogy to the aggregation of colloidal particles, or propagate indefinitely in a polar manner by addition of monomeric Ure2p molecules to both ends of the fibrils. This scenario accounts for the decreased exposure to the solvent upon assembly of sequence 12–37 (14); the importance of the region extending from amino acids 15 to 42 in the assembly process; and the

6 L. Bousset and R. Melki, unpublished data.

7 N. Fay, L. Bousset, and R. Melki, unpublished data.
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FIGURE 7. Molecular model for the assembly of Ure2p into fibrils. The proposed model is based on the following previously made observations. 1) Dissociation of the Ure2p dimer occurs prior to assembly into fibrils, as the Ure2pC2Z1 variant, which can be cross-linked as a native dimer, is unable to assemble into fibrils in its cross-linked form (18). 2) Ure2p in its fibrillar state binds glutathione and exhibits glutathione peroxidase activity (10, 11). 3) The C-terminal domain of Ure2p is tightly involved in the fibrillar scaffold (14). 4) Amino acids 15–42 are essential for assembly (23). 5) Assembly into fibrils is accompanied by a decreased exposure to the solvent of cleavage sites located in region 12–37 and an increased exposure of those located in region 66–86 (14). The model integrates the findings reported in this study showing that 1) soluble Ure2p cross-linked via its N-terminal domain is unable to assemble into fibrils, whereas the form in which amino acids 6 and 137 are covalently bound via an intramolecular bond assemble into fibrils in a manner indistinguishable from native Ure2p; 2) amino acids 6 and 137 are in close vicinity within Ure2p fibrils; and 3) amino acids 6 and 137 from two neighboring Ure2p molecules within the fibrils establish neither 6–6 nor 137–137 covalent bonds. Native dimeric Ure2p is in equilibrium with the monomeric form of the protein. The dissociation of the native Ure2p dimer exposes a hydrophobic surface area to the solvent (red). This area can establish a large number of hydrogen bonds with the N-terminal domain (blue) from another Ure2p polypeptide chain following the establishment of intramolecular interactions between the regions centered around amino acids 6 and 137 and the subsequent limited conformational rearrangement of the flexible N-terminal domain of Ure2p. This is at the origin of strong intermolecular interactions between Ure2p molecules through non-overlapping areas and leads to the formation of nucleation units that can grow indefinitely by addition of Ure2p monomers to both of their ends given that each additional monomer exposes a binding site similar to that masked by its association. The oligomers generated are helical because two neighboring monomers have the same relative orientation. A top and side view of the oligomeric/fibrillar scaffold are represented. Finally, the GSH-binding site (pink) is maintained in a native conformation in the fibrils and is available for binding its ligands.

In the present model, the N-terminal domain of Ure2p in its assembly-competent state remains in the same conformation within the fibrils as witnessed by the establishment of a covalent bond between amino acids 6 and 137. In contrast, the reduction of the degree of freedom of the N-terminal domain of Ure2p does not allow the establishment of a covalent bond between two cysteine residues at position 6. This accounts for the finding that the C-terminal domain of Ure2p is tightly involved in the fibrillar scaffold (14).

Further characterization at the molecular level of the conformational rearrangements that lead to Ure2p assembly and of the fibrillar state of Ure2p will come from solvent accessibility studies using hydrogen/deuterium exchange monitored by mass spectrometry. Indeed, a peptide mass fingerprint of the soluble protein with good coverage of the primary structure will allow the identification upon assembly of regions buried within the fibrils and highly protected from hydrogen exchange and the mapping of the fibrillar surfaces exposed to the solvent.

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